CHAPTER – 1

LITERATURE REVIEW

1.1 LEAD AND CADMIUM

1.1.1 Biosphere

Biosphere is the natural environment of living objects. It envelops the earth and contains superficial part of the lithosphere, the lower part of atmosphere and the hydrosphere. A relatively homeostatic environment is essential for the survival of an organism in a given ecosystem. Biosphere contains everything that exists in this environment including all types of contaminants, amongst which heavy metals are given prime importance.

Lead and cadmium, the typical toxic elements not essential for life, may be present as contaminants in the environment. The toxic effects of lead and cadmium have long been recognized (Lin-Fu, 1982) and to date remain a major public health problem (Graziano, et al., 1985). It has been reported that primary sources responsible for lead and cadmium exposure include food, water and inhalation (Bryce-Smith & Stephens, 1981; Friberg et al., 1979). The important fact is that once the ecosystem is contaminated with heavy metals, they remain as a potential threat for a long time. It has been known for more than a century that lead and cadmium can cause acute poisoning in man. The chronic symptoms after long term exposure to lead and cadmium are well documented (Hager, 1958; Cramer and Selander, 1965; Friberg, 1949, 1950).

Researchers conducted detailed examinations of lead and cadmium exposed workers and found their toxic effects in multiple organ systems. These include the effects of lead on hematopoiesis (Sasa et al., 1973), central and peripheral nervous system (Grandjean, 1978; Seppalainen et al., 1975), behavior (Silbergeld & Goldberg, 1973), spermatogenesis (Lancranjan et al., 1975), fetal development (Ferm & Carpenter, 1967), cardiovascular responses (Williams et al., 1977; Kopp et al., 1980) and bone and vitamin D metabolism (Anderson & Danychuk, 1977; Sorrel et al., 1977).

In case of cadmium, the itai-itai disease episode, pulmonary edema (Friberg, et al., 1971), hypertension (Balaraman, 1986), congenital or acquired renal tubular dysfunction (Butler and Flynn, 1958) have been reported.

In mid 90s, much attention was given to chronic low-level lead and cadmium exposure (Needleman, 1980, Balaraman, 1986) since it is self-sufficient to cause recognizable clinical poisoning and may be associated with harmful effects. The metabolic peculiarities of lead and cadmium like the slow selective accumulation in the organs and extremely long biological half-life make it difficult to extrapolate the results obtained in short term animal studies to humans.

1.1.2 Occurrence

The concentration of lead in earth's crust has been estimated at 12.5 ppm, ranking it as the 36th element in order of abundance (Soni, 1990). In soil forming rocks, lead level upto 150 ppm has been reported. The usual range of lead concentration in soil has been estimated at 2 to 200 ppm with an average value of 10.0 ppm (dry weight) (Bowen, 1966). Shacklette et al. (1971) reported a range of <10 to 700 ppm and an average of 20 ppm of lead in soil.

In natural water, lead is generally present in very low concentrations (Soni, 1990). Hart (1982) reported a concentration range of 0.0003 to 0.003 ppm in natural freshwater, while Forstner and Wittmann (1979) reported the likely levels as 0.002 ppm. Bowen (1966) had shown that the lead concentration in natural water ranges from 0.0006 ppm to 0.12 ppm with a median value of 0.005 ppm. In survey of stream water in the USA, Kopp and Kroner (1970) found an average concentration of 0.023 ppm lead. In Canada, river and lake water samples collected between 1972 and 1977 were reported to have lead levels varying from 0.001 to 0.05 ppm (dissolved lead), and less than 0.001 to 0.1 ppm (extractable lead). Moore and Ramamoorthy (1984) reported lead levels varying from 2.0 to 50.0 ppm in sediments collected from pollution free freshwater in USA.

In animals (except humans) the reported lead levels vary from 0.001 to 130.0 ppm (dry weight). Human tissues (dry) showed lead levels up to 170 ppm. The lead levels in human nails, hair and bones are greater by several orders of magnitudes than in any other tissues. In human, blood lead levels vary from 0.1 to 0.46 ppm (Bowen, 1966).

Cadmium ranks 64th in order of abundance in the earth's crust with an average concentration of 0.2 ppm (Taylor, 1964), which is less than that of chromium, zinc, copper and lead. Cadmium occurs in various other natural materials such as

magnetic rocks (0.026 to 0.130 ppm), shales (0.300 ppm), sandstones (0.020 ppm), limestones (0.035 to 0.09 ppm), and sediments (0.39 to 0.57 ppm) (Forstner, 1980). It has been reported that the cadmium levels in natural unpolluted freshwater varies from 0.01 ppb to 0.4 ppb (Hart, 1982). Similarly, Moore and Ramamoorthy (1984) reported the cadmium levels from 0.00001 to 0.001 ppm in a remote stream of California. The cadmium concentration up to 0.003, < 0.003, < 0.25 and 0.005 ppm were reported in the rivers of North America, Asia, Europe and U.K. respectively (UNEP, 1987). Similarly, cadmium concentrations varying from 0.00008 to 0.01 ppm were reported in selected rivers draining major watersheds in Canada, Australia, France, Germany, Netherlands and Switzerland (UNEP, 1987). The average concentration of cadmium in water from public water supplies in seven large cities of the European community was 0.0011 ppm with a range of 0.0002 to 0.004 ppm.

In uncontaminated sediments, the level of cadmium is generally < 0.5 ppm. Forstner (1980) reported cadmium levels varying from 0.04 to 0.84 ppm in 72 sediment samples collected from various unpolluted lakes from South America, Africa and California. In air, the natural background cadmium levels lie below 0.1 ng cadmium/m^3.

1.1.3 Usage Pattern

Lead is a widely distributed heavy metal, which has been and continues to be a persistent source of toxicological concern. The sources of lead are both natural and man-made. The environmental significance of lead reflects both its use and its abundance as compared to most other toxic heavy metals. Lead in various forms has been known and used by man from ancient times. The world production of lead is approximately 4 million tons, which is more than any other toxic heavy metal. In the United States, lead is consumed mainly as lead metal products, such as lead solder, lead pipes, lead sheeting, storage battery components and in synthesis of organolead fuel additives such as tetraethyl lead. In a modern industrial society, lead is present in food, water, air, soil, dust fall, and other materials with which man and other animals come in contact. Lead-using industrial activity and high-density automobile traffic associated with lead emissions generate appreciable contamination of the air and soil. Deteriorating urban housing also presents a

potentially high source of lead exposure because of lead-based paint used in these houses. Lead does not possess any known beneficial biological effects and is viewed purely as a deleterious agent. The normal blood level of lead has been a subject of controversy (Patterson, 1965; Goldwater and Hoover, 1967). The overall body burden of the metal depends on the industrialization of that particular area. Piomelli et al. (1980) have reported that lead concentrations in blood and air in a remote Himalayan population were much lower than in New York City. In short, lead is mainly used in industries involved in storage batteries, anti-nock agent in petroleum/ gasoline, paints, ammunition, glassware, ceramics, protection from radiation, bearing, alloys, rubber processing and printing press.

The uses of cadmium are in alloys, batteries, fungicides, nuclear control rods, ceramics, electroplating, solar cells, and alloy of telephone wires and others.

1.1.4 Lead and Cadmium in Environment

Environmental pollution with metals perhaps began with the discovery of fire and gradually aggravated to its present alarming level with industrial development and advancement of society. Human activities, such as mining, smelting, refining, energy production, industrial and vehicular emission, agricultural operations, sewage discharge and disposal of waste are rapidly increasing its pollution. The global estimate of lead emissions per annum through natural and man made sources is about 24.5 X 10^3 and 500 X 10^3 metric tonnes respectively. It has been estimated that the lead emissions into the atmosphere have increased sharply from 2420 X 10^3 metric tones to 4265 X 10^3 metric tonnes during pre 1970 to 1971-1980 (Soni, 1990).

Generally, cadmium is always found in association with around 5 percent levels of zinc. It has been estimated that approximately 500 tonnes of cadmium enters the environment annually as a result of natural weathering and about 2000 tonnes as a result of human activities. About 20 percent of released cadmium comes from zinc mining and smelting operations with another 30 percent from the manufacturing, use and disposal of cadmium products. The remaining 50 percent is dispersed as a contamination in other substances, including phosphate fertilizers, sewage effluent and sludge. Moreover, it is also released during combustion of fossil fuels. Following points summarizes the potential of these metals in the environment.

1.1.4.1 In Air

The ambient air that we breathe comes from the lower atmosphere (below 80 km). Pollutants such as dust, smoke, soot, industrial and automobile exhaust and gaseous and particulate matter, are found in varying concentrations depending upon factors, such as population, vehicular density, location and type of industrial units in that area. Metal compounds though not a normal constituent of air, are found in great variety, particularly in urban areas. With some exception, they occur mostly in the particulate phase. Particles of 0.1-10μm in diameter settle very slowly and remain suspended in the air for a longer duration of time. The typically small size (especially the particles <1μm in diameter) of these particulates allows them to be inhaled deeply into the respiratory tract. Metal ions are absorbed by the lungs ten times more efficiently than the intestine.

Data on the concentration of lead and cadmium in air of United States collected by National Air Sampling Network have been summarized by Tabor and Warren (1958), and by Shroeder (1970). The atmospheric concentration range of lead and cadmium in different parts of the world such as Greenland is 0.003-0.63ng of Cd/m^3 and 15-22ng of Pb/m^3, North America 21ng of Cd/ m^3 and 2700ng of Pb/m^3, in Europe it is 20ng of Cd/m^3 and 120ng of Pb/m^3. In the same study it is reported that in North India the concentration of these metals is 330-21000ng of Cd/m^3 and 25.3-677ng of Pb/m^3.

The World Health Organisation (WHO) guidelines for the maximum permissible concentration of metals in air are 10-20ng/m^3/yr for cadmium and 0.5-1μg/m^3/yr for lead (Soni, 1990). National Air Surveillance Network conducted a survey in mid seventees on the mean annual lead concentration in U. S. cities and reported that the lead levels were more than 2000ng/m^3 in 5 percent of stations. However, lead levels in air exceeding even 3000ng/m^3 were observed in 1 percent of surveyed sites (Shukla and Singhal, 1984) of Canada. National Air Pollution Surveillance of Environment, Canada reported mean lead levels in major Canadian towns and cities during 1974 and 1975 which were 700ng/m^3, ranging from 200ng/m^3 in Lethbridge, Alberta to more than 2000ng/m^3 in Montral. Data was collected in 1966 (Schroeder, 1970) for 58 cities and 29 non-urban areas giving range of concentration of cadmium (ng/m^3): 2-370 for urban areas and 0.4-26 for

non-urban areas. Friberg et al. (1971) quoted weakly means of cadmium 500ng/m^3 at a distance of 100 meters and 200ng/m^3 at a distance of 400 meters from Japanese smelter. The data of Langerwerff (1971) indicates that plants near highways take up half of the lead and cadmium from air borne sources.

1.1.4.2 In Soil

Analysis of uncontaminated soils indicates that normal content of lead and cadmium are less than 1 ppm perhaps about 0.8 ppm of lead and 0.4 ppm of cadmium on the average (Fleischer et al., 1974). The data of Langerwerff (1971) clearly shows the extent of soil contamination near highways. Contamination from cadmium occurs in various other natural materials such as magnetic rocks (0.026-0.130 ppm), shales (0.300 ppm), limestones (0.035-0.09 ppm) and sediments (0.39 – 0.57 ppm). The contents are significantly higher in the industrial and airport zone than in the residential areas.

1.1.4.3 In Water

Lead finds its ways into aquatic environment. Bertine and Goldberg (1977) estimated that worldwide weathering phenomenon mobilize approximately 21000 to 110000 tonnes of lead each year. Various pathways through which lead enters aquatic environment include: sedimentation and rainfall containing atmospheric lead, urban storm water runoff (specially from industrial and highway sectors), industrial effluents arising from plating units and from paper, rayon, dye, and pigments, chemical, fertilizer, ghee and battery industry and mine drainage. Sittig (1976) has reported that the industrial effluents contain high amount of lead e.g. battery manufacture (0.5 to 48 ppm) plating (140 ppm lead and 50 ppm cadmium), T. V. tube manufacturing (400 ppm) and mine drainage (0.02 to 2.5 ppm lead and 1000 ppm cadmium).

Most fresh waters contain less than 1 ppm cadmium. The chemistry in surface and ground waters has been reviewed by Hem (1972) who gives calculations of equilibrium solubility with $Cd(OH)_2$ or $CdCO_3$, showing minimum solubility at pH 9.0-10.0.

The surface water that contains more than few ppb cadmium near urban areas have almost certainly been contaminated by industrial wastes from metallurgical plants, plating works, or plants manufacturing cadmium pigments, cadmium stabilized plastics or nickel-cadmium batteries or by effluent from sewage treatment.
Soni (1990) has reported that the industrial effluent of dye and pigments contains 0.168-1.25 ppm lead and 0.004-0.009 ppm cadmium, whereas chemical industry effluent contains 0.007-0.025 ppm lead and 0.015-0.075 ppm cadmium. The buildup of lead and cadmium into water, and sediments in higher than natural levels results in bioaccumulation of the metal in various pockets of the food chain. The concentration of lead and cadmium in plant food is given in table- 1.1.

Table-1.1: Metals in common plant food

Plant Food	Concentration (mg/kg fresh fruit)	
	Lead	**Cadmium**
Cereals (wheat, rye, rice)	0.035-0.18	0.016-0.75
Potatoes	0.09	0.03-0.05
Vegetables (lettuce, cabbage, tomatoes, spinach, celery, cucumber)	0.07-0.2	0.04-0.067
Fruits (peaches, bananas, apple, citrus fruits)	0.00-0.12	0.008-0.011
Mushrooms	0.3	0.46
Tea	0.73	0.21
Coffee	0.20	0.17

High levels of these metals are reported in algae, freshwater invertebrates and fish, that are responsible for its toxicities in humans.

1.2 NORMAL HUMAN INTAKE

1.2.1 Lead

The characteristics of uptake of lead by man from environmental sources exist or are suspected to exist. Thus in the general adult population, respiratory intake via ambient air and oral intake via food, water and beverages are the major sources (Lee et al., 1989). But their relative importance has been a matter of considerable uncertainty. It is relatively simple to determine the range of air lead concentration

to which people are exposed and the concentration of lead in various foods and beverages, but it is more difficult to translate these concentration terms into units of lead inhaled or ingested per unit of time. But the greatest problem of all is determination of the fractional absorption, i.e. the relationship between the internal dose and the external dose.

Mercer (1975) conducted experimental studies mainly in animals in man which clearly indicates that great variations occurs both in total fractional aerosol deposition and in anatomical distribution pattern, depending on particle size. Further, the fraction of deposited lead, which ultimately is transferred to the systemic circulation, varies greatly, depending on the site of airway deposition and on the lead aerosol. Kehoe (1961), Nozaki (1966) and Mehani (1966) have reported studies of total airway deposition of lead aerosols in man. Results are in general agreement with predictions derived from animal studies.

An estimate has been made of the relationship of ambient air lead to total intake by kinetic analysis of the labeling of blood, bone and excreta following oral administration of the stable isotope ^{204}Pb to two volunteers by Robinowitz (1974). It was estimated that at an ambient air lead concentration of 2 $\mu g/m^3$ i.e. about 40 percent of the total daily internal dose, originated from the air. Input from the other compartments, e.g. bone was estimated from very sketchy data. Hence, the conclusions are questionable. Air lead exposure has been estimated in a large population of city-dwelling adults using personal air sampling devices. The analysis of air Pb and PbB data, it was estimated that the contribution of air lead to PbB was about 1 μg Pb/100gm blood per 1 μg Pb/m^3 of air, within the ambient air lead concentration range encountered by city population. Even in densely populated cities where the average ambient air lead concentration is of the order of 1-3 $\mu g/m^3$, only a small fraction of the internal dose of lead can be attributed to air, since blood lead levels are of the order of 15-25 μg/100g which are considerably greater than that of air sources. Probably, a blood lead level in the general population is the result of uptake of lead through gastrointestinal tract. The absorption of lead from dietary constituents and water was established years ago as being about 8 percent for adults, based on long-term balance studies conducted by Kehoe (1961), which was confirmed later on by the experiments of Robinowitz (1974). In contrast to the low absorption of lead in adults, the absorption of dietary

lead in normal infants and young children has been found to be approximately 50 percent. The accuracy of this estimate is questionable. Nevertheless, Kostial et al. (1971) and Forbes and Reina (1972) documented this in their experimental studies on animals with tracer doses of radioactive lead and confirmed that absorption is much greater in the young than in adults.

It has long been known that dietary calcium deficiency enhances the absorption of lead (Sobel et al., 1938), a fact which was reconfirmed by Six and Goyer (1970b). It seems unlikely that this interaction involves a competition for an active transport mechanism (Gruder, 1975). Other factors have been shown to influence materially the absorption of lead in animals. Thus, milk enhances the absorption of lead (Kello and Kostrial, 1973), while proteins decrease lead absorption.

It has been found that lead retention following oral administration is greater in iron deficient animals (Six and Goyer, 1970a). But it is not clear whether this interaction involves an increase of lead absorption or an increase in lead retention. Fasting also seems to enhance lead absorption (Garber and Wei, 1974). The availability for absorption of lead in dried paint films is of special interest in view of the importance of lead-based paint as a source of pediatric lead poisoning. Lead chromate and lead naphthenate incorporated into dried paint films are about one half to one third as available for absorption as are lead naphthenate in oil or lead nitrate in aqueous solutions (Gage and Litchfield, 1968 and 1969). Thus the matrix effect of paint on the availability of lead for absorption is not great. Inorganic salts of lead do not readily penetrate the intact skin. By contrast, lipid soluble forms e.g. tetraethyl lead and lead naphthenate penetrates to a significant degree.

1.2.2 Cadmium

The principal source of cadmium would appear to be food rather than air and water (this may not be true in smokers). Cadmium occurs in small amounts in all foods used by man or animals although little is known about its chemical form or binding. Friberg et al. (1971) have reviewed data on cadmium in food in various countries and there seems to be general agreement that food average about 0.05 ppm cadmium (net weight), of course, with wide variation depending on the source. There have been relatively few comprehensive studies of the total human

intake via foods, but the available data declines to put the average of 50μg/day or less with considerable variation. Murthy et al. (1971) reported 27-64μ g/day cadmium intake via food. Further evidence of approximate correctness of the figure of 50μg/day is provided by data on daily fecal excretion of cadmium in the general population. Thus, Tsuchiya (1969) reported that daily fecal excretion in four non-occupationally exposed men was 57 μg.

The studies of water supplies indicate that except for unusual instances of contamination, the intake via water is probably negligible (Kopp, 1970). The average intake from drinking water is about 1 or 2 μg/day. Little seems to be known as to the contribution of particulate content of water to the total cadmium content. Airborne particulate or aerosols provide an additional source of cadmium to the body. A large amount of data from 35 stations gave an average airborne cadmium concentration of 0.002 $\mu g/m^3$ (Fleischer et al., 1974). However, work by Lewis et al. (1972a) on cigarette smoking as a source of cadmium suggests that this may be an important item. Autopsies were performed on 172 adults, including 45 male smokers whose approximate cigarette consumption was known, and 23 non-smoking males. The mean age at death for each group was 60 years. Cadmium levels in lungs, liver, and kidney were determined by using atomic absorption. The estimated body burden of cadmium in non-smokers averaged 6.63 mg and was double the amount, 15.8 mg in the smokers. Lewis et al. (1972b) estimate that their data point to non-smoker retention of 1 μg or less/day, compared with about 2.5 μg/day for smokers. Szadkowski (1969) reported that about 1.4μg of cadmium found in a cigarette would be in particulate phase and 0.03 μg in the gaseous phase. About 0.1-0.13 μg might be inhaled per cigarette. The respiratory intake from two packs per day would be about 4-6 μg or 10-20 times the intake from the reported levels in the air of lower Manhattan.

In summary, it can be stated that intake for men under ordinary circumstances is principally from food, and most estimates would put this at about 20-50 μg/day. Due to poor absorption from intestinal tract it is possible that only about 2 μg/day or less is actually assimilated. The intake from drinking water is presumably 1 or 2 μg/day on the average due to poor absorption. The intake from ambient air is probably very low. The daily assimilation of cadmium from ambient air is approximately 0.02 μg.

For non-smoking, non-industrially exposed U.S. adult, the likely daily assimilation can be summarized as follows: 0.02 μg from air and 0.1 μg for a total of 2.12 μg from water. This value approximates the reported daily excretion of cadmium, suggesting that adults in general population be approximately in cadmium balance.

1.3 DISPOSITION

1.3.1 Lead

There are three major routes of lead exposure within the environment. In the general population the greatest exposure takes place via oral intake (food and drink), although in specific circumstances the inhalation of air contaminated with lead or uptake of dust from the industrial processes may represent important exposure routes. Inorganic lead salts do not rapidly penetrate the intact skin (Rastogi and Clausen, 1976), but lipid soluble forms such as tetraethyl lead penetrate significantly.

Inorganic lead is absorbed primarily in the duodenum, at least in the rat. The total body burden of lead does not seem to affect lead absorption (Conard and Barton, 1978). In human adults not occupationally exposed to lead, and in children older than 6-8 years, dietary lead probably comprises the major fraction of daily intake, recent estimates being 100-500 μg per day. The intestinal absorption of lead in adults is about 10 percent (Robinowitz et al., 1976), whereas normal infants and children 5 years of age or younger absorb approximately 40 percent (Ziegler et al, 978). Although only 10 percent of ingested lead is absorbed in adults, up to 40 percent of inhaled lead will be taken in by pulmonary route (Moore et al., 1980), provided the particle size is small. Nutritional factors influence the extent of lead absorption from the gastrointestinal tract. In particular, a low dietary level of calcium (Six and Goyer, 1970b) has been shown to increase the body burden of lead in rats. A study by Barton et al. (1978b) showed that both lead and calcium compete for similar binding sites on intestinal mucosal proteins, which are important in the absorptive process. However, these authors also reported that dietary calcium had no significant effect on the absorption of lead, but calcium-deprived rats showed decreased excretion and thus increased body retention of

lead. Mykkanen and Wasserman (1981) investigated in more detail the mechanism of lead transport by the gastrointestinal tract and, particularly, the similarities or dissimilarities between lead and calcium in this process. The absorption of these metals was determined in 3-week-old while Leghorn cockerels, raised on a commercial diet or special diets, using an *in vivo* ligated loop procedure. It was shown that lead is rapidly taken up by the intestinal tissue and only slowly transferred into the circulation, whereas calcium, also accumulated rapidly by the intestine, is released much faster from the tissue in the serosal direction. Their data imply that, in spite of the similarities in the response of lead and calcium absorptive processes to various treatments, there is no direct interaction between these cations in the intestine of the chick.

Vitamin D administration also enhances lead absorption in rats (Smith et al., 1978; Hart and Smith, 1981). In a more direct study, Barton et al. (1980) demonstrated that the manipulation of dietary vitamin D content had no significance on the absorption of lead from isolated gut loops, and parenteral vitamin administration did not affect lead absorption in rachitic animals. In contrast, dietary vitamin D deficiency and repletion resulted in increased absorption in intact animals because of prolonged gastrointestinal transit time. It has also been found that retention of orally administered lead is greater in iron-deficient animals (Six and Goyer, 1970a). The lead absorption is affected by the state of iron repletion; endogenous iron is available to block absorptive sites utilized by lead in the intestinal mucosa (Barton et al., 1978a). Dietary constituents such as ascorbic acid and sulfhydryl group of amino acid, increase lead absorption (Conrad and Barton, 1978). Lactose also facilitates the intestinal absorption of lead in weanling rats (Bushnell and DeLuca, 1980).

Once absorbed, lead is transported in the blood with approximately 95 percent binding to erythrocytes. In the blood, lead has a biological half-life of approximately 25 days. There are three discrete lead compartments in the body: circulating blood, relatively labile soft tissue fractions and an inert but appreciable portion deposited in the skeleton. Lead retained in the soft tissues has a half-life of a few months, but the half-life in the brain may be somewhat longer (Grandjean, 1978). More than 90 percent of the body lead burden is accumulated in the skeleton. The level of lead in the human skeleton increases until the age of 40 years (Barry and Mossman, 1970). The half-life of bone lead in man is estimated to

be about 10 years. Blood sample analysis is most commonly used to determine lead retention in the body.

It has been long known that predominant site for localization of lead is the calcified matrix of the skeleton. But the major concern has been with the disposition of lead in the other, more toxicologically significant tissues, e.g. brain, kidney, and the hemopoietic system.

In past some years the studies of the concentration of lead in the human tissues indicated that the total body burden of lead increases throughout life (Barry, 1975; Gross et al., 1975) and it is most pronounced in densely calcified matrixes of bones. Many soft tissues do not show increase after the second decade of life but kidney shows a progressive increase. This may be due to replacement of parenchymal cells having a high affinity for lead by connective tissue.

The subcellular distribution of lead has been studied in animal systems, mainly by cell fractionation technique. All such studies must be viewed with a certain amount of notion because they are essentially *in vitro* studies in which redistribution during the preparation of cell fractions is usually not ruled out. Nevertheless, functional and ultrastructural changes are noted following lead exposure, suggesting penetration to the intracellular milieu (Goyar and Krall, 1969).

The concentration of lead in peripheral whole blood and blood serum, which probably reflects the concentration in the readily exchangeable pool, does not change systematically with age (Butt et al., 1964). As a matter of fact, the levels of lead in blood which are usually expressed as μg lead/100g blood (PbB) of newborn children and of their mother differs only slightly, with PbB generally being reported to be slightly lower in the infants (Scanlon, 1971).

The response of PbB to sudden changes in the external lead exposure level has been studied in man. Workers newly introduced into the treading of lead and related products show a pronounced rise in PbB that attains a new plateau after about 50-60 days (Tola et al., 1973). In a more closely control of air lead exposure; the time for attaining an apparent new equilibrium of PbB was about 100 days (Griffin et al., 1975). This is in agreement with studies involving the introduction of ^{204}Pb into the diet on a continuous basis, wherein the labeling of the blood with ^{204}Pb was monitored (Robonowitz, 1974).

The discrepancy between rates of fall of PbB following short-term versus long-term elevated exposure has not been studied systematically. But it is reasonable to suppose that short-term clearance of lead from the blood involves a greater degree of redistribution to other compartments within the body than does long-term clearance. Following a single dose of ^{210}Pb in dogs, the curve for disappearance from the blood was analysed as three separate exponents with the last one having a half time of 103 days (Hursh, 1973). The calculations were made on the basis of blood analyses carried out to 280 days. The biological half-life for ^{210}Pb in the body was estimated at 1940 days. Thus, net transfer of lead to other compartments must be occurring even at 280 days.

Some investigators have advocated the use of chelating agents for assessing the degree of industrial lead exposure (Teisinger and Srbova, 1959). The procedure has also been used as a means of determining whether exposure to lead in early childhood might be involved in renal disease of young and middle-aged adults (Emmerson, 1963) or in hyperactivity of school children (David et al., 1972). In this procedure the amount of lead mobilized from tissues and excreted in the urine is measured following a standard dose either of calcium disodium ethylenediaminetetraacetate (EDTA) or of D-penicillamine (PCA). In both animals (Hammond and Aronson, 1960) and humans (Selander et al., 1966), there is a high correlation between pre-treatment PbB and the amount of lead excreted in response to these agents. Contrary to the impression conveyed in some textbooks, most of the metal mobilized in this manner originates from osseous, rather than soft tissue (Hammond, 1971; Momcilovic and Kostial, 1974; Barry, 1975). When given i.v. in comparable doses, EDTA is more effective than PCA (Hammond, 1973; Ohlsson, 1962; Selander, 1967). When given orally, the order of effectiveness is reversed (Ohlsson, 1962; Selander, 1967), probably because of the superior gastrointestinal absorption of PCA. The routine use of these agents for prophylactic and diagnostic purpose is somewhat hazardous. Signs of poisoning in children may be transiently aggravated using EDTA (Chisolm, 1968), and a nephrotic syndrome has been reported using PCA (Adams et al., 1964). But the EDTA effect probably occurs only when frank clinical signs are present and the PCA effect probably occurs only with long term use at high doses.

The use of lead mobilized by chelating agents, as a measure of internal exposure offers no clear advantage over other methods of assessment. Elevated excretion of

lead can be obtained by this technique many years after high exposure has terminated, but in such cases PbBs also remain elevated, and the correlation coefficient for the two parameters is about 0.7 (log-log) as calculated from data in the literature (Prerovska and Tesinger, 1970).

The major route of elimination of ingested lead that has not been absorbed from the gastrointestinal tract is fecal excretion. Primarily, the absorbed fraction of lead is excreted in urine, although lesser amounts are excreted in sweat. The kidney excretes lead in two ways: glomerular filtration and transtubular flow (Goyer and Mahaffey, 1972). A small portion of absorbed lead is excreted in the bile in most animal models studied (Klassen and Shoeman. 1974). Rate of lead elimination from femur and whole body was apparently less rapid in young than in adult mice (Keller and Doherty, 1980).

1.3.2 Cadmium

Although there is little doubt that man and other mammals absorb cadmium through the lung and mouth, there have been very few studies designed to estimate quantitative human uptake from the environment.

Tipton and Stewart (1970) described a balance study in three normal subjects over a period from 140 to 347 days. Using atomic absorption methods, the amounts of cadmium and a number of other metals were determined in the diet, and the amount excreted in the urine and feces were measured. The subjects were reported to have ingested an average of 170 μg Cd/day from the diet and to have excreted about 42 μg/day in the feces and 94 μg/day in the urine. Friberg et al. (1971) pointed out that these results indicated absorption of 75 percent of the intake much higher than the percentage reported by others. The urinary excretion also seemed very high and it seems possible that analytical errors resulted from sodium chloride interference.

Bostrom and Wester (1969) studied the intake in human for a period of 5 days. They found cadmium intake level of 12 μg/day and fecal excretion of about 5 μg/day. The short duration of the study would make interpretation of absorption uncertain. Various animal studies suggest that the absorption of cadmium from the gastrointestinal tract is poor. For example, Decker et al. (1957) found 2.6 percent

of a single oral dose of ^{115}Cd in the liver and kidney of rat three days later. At 7 and 15 days after administration, 2 percent of the dose was present in these organs. Thus, at least 2.6 percent of the dose had been absorbed, but probably not much more than that, in view of the fact that little or no radioactivity could be detected in muscle, lung, bone, spleen and urine. Decker et al. (1958) measured cadmium levels in the kidney and liver. These contained about 0.3 to 0.5 percent of the dose of cadmium ingested in one year. Since 50 to 75 percent of the body burden will be in these organs it is clear that overall retention probably was only 1 percent or less of the dose. Lucis et al. (1969) studied the metabolism of ^{109}Cd administration to rats orally. The results of this experiment indicated low excretion rate and storage mainly in liver and kidney. Friberg et al. (1971) have also reported that monkeys retained only about 3 percent of an ingested dose 10 days after ingestion. Another method of estimating absorption in humans is to determine body burden at autopsy and estimate total intake over a half-life. Though subject to many errors, such calculations are compatible with an absorption rate of about 3 to 8 percent (Friberg et al., 1971).

There is strong evidence that cigarette smoking contributes substantially to the cadmium body burden of smoker (Lewis et al., 1972a; 1972b). Rough calculations suggest that the retention of cadmium inhaled in cigarette smoking is substantial. Lewis et al. (1971a; 1972b) estimated that the body burden of cadmium due to cigarette smoking is 0.36 percent mg/pack-yr (where 1 pack-yr denotes one pack smoked per day for one year). On estimating the sum of cadmium in liver, kidney and lungs attributable to cigarette smoking as 25mg/100 pack-yr and substracting 7mg from other sources giving 18 mg. As the total body burden is assumed to be twice the burden in these organs, yielding (18mg X 2)/100 pack-yr or 360μg/pack-yr. Menden et al. (1972) reported that approximately 2μg Cd is inhaled per pack implying that 1 pack-yr provides 730μg Cd inhaled (2 X 365 = One smoked per day for one year).

Prodan (1932) studied the accumulation of cadmium in cats exposed to fume, oxides and sulphides for short periods. The largest percentages were found in the lungs, liver and kidney, and the estimated retention to vary from about 17 to 30 percent. In the case of sulphide exposure, virtually all the cadmium was found in the lung. Friberg (1950) exposed rabbits to a mixture of cadmium and iron oxide

dust and found the principal amounts in the lung, liver and kidney and an estimated pulmonary absorption of about 30 percent.

Cadmium is well absorbed from the injection site and again seems to be stored mainly in liver and kidney (Friberg et al., 1971). The exact mechanism by which cadmium is transported through intestinal mucosa is unknown although there has been some speculation that it may involve mechanism similar to those of copper and iron transport. There is now substantial evidence that once it reaches the liver, its presence stimulates the formation of an unusual protein of low molecular weight discovered by Margoshes and Vallee (1957) and named metallothionein. It was originally found in equine kidney but now is known to be present in the liver and kidney of many mammalian species. It usually contains about equal molar concentration of cadmium and zinc and has a molecular weight of about 10500. It may contain as much as 5.9 percent Cd and also has the ability to bind mercury. The work by Nordberg et al. (1971) has resulted in the finding of 60 percent of ^{109}Cd related to a similar protein in mouse blood after repeated subcutaneous injections over a 6 months period. The protein was attached to the red cells, but clearly separable from hemoglobin. The plasma levels were too low to allow separation and identification of the binding protein. Some portion of plasma binding was thought to be on a low molecular weight protein. This fraction may be filtered in the glomerulus and thus perhaps represents the pathways for tubular reabsorption, storage, and urinary excretion. The excretion of cadmium in the urine has been approximately 1-2μg/day in the general adult population (Imbus et al., 1963; Lehnert et al., 1969). Little is known concerning the relative importance of urinary and fecal excretion in man. The only study that provides any information indicates that 30 days following oral dose of ^{115}Cd, the rate of fecal excretion was approximately half the rate of urinary excretion (Rahola et al., 1972). There is also some evidence for excretion through the intestinal tract following injection in rats in feces (Decker et al., 1957). The correlation between the concentration in blood and other organs has not been established for man. Thus, blood cadmium has not yet been shown to be a reliable index of exposure. Concentrations in other organs are considerably lower than in either liver or kidney. The body burden of Cd in an adult is estimated to be about 30 mg. The newborns are said to contain only about 1 μg of Cd, so there is a gradual increase with age. Of the total body burden, 50-75

percent will be in the liver and kidneys, about $1/3^{rd}$ of it in the kidney. The concentration of cadmium in the kidneys is of special interest. Industrial experience suggests that the kidneys are especially sensitive to the toxic effects of cadmium. The threshold concentration of cadmium in kidney above which renal damage is likely to occur is estimated to be 200 ppm in outer cortex (Friberg et al., 1971). This is approximately four times the concentration reported for adults in the general population in one study (Schroeder and Balassa, 1961) and about six times the concentration reported for the category of moderate smokers of advanced age in other study (Lewis et al., 1972a). The margin of difference might be considerably smaller for moderate smokers in middle age, since the concentration of cadmium is known to fall in advanced age.

1.4 TOXIC EFFECTS IN MAN

There are a number of well-documented studies of acute and chronic effects of lead and cadmium in man. Animal studies indicate a variety of toxic effects, the significance of which has not yet been demonstrated for man. The toxic effects of these metals in man can be classified according to the route of exposure and period of exposure.

Lead poisoning in young children living in deteriorating inner city housing emerged as an urban problem in the United States as early as the second and third decades of last century (Williams et al., 1952). Thomas and Blackfan (1914) of the Johns Hopkins Hospital of Baltimore were the first to describe in American literature the clinical and pathological effects of lead-induced encephalopathy in children. Williams et al. (1952) pointed out that prior to 1951 multiple cases of lead poisoning had already been reported from 19 separate communities, and it was decided early that this problem was related to pica and habits of chewing cribs, eating of painted plasters and fallen paint flakes. Sixty percent of the affected children were over 5 years of age. During the 20-year period 1931-1951 in Baltimore, 293 cases were identified as having clinical lead poisoning identified by a blood lead greater than 50μg/100ml coupled with a clinical manifestation of lead toxicity (anemia or CNS symptoms). Eighty-five cases were fatal.

Following the early Baltimore experience public health departments of other major American cities began efforts to determine the extent to which lead poisoning did exist in the urban community. Generally, two steps were taken. First, lead poisoning was made a reportable disease, and second, separate programs for control of lead poisoning were established. A report of the result of these measures in Philadelphia from 1955 to 1960 (Ingalls et al., 1961) had noted that about 50 cases of lead poisoning were reported each year. From 10 to 20 percent of diagnosed patients died in the period, and neurological disorders including mental retardation persisted as sequelac in those who survived therapy (Perlstein and Attala, 1966). Other studies (Ingalls et al., 1961; Thomas and Blackfan, 1914; Williams et al., 1952) however indicated that 9.1-45.5 percent of children surveyed had blood lead levels above 40 μg/dl and up to 12.5 percent had above 60μg/dl. The Bureau of Community Environmental Management and National Bureau of Standards reports estimate that about 23 or 24 percent of children at risk had elevated lead levels (above 40μg/dl), and about 5 percent of these children had clinical symptoms. Greenberg et al. (1986) reported the detailed evaluation of renal function in a cohort of 34 workers exposed to two nephrotoxins: lead and cadmium. A highly significant increase in blood lead levels have been recorded in 92 lead acid battery factory workers in Sudan along with CNS symptoms (insomnia, fatigue, weakness and drowsiness) and blue line gums showing adverse health effects of occupational exposure to lead (El-Karim et al., 1986). Acute nausea and vomiting with 15-30mg of cadmium is well documented by Fleischer et al. (1974). Actual oral lethal dose in man has not been established, but estimates have been made that it is probably in the neighborhood of several hundred milligram (Fleischer et al., 1974). Friberg et al. (1971) reported acute pulmonary edema by inhalation of metallic fumes or cadmium oxide dust. Fatalities were recorded from a 5 hour exposure at about 8mg/m^3 although in one instance recovery was reported after exposure to 11mg/m^3 for 2 hour. The pulmonary changes seen in man have been reported in experimental animals (Nordberg et al., 1971). Chronic exposure to cadmium through the respiratory tract produces a number of toxic effects, the most important of which is the chronic emphysema first described in the classical report of Friberg (1950), in accordance with this, it is also reported that with emphysema, protein, glucose and calcium excretion was

also noted in urine by Friberg et al. (1971). Ahlmark (1960) reported a high incidence of renal stones with long exposure to cadmium dust. The form in which cadmium is excreted in urine is not known although it is possible that it may be in the form of a metallothionein complex.

Anosmia has been reported in a group of alkaline battery workers who were exposed to both cadmium and nickel dust (Adams and Crabtree, 1966). In 1955, a somewhat unique disease was described in the vicinity of a mine in Toyoma Prefecture, Japan. The disease was epidemic among elderly woman who had borne many children (average of 6). The outstanding features of the disease were lumber pain and myalgia, spontaneous fractures with skeletal deformation. Pain was readily elicited from pressure applied to bones. Extensive epidemiological studies were instituted after it was demonstrated that the water, rice and fish in the endemic area were found to contain high concentration of cadmium and other metals probably due to contamination of the local river by the effluent from a zinc lead-cadmium smelter. Their studies continued and were extended to include other areas in Japan where similar mining operations exist. Friberg et al. (1971) have summarized results of these investigations. The evidence available to date strongly indicates that this syndrome, termed as itai-itai, is due to long-term cadmium exposure. It is the first likely instance of cadmium poisoning in man due to general environmental contamination. The characteristics skeletal changes found in the older women are not usually observed in industrial cadmium poisoning. The neuro-muscular signs and skeletal defects described in itai-itai, however, have also been observed in a series of cases of cadmium poisoning in France during World War II. Four women and two men were affected. All had been exposed for at least 8 years (Nicand et al., 1942). Similar cases of industrial cadmium poisoning have also been reported (Bonnell, 1955; Gervais and Delpech, 1963). Thus, the musculoskeletal features of itai-itai are far from unique as manifestations of excessive cadmium exposure. Itai-itai is not solely a musculoskeletol disease. The more classical renal effects of cadmium seen in industrial poisoning: proteinuria was always found in clinical cases of itai-itai. Glucosuria and aminoaciduria were also usually present (Friberg et al., 1971). Further, the incidence of proteinuria and glucosuria was much higher between older women and men in the endemic area than elsewhere in Toyoma Prefecture. The urinary excretion of cadmium also was three times greater among people in the endemic area than in the non-endemic area

of Toyoma prefecture. Studies conducted in Japan indicate that excessive exposure to cadmium may be more widespread in that country than had been previously thought (Friberg et al., 1973). Unfortunately, due to faulty experimental design, these studies could not provide any significant new information in regard to dose-response relationship.

Recent studies (Burglund et al., 2000) indicate that lead and cadmium may exert both direct and indirect actions on bone turnover, indirectly via kidney dysfunction, and directly on osteoblast and osteoclast function. Increased blood lead concentrations, most likely as a result of an increased bone turnover, have been detected in pregnant, lactating, and menopausal women. Lead exposure has also been negatively associated with children's growth in stature. Both lead and cadmium are nephrotoxic and can disturb vitamin D metabolism. Cadmium has been shown to induce kidney damage and osteoporosis/osteomalacia at long-term high-level exposure. A negative association between cadmium dose and bone mass has recently been detected in both occupationally and environmentally exposed people at relatively low cadmium exposure.

Lead and cadmium have shown immunomodulatory activities (Krocova et al., 2000). The viability of both lymphocytes and macrophages was affected by heavy metals in a dose- and time-dependent manner. In the case of lead, the depression of N-oxide production closely correlated with increased blast transformation of spleen cells induced by concanavalin A (ConA). On the contrary, cadmium suppressed the production of N-oxides but stimulated significantly the proliferation of spleen cells. The production of cytokines by lymphocytes and macrophages was dependent on the *in vitro* model used. Generally, the treatment of macrophages with lead results in disregulation of the production of proinflammatory cytokines tumour necrosis factor alpha (TNF- alpha), interleukin-1 alpha (IL-1 alpha) and interleukin 6 (IL-6)] and preferential production of Th1 type of cytokines (IFN-gamma and IL-2). Cadmium seemed to trigger the Th2 cytokine regulatory pathway [interleukin 4 (IL-4), interleukin 10 (IL-10)]. The results suggest the metal-induced changes in immunoregulatory mechanism of host with potentially severe clinical consequences.

Telisman et al., (2000) have conducted a study based on measurement of blood lead (BPb), activity of delta -aminolevulinic acid dehydratase (ALAD), erythrocyte protoporphyrin (EP), blood cadmium (BCd), serum zinc (SZn), seminal fluid zinc

(SfZn), serum copper (SCu), and parameters of semen quality and of reproductive endocrine function. The parameters were measured in 149 healthy male industrial workers, 20-43 years of age. The group contained 98 subjects with slight to moderate occupational exposure to Pb and 51 reference subjects. All of the subjects lived in Zagreb, Croatia. Significant ($p < 0.05$) correlations of BPb, ALAD, and/or EP with reproductive parameters indicated a Pb-related decrease in sperm density, in counts of total, motile, and viable sperm, in the percentage and count of progressively motile sperm, in parameters of prostate secretory function (SfZn, acid phosphatase, and citric acid in seminal fluid), and an increase in abnormal sperm head morphology, serum testosterone, and estradiol. These associations were confirmed by results of multiple regression, which also showed significant ($p < 0.05$) influence of BCd, SZn, SCu, smoking habits, alcohol consumption, or age on certain reproductive parameters. These effects were mainly of lower rank and intensity as compared to Pb-related reproductive effects, whereas BCd contributed to a decrease in sperm motility and an increase in abnormal sperm morphology and serum testosterone. No significant Pb- or Cd-related influence was found on levels of the lactate dehydrogenase isoenzyme (LDH-C-4) and fructose in seminal fluid or on follicle-stimulating hormone, luteinizing hormone, and prolactin in serum. The seminal fluid concentrations of Pb (SfPb) and Cd (SfCd) were measured in 118 of the 149 subjects, and a highly significant ($p < 0.0001$) correlation was found between BPb and SfPb levels ($r = 0.571$) and between BCd and SfCd levels ($r = 0.490$). The overall study results indicate that even moderate exposures to Pb (BPb < 400 mu g/L) and Cd (BCd < 10 mu g/L) can significantly reduce human semen quality without conclusive evidence of impairment of male reproductive endocrine function.

1.5 PHYSIOLOGICAL RESPONSES OF ORGAN SYSTEMS

1.5.1 Lead

Acute cases of lead poisoning are rare in adults. Depending on the severity and duration of exposure, the effect may range from lead colic to encephalopathy and death. Primarily, the more common responses caused by chronic lead exposure are cited ahead.

Anemia from lead poisoning is probably the most well-known and best-understood effect in man. A decrease in blood hemoglobin appears at relatively low exposure levels. The anemia caused by lead exposure is attributed to two different actions of lead: lead inhibits heme synthesis, but it also causes increased fragility of the red blood cells and, consequently, an increased rate of destruction. The mechanism for shortened erythrocyte survival is not well understood, but it has been demonstrated that an inhibition of erythrocyte membrane Na^+-K^+-ATPase occurs in people with only moderately elevated lead exposure (Hernberg, 1976). The decreased erythrocyte life span is probably caused by a leak of potassium associated with an inhibition of Na^+-K^+-ATPase, and this leads to an increased mechanical fragility of the cell. Interference with the heme biosynthetic pathway by lead occurs in two steps: a) Inhibition of the activity of the enzyme aminolevulinic acid dehydrase (ALAD), which mediates the condensation of two units of aminolevulinic acid (ALA) to form parphobilinogen, and b) the insertion of iron into protoporphyrin IX to form heme, the final step in heme synthesis. Most sensitive to a low level of lead is the enzyme ALAD (a zinc activated enzyme). The mechanism of inhibition probably involves direct competition of lead with zinc for the binding to the sulfhydryl group in close relation to the active site of the enzyme. The inhibition is primarily of the noncompetitive type and can be detected in the erythrocytes at blood lead levels as low as 10-15μg/dl (Sasa et al., 1978). This enzyme inhibition occurs in a large proportion of people in industrialized societies. The correlation between blood lead and degree of ALAD inhibition is established so sufficiently that the ALAD activity may be employed as an accurate estimate of blood lead levels. There is controversy over the clinical meaning of moderate inhibition of ALAD by lead, but significant inhibition, to the point that urinary excretion of the substrate ALA is increased, is generally accepted as an indication of significant physiological impairment.

Ferrochelatase, the final mitochondrial enzyme in heme synthesis, is less sensitive to lead than ALAD. When lead exposure is high, its substrate protoporphyrin IX accumulates in red blood cells because heme is not found due to inhibition of heme synthesis by lead. Instead, zinc protoporphyrin (ZPP) that is found in large amounts occupies the molecular site reserved for iron. Because ZPP is stable and firmly bound to hemoglobin, the ZPP level in the blood is also an indication of the

average toxicity of lead in the bone marrow during the 3 months life span of erythrocyte (Lamola et al., 1975). Higher concentration of lead may also interfere with other steps in the heme synthesis by depleting cellular protein content by *de novo* induction of microsomal enzyme heme oxygenase, resulting in increased catabolism of heme (Maines and Kappas, 1977).

Next to hematopoietic system, the nervous system is most affected by lead exposure. The physiological responses to lead are evident in both the peripheral and central nervous system.

Earlier studies have demonstrated that lead damages peripheral nerves; the pathological change is mainly axonal degeneration (Krigman et al., 1980). The degeneration of spinal nerve roots after lead exposure is questionable (Hyslop and Krans, 1923). Segmental demyelination is not well established in human lead neuropathy (Behse and Carlsen, 1978) but is common in animals (Fullerton, 1966). A degenerative change in the sympathetic nerves and ganglia as well as submucosal and myenteric plexuses of the gut after lead exposure has been reported (Cantarow and Trumpa, 1944). Chronic low-level lead exposure in humans also affects nerve conduction velocity (Seppalainen and Hernberg, 1972). Lead exposure has been a cause of motor neuron disease (Campell et al., 1970). The neurophysiological manifestations of lead toxicity are best demonstrated in the peripheral nervous system. The amount of acetylcholine normally released by the action has a potential at the presynaptic junction is reduced by lead in both *in vitro* and *in vivo* (Silbergeld et al., 1974). This effect of lead is reversed by the addition of calcium to the system, indicating that lead probably complexes with calcium in the presynaptic membranes (Silbergeld and Adler, 1978).

The deleterious effects of lead on the central nervous system (CNS) may range from behavioral dysfunction to encephalopathy, particularly in growing animals. An association between lead and at least some form of minimal brain dysfunction or hyperactivity in children has been suggested (Sobotka and Cook, 1974; Lin-Fu, 1972). David et al. (1972) reported that concentration of lead in blood and urine were found to be significantly elevated in the greater proportion of a group of children who had minimal brain dysfunction as compared with those of a control group. Lead induced hyperactivity has been reported in mice (Silbergeld and Goldberg, 1973) and monkeys (Allen et al., 1974). Lead was given in drinking water or food to nursing mothers or directly to pups immediately after birth and

before weaning. These animals exhibited significant increase in motor activity and poor learning performance (Brown, 1975). Pharmacologically, these animals responded to certain drugs in a manner comparable to that of hyperactive children (Silbergeld and Goldberg, 1975; Laporte and Talbott, 1978). In such hyperactive mice, the increased motor activity was suppressed by the administration of amphetamine, methyl phenidate, cholinergic agonists, and aminergic antagonists and aggravated by aminegic agonists and the anticholinergic agents atropine (Silbergeld and Goldberg, 1975). This concept is not universally accepted because of lack of reproducibility in rats (Sobotka and Cook, 1974; Memo et al., 1980). The behavioral and pharmacological consequences of chronic lead exposure in mice and their similarity to those seen in hyperactive children with increased lead burden prompted relatively extended studies on the neurochemical effects of postnatal lead exposure in animals. Squrhoff and Michaelson (1973) initially reported that neonatal lead exposure in rats caused a 20 percent decrease in dopamine (DA) and no change in norepinephrine levels in brain as compared to control.

The interaction of lead and calcium metabolism has been known for a long time. It was shown that low dietary calcium enhances lead retention (Lederer and Bing, 1940). This phenomenon was reinvestigated and the finding supplemented by the work of Six and Goyer (1970b) and Mahaffey et al. (1973) who demonstrated the improvement in lead toxicity upon decreasing dietary calcium. It has been observed in rats that ingesting Pb (2mg/kg/day for 7 days) leads to decreased active transport of calcium across the duodenal wall as determined by the *in vitro* inverted gut sac technique (Gruder et al., 1974; Gruder, 1975). On the other hand, when total calcium retention of orally administered ^{47}Ca suggested the ingestion of a high dose of lead (20mg/kg/day for 7 days). Barton et al. (1978b) demonstrated that both lead and calcium compete for similar binding sites on intestinal mucosal proteins, which are important for absorptive process. These authors have also shown that lead is bound mainly to a high molecular weight mucosal protein, whereas calcium is bound to low molecular weight protein (calcium binding protein). Barton et al. (1978b) have also shown that dietary calcium have no significant effect on lead absorption, but calcium-deprived rats has decreased excretion resulting in increased body retention of lead. Prolonged dietary calcium deficiency stimulates parathyroid hormone secretion, and the acute administration

of parathyroid hormone in rats has been shown to increase the renal lead accumulation (Mouw et al., 1978a). The interaction of lead and vitamin D metabolism, which is intimately associated with calcium metabolism, has also been studied (Sorrel, et al., 1977). Fraser and Kodicek (1970) first reported that lead burdened children have low levels of serum 25-hydroxyvitamin D, which is an intermediate metabolite of vitamin D produced in the kidney and presumed to be the most active metabolite of vitamin D. Omdahl et al. (1971) also noticed the same type of observations. Acute intravenous administration of lead acetate results in transient hypercalcemia and hyperphosphatemia in rats (Kato et al., 1977) as a result of a direct action of lead on bone minerals.

The effects of lead on the kidney are manifested in two ways in both adults and children: namely, reduced glomerular function and proximal tubular damage. In mammals, lead concentrates in the kidneys causing hyperaminoaciduria, glycosuria and hyperphosphaturia- all reflecting decrease in the renal tubular reabsorption process (Goyer, 1968). Because lead poisoning impairs mitochondrial functions (Alvares, 1978), it is possible that a deficiency of ATP is responsible for the reduction of tubular functions. Lead poisoning in adults is sometimes associated with gout, hyperuricemia and decreased renal urate clearance. The findings are indicative of either increased tubular reabsorption or decreased tubular secretion of urate. Cases of gout and associated effects occur in conjunction with other renal effects involving a gross reduction in glomerular filtration and progressive renal failure (Lilis et al., 1968; Wedeem et al., 1975). Bantman et al. (1981) demonstrated that hyperuricemia, renal insufficiency and nephropathy probably resulted from lead poisoning in gout patients.

Acute administration of lead in dogs increases the urinary excretion of sodium, potassium, calcium and water, despite a constant glomerular filtration rate. Similar changes, as well as an increase in plasma renin activity, are seen in rats (Mouw et al., 1978b). The levels of lead exposure that causes chronic lead nephropathy with associated glomerular and vascular changes in man are not known. In rats, however, Fowler et al. (1980) observed that chronic lead exposure at 5 ppm for a month in males and 25 ppm in females produced nuclear inclusion bodies and increased number of iron positive granules within renal proximal tubular cells. Goyer (1971) has reported the same fact. Chang et al. (1980) has shown that the

organolead compound, tetraethyl lead, a gasoline additive, is just as nephrotoxic as inorganic lead compounds.

Relationships between cardiovascular diseases and elevated blood lead concentration have been suggested (Beevers et al., 1976). Electrocardiographic abnormalities observed in lead treated animals and in lead poisoned humans have been attributed to a disturbance in the function of the ANS (Hejtmanick and Williams, 1979; Webb et al., 1981). It has been postulated that lead plays a role in human hypertension also (Beevers et al., 1976; Morgan et al., 1966). Mouw et al. (1978b) demonstrated that acute intravenous administration of lead in anesthetized dogs causes an increase in plasma renin activity. In a later study, Goldman et al. (1981) demonstrated that lead may increase renin secretion in animals otherwise unstimulated to secrete, but the major mechanism for the short-term rise in plasma renin activity other than lead is the elimination of hepatic removal of renin. Lead also prevents angiotensin II from rising proportionately with plasma renin activity, presumably by inhibiting angiotensin-converting enzyme (Goldman et al., 1981). It is apparent that lead induced hypertension may not be related to the renin-angiotensin system. Williams et al. (1977) have demonstrated that rat pups exposed to lead during the neonatal period and subsequenly raised on a lead free diet for 4 months showed significantly more cardiac arrhythmias in response to norepinephrine. In a later study, Hejtmancik and Williams (1979) showed that, whereas bilateral vagotomy or atropine pretreatment decreased the frequency of cardiac arrhythmia, norepinephrine still caused significantly more extrasystolic lead exposed than in control rats. Isolated perfused hearts from lead exposed animals exhibited more irregularities in rhythm after norepinephrine then hearts from animals, indicating that some direct cardiac effects are also involved. Kopp et al. (1980) demonstrated significant metabolic changes in cardiac function after chronic low-level lead feeding in rats.

Another interesting aspect of lead toxicity is manifested by the immune system of the host. Prolonged exposure to lead has been shown to suppress the immune system and increase susceptibility to infection, which, in a normal condition, the host is able to withstand (Hemphill et al., 1971).

Seyle et al. (1966) demonstrated that a single normally well-tolerated intravenous injection of lead acetate increases the sensitivity of the rat to the endotoxins of various gram-negative bacteria about 100000 times above normal. Trejo et al.

(1972) demonstrated that, whereas lead acetate induces endotoxin hyperactivity by impairing the phagocytic as well as endotoxin detoxifying properties of the macrophages, hepatic parenchymal cell dysfunction might also be a contributing factor. Lead exposure in humans has been suspected to affect spermatogenesis (Lancranjan et al., 1975).

1.5.2 Cadmium

Evidence of a possible relation of cadmium to hypertension has been reviewed (Schroeder and Vinton, 1962; Schroeder et al., 1963; Schroeder, 1965; Perry et al., 1977; Balarman, 1986). This was based primarily on a series of experiments and autopsy studies showing increased cadmium or cadmium/ zinc ratios in the kidney of hypertensive animals and subjects. However, workers like Hammer et al. (1972) found no evidence of hypertension in workers exposed to cadmium containing superphosphate dust.

The induction of hypertension in animals by feeding cadmium was first reported by Schroeder and Vinton (1962). Certainly parenteral administration of cadmium could induce acute transient hypertension in animals (Schroeder et al., 1966; Perry and Erlanger, 1971b). During the next years, Schroeder extended his initial obervations in numerous reports (Schroeder, 1964; Schroeder and Buckman, 1967; Schroeder et al., 1968a; 1968b; 1970; Kanisawa and Schroeder, 1969a; 1969b). Perry and Erlanger (1971a) confirmed that chronically fed cadmium could raise systolic pressure. Schroeder et al., have reported very marked hypertension whereas Perry et al., could induce a mild elevation in blood pressure in experimental animals.

Schroeder et al. (1966) gave first report of chronic hypertension after the injection of cadmium. Cadmium acetate (2mg/kg, i.p.) was administered to female rats of the Long-Evans strain. Three weeks after a single injection of cadmium 31 percent of the rats exhibited elevation of blood pressure. When a second dose of cadmium 1mg/kg was given to the normotensives, all exhibited hypertension after one week. When cadmium was given intraperitoneally (2mg/kg) to another groups of rats with partial constriction of left renal artery, 71 percent of the rats showed severe hypertension. The interest in renal effects (aside from hypertension) arises mainly from two sources, the proteinuria of low molecular weight type after long exposure

to excessive concentrations of cadmium by inhalation., and the storage of cadmium in kidney in the form of cadmium binding proteins (CDBP) known as metallothionein (Friberg, 1971). The proteinuria is considered to be the first sign of tubular dysfunction and is said to occur when renal cortical levels of cadmium reach about 200 ppm (wet weight) compared to normal levels of about 50 ppm in adults (Friberg, et al., 1973). Renal cortical cadmium appears to rise from about zero at birth to 50 ppm in adults, followed by some decline after 50 years of age (Friberg, 1971). Zinc seems to parallel cadmium on a molar basis as regards storage in the kidney, but additional mobile zinc is also present (Friberg, 1971). Functional effects on the kidney have been examined. Infusion of cadmium chloride in dogs (plus cysteine to prevent effects on blood pressure etc) caused a drop in the sodium excretion and increased reabsorption of sodium in the proximal tubule. In addition to proteinuria, some cases have shown glycosuria; this has been thought to be of renal origin. Glucose tolerance curves in rats getting 17μg cadmium/ml of drinking water for 4 weeks (zinc and copper levels were varied) varied directly with serum and dietary zinc levels. Cadmium tended to lower serum zinc. Glucose tolerance was affected. Insulin levels also varied with the zinc levels. Increasing intake of zinc prevented these adverse effects of cadmium (Fassette, 1975). Rats given cadmium chloride i.p. at 1mg/kg daily for 45 days showed a drop in hepatic glycogen and increased blood glucose and urea levels. Four enzymes involved in gluconeogenesis appeared to increase in the liver and renal cortex. The changes persisted for a month (Fassette, 1975). It is difficult to interpret such studies in the cortex of human exposures, which thus far have not suggested any direct effects on carbohydrate metabolism.

Much attention is being given to CDBP because the nature of the binding appears to be of critical importance in the understanding of metabolism and in interpretation of toxic effects. It has been reported that cadmium chloride given intravenously in rats is excreted in the bile in proportion to the dose. The retention in liver is in proportion to the dose up to 1mg/kg; at higher doses biliary excretion predominates. Under these conditions, the biliary cadmium is in the form of glutathione complex, and cadmium in liver and kidney supernatants is bound to high molecular weight proteins. When CDBP were induced by injection of cadmium 24 hours previously, liver retention increased and biliary excretion

decreased. The cadmium was now bound to low molecular weight proteins in liver and kidney supernatants (Fassette, 1975). In other words, these studies show that induced synthesis or dosing with CDBP have profound but completely different effects on the distribution and excretion of cadmium. It is unlikely that correlations can be made directly with the quantities of cadmium present in damaged tissues, but that the nature and properties of the bound fractions are essential. In addition to the evidence for CDBP in vertebrates and invertebrates, such proteins are found in microorganisms.

It has been known for some time that cadmium effects can be profoundly altered or abolished by zinc, selenium and calcium in experimental studies, although with the possible exception of calcium, this has not been established for humans. Report of increased prostatic cancer in small numbers of exposed workers have not been followed by other reports of increased cancer in man from exposure. There was a low incidence of fibrosarcoma at the injection site; however, cancer was not related to long-term feeding of rats and dogs (Gunn et al., 1964). Friberg (1979) has very well explained the itai-itai episode the only large level example of cadmium toxicity in humans.

1.6 BIOCHEMICAL AND METABOLIC EFFECTS

1.6.1 Lead

The best-known effect of lead is the inhibition to some degree, of nearly all enzymatic steps that lead to heme synthesis (Stankovic, 1971). One of the primary reactions to lead is with immature erythrocytes (Albahary, 1972). Lead appears to increase their production and reacts both on the reticulocyte membrane and intracellularly. Nearly all of the blood lead is found in red cells. One clinical characteristics of plumbism is the appearance of basophilic stippled cells in both bone marrow and peripheral circulation. These contain mitochondria and resemble reticulocyte. The stippling is due to the agglutination of ribosomes (Jensen et al., 1965). Another way in which lead affects the metabolism of reticulocyte is by inhibiting the uptake of iron from transferrin. This is in addition to the more direct reaction of the ferrochelatase in effecting iron utilization (Moragan and Baker, 1969). There is also evidence that globin synthesis is inhibited in the presence of

lead, although it is unclear what is the minimal amount of lead required for this inhibition to begin and whether it is related to stippling, which involves ribosomes. The decrease in survival time of erythrocyte is noticeable although the exact cause is not known. The initial reaction in heme synthesis is the combination of succinyl-COA with glycine to form δ-aminolevulinic acid. This reaction is catalyzed by the enzyme δ-aminolevulinic acid synthase (ALA-synthase), which is a mitochondrial enzyme. One major source of toxicity of lead is the inhibition of this enzyme. However, the next step is a combination of the two δ-aminolevulinic acid molecules into porphobilinogen, which is catalysed by δ-aminolevulinic acid dehydrase and is inhibited to an even greater extent by lead, which occurs in cytoplasm. This inhibition causes urinary excretion and increase blood level of δ-aminolevulinic acid (δ ALA) (Fassette, 1975).

Brain is one of the major target organs where severe neurologic alterations may be triggered after exposure. The primary effects of lead on brain function are thought to be damage to the nervous system microvasculature. However, the mechanism of this toxicity is poorly understood. It is thought that, nitric oxide synthase may be a target for lead and changes in its functions can result in a cascade of pathophysiological effects observed by Garcia-Arenas et al. (1999) as evidenced in isolated capillaries and synaptosomes. It was observed that, dose dependent deposition of lead in capillaries and synaptosomes along with inhibition of nitric oxide production might be one of the main subcellular mechanisms of lead toxicity. This is probably because of ability of lead to displace the calcium from various calcium binding proteins such as calmoduline (Hoberman et al., 1983).

Mittal et al. (1995) have shown that brain cytosolic preparations preincubated with lead, cadmium or mercury produced significant inhibition of nitric oxide synthase. They suggested that while calcium modulates nitric oxide synthase activity, inhibitory influence of toxic heavy metals might be exerted on the catalytic site by direct binding or by interference with electron transfer during catalysis. There are reports (Cory-Slechta, 1995) showing that lead exposure produces learning impairment in rodents. This effect could be related to a specific inhibition of neuronal nitric oxide synthase as it s essential for hippo-campal long-term activation, a process frequently associated with learning in rodents (Xu et al., 1998). Similarly, Kronke at al., (1997) observed increase in nitric oxide synthase

activity, and a cause of cell toxicity, as it is enhanced in inflammatory deleterious processes.

1.6.2 Cadmium

The observation of resulting hyperglycemia and glycosuria in cadmium treated animals (Ghafghazi and Manner, 1973) encouraged studies on carbohydrate metabolism in liver and kidney cortex. Cadmium exposure produced histopathological changes, decreased glucogen levels and aldolase activity as well as an increase in hepatic phosphorylase enzyme (Stowe et al., 1972). Elevated levels of blood glucose and urea, and decreased contents of hepatic glycogen were observed in cadmium chloride exposed adult as well as neonate animals (Merali et al., 1975; Merali and Singhal, 1980). It was also showed that the levels of hepatic gluconeogenic enzymes were increased in a dose dependent manner. More pronounced changes were observed in various parameters in the neonatal rats exposed to cadmium from birth. However, withdrawal of cadmium exposure for 2 weeks could not restore the altered biochemical changes to normal limits (Singhal et al., 1974). Merali et al. (1975) found elevated levels of hepatic adenylate cyclase and cyclic AMP in cadmium treated animals, which may lead to stiumulation of gluconeogenic enzymes. Rastogi et al. (1975) reported increased synthesis of adrenal catecholamines responsible for activation of enzyme phosphorylase, resulting in enhanced conversion of glycogen stores into glucose. Further, Ghafghazi and Menner (1973) suggested the association of hyperglycemia with pancreatic function, as they observed reduced glucose tolerance, serum immunoreactive insulin concentration and insulogenic indices in cadmium treated mice. Merali and Singhal (1975) reported an increase in blood glucose and a decrease in resting insulin levels as well as in glucose stimulated serum immunoreactive insulin levels in cadmium-exposed rats. Furthermore, a smaller increase in phentolamine induced immunoreactive insulin levels in metal exposed animals compared with that of control animals indicated that reduced functional activity of pancreas was not the result of increased catecholamine release alone. A decreased *in vitro* release of insulin in response to high glucose concentrations was observed in islets isolated from cadmium treated rats (Merali and Singhal, 1980).

In experimental animals, cadmium produces profound effects on testicular tissue. It is believed that certain biochemical changes precede and may be responsible for the increased vascular permeability, which leads to hemorrhagic necrosis in the testes. Lee and Dixon (1973) reported reduced male fertility and thymidine uptake into spermatoginial cells after lower level cadmium exposure of animals. Cadmium altered cyclic AMP metabolism in both primary and secondary sex organs, and the changes persisted even 28 days after the withdrawal of metal exposure (Sutherland et al., 1974). It is known that cyclic nucleotides play an important role in the process of reproduction through hormonal regulation. However, the contribution of cadmium-induced changes in cyclic AMP metabolism is difficult to assess at present. Although, experimental data show a great deal of vulnerability of the male reproductive system to cadmium toxicity, the testicular necrosis has not been seen in human so far.

It has been found that neonatal animals are more susceptible to the neurotoxic effects of cadmium compared with adults. Rohrer et al. (1978) reported changes in cerebral vasculature of the foetus exposed to cadmium. Cadmium accumulates in adult brain although it penetrates the blood brain barrier with more ease in fetal rats. Greater concentration of cadmium was found in brains of newborn rats after intravenous administration indicating that the blood brain barrier to cadmium is not fully developed in the neonates (Wong and Klaassen, 1981). Rozear et al. (1971) reported decreased spontaneous neural firing after administration of the heavy metal into cerebral cortex or brain stem of cat. Further, *in vitro* addition of this metal was found to act as a synaptic blocking agent at both adrenergic and cholinergic synapses (Cooper et al., 1978).

Ribas-Ozonas et al. (1974) reported an increase in 5-hydroxytryptamine and 5-hydroxyindole acetic acid in different regions of rat brain following 60 min of a single intraventricular injection of cadmium. Shukla and Singhal (1984) have shown that the administration of 0.5mg cadmium/kg/day i.p. to 22 day old rats for 30 days was found to increase NA in the midbrain and pon-medulla, and 5-HT in the hypothalamus region without affecting dopamine levels. However, a dose of 0.1mg cadmium/kg did not produce any change in the levels of monoaminase but decreased GABA contents in the cerebellum and hypothalamus regions. In contrast the administration of 0.5mg cadmium/kg/day i.p. to adult rats has been reported to increase dopamine and decrease 5-HT levels in the whole brain (Shukla and

Chandra, 1981). Further, *in vitro* addition of cadmium inhibited synaptosomal uptake of ^3H dopamine and ^3H norepinephrine in a concentration dependent manner (Hobson et al., 1983). Exposure to cadmium has also been found to alter the behavioral responses to apomorphine in rats suggesting the existence of dopamine supersensitivity in metal exposed animals (Smith et al., 1983a). It was proposed that the cadmium-induced increase in spontaneous locomotor activity might be related to increased adrenergic activitiy. However, the precise mechanism by which cadmium alters behavior and produces neurotoxic manifestations remains to be elucidated.

1.7 OXIDATIVE STRESS

Oxidative stress is caused by exposure to reactive oxygen intermediates, such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) that can damage proteins, nucleic acids and cell membranes. Increasing evidence suggests that the cumulative damage caused by reactive oxygen species contributes to numerous diseases (Aruoma and Halliwell, 1998).

Recent studies also suggest that the effects of these oxidants are integrally linked to the damage caused by hypochlorous acid (HOCl) and the reactive nitrogen intermediates nitric oxide (NO), Peroxynitrite (HOONO) and Nitrosothiols (RSNO). To counter oxidative stress, cells constitutively express enzymes that detoxify the reactive oxygen species and repair the damage caused by them. In addition, bacterial, yeast and mammalian cells all have adaptive responses to elevate levels of reactive oxygen species and transduce the signal into increased expression of defense activities (Storz and Imlay, 1999).

Oxidative stress is an unavoidable by product of the aerobic lifestyle because of superoxide and hydrogen peroxide radicals, which are formed wherever molecular oxygen chemically oxidizes electron carriers. Reduced flavoproteine in particular have been implicated in this process. The flavin of NADH denydrogenase II is the primary site of electron transfer to oxygen in aerobic respiratory chain; contrary to expectation, little or no $O_2^{-\bullet}$ or H_2O_2 are formed by quinone oxidation or during oxygen reduction of the cytochrome oxidases (Storz and Imlay, 1999). This superoxide concentration is tolerable, about half what is necessary to diminish the activities of vulnerable enzyme and inhibit cell growth. Thus the defenses

maintained in the cell are calibrated to just avoid toxicity from endogenous oxidants. These defenses are inadequate, however, if the rates of intracellular $O_2^{-\bullet}$ and H_2O_2 formation are accelerated. $O_2^{-\bullet}$ and H_2O_2 have different chemical reactivity and generate distinct type of damage inside cells.

The cells that lack cytosolic superoxide dismutase cannot grow in air without amino acid supplements, cannot catabolize non-fermentable carbon sources and exhibit high rates of spontaneous mutagenesis (Carlioz and Touati, 1986).

Most of these phenotypes have been traced to a single type of injury, the oxidative inactivation of a family of dehydrogenase. These enzymes utilize exposed iron-sulfur clusters (4Fe-4S) to bind and dehydrate substrates; dehydratase oxidation by $O_2^{-\bullet}$ provokes cluster disintegration and a loss of enzyme activity (Flint et al., 1993).

The auxotrophy of superoxide dismutase mutants for branched-chain amino acids and their inability to catabolize non-fermentable carbon sources reflect the inactivation of dihydroxy acid dehydratase and of aconitase and fumarase respectively (Fridovich, 1995).

Although H_2O_2 can inhibit cell growth, the causal lesions have not been clearly demonstrated. H_2O_2 however, efficiently oxidizes enzyme thiols, and thus is likely to inactivate enzymes such as glyceraldehyde-3-phosphate dehydrogenase, that rely upon active site cysteine residues for catalytic function. H_2O_2 also reacts with adventious Fe^{2+} to form $HO^{-\bullet}$, a powerful oxidant that reacts at diffusion-limited rates with most biomolecules. Because iron can localize along the phosphodiester backbone of nucleic acids, DNA is a particular target of $HO^{-\bullet}$ and most of the cell death that occurs upon H_2O_2 exposure is probably due to DNA damage (Imlay and Linn, 1988). A wide variety of DNA lesions are formed (Aruoma and Halliwell, 1998). Since some of the base damage can result in miscoding, lesions formed by endogenous oxidants may be significant or even preponderant source of spontaneous mutagenesis in the aerobically growing cells.

Fortunately, the human body makes several important antioxidants. The most important are ubiquinol and glutathione. Enzymes such as superoxide dismutase, catalase and glutathione peroxidase also destroy free radicals. Antioxidants may act at different levels in the oxidative process by scavenging initiation of free radicals, binding metal ions, scavenging peroxyl radicals and removing oxidatively

damaged bio-chemicals. Some antioxidants must be provided as micronutrients; they include ascorbic acid, beta-carotene, and vitamin E and trace metals such as selenium.

1.7.1 Free Radicals in Tissue Injury

Free radicals have now been accepted into the biochemical and medical orthodoxy. For many years they were dismissed as either non-existent in biological systems or simply an unimportant curiosity. Some misgivings and misunderstanding persist, of course. The elusive nature of free radicals is probably responsible; other reactive short-lived biochemical entities exist but none so intangible as free radicals. Many of the substances (e.g. prostaglandin, histamine, catacholamines etc.), can be synthesized, stored, treated, prepared in solutions at specific concentrations, administered in experimental and even clinical situations and specifically measured in quantitative terms. Most free radicals (there are exceptions) are so short-lived that all of the above are exceedingly difficult, if not impossible.

A free radical can be defined as a chemical species possessing an unpaired electron. It can also be considered as a fragment of a molecule. As such, free radicals can be formed by three different ways: a) by homolytic cleavage of a covalent bond of a normal molecule, with each fragment retaining one of the paired electrons; b) by the loss of a single electron from a normal molecule; c) by the addition of a single electron to a normal molecule. The latter, electron transfer, is a far more common process in biological systems than is homolytic fission, which generally requires high-energy input from high temperatures, UV light or ionising radiation. Heterocyclic fission, in which the electrons of the covalent bond are retained by only one of the fragments of the parent molecules, does not result in free redicals but in ions, which are charged. Free radicals can be positively or negatively charged, or electrically neutral (Cheeseman and Slater, 1993). Writing it with a heavy superscript dot conventionally indicates the unpaired electron and the radical nature of a species. The process by which free radicals and ions are formed are illustrated below:

Radical formation by electron transfer

$$A + e^{-\bullet} \longrightarrow A^{-\bullet}$$

Radical formation by homolytic fission

$$X:Y \longrightarrow X^{\bullet} + Y^{\bullet}$$

Ion formation by heterolytic fission

$$X:Y \longrightarrow X:^{-} + Y^{+}$$

It can be a source of confusion that the electrons in one of the most important molecule in free radical biochemistry, oxygen, are distributed in such a way that two of the electrons are unpaired. Thus, oxygen is sometimes considered a di-radical. While the di-radical nature of the oxygen does enable it to react with many other free radicals, in general it reacts relatively slowly with non-radical species. When considering its reactions in the context of free radical biochemistry, it is usually easiest to simply consider it as a normal molecule that can readily add to free radicals or accept a single electron from them, while not being a free radical itself. Normally a balance between oxidative events and antioxidative forces maintains the *status quo* within living cells. A variety of enzymes help to maintain in a reduced state despite the presence of aerobic environment. Thus, major cellular reducing agents, such as ascorbate, glutathione and tocopherol are present predominantly in their reduced forms. In addition, a number of enzymes scavenge and remove these reactive chemical species. When normal balance is upset, either by loss of reducing agent or protective enzymes, or by increased production of oxidizing species, or by both events simultaneously, the tissue is considered to be *in* oxidant stress. An antioxidant is any substance that, when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate. The term oxidizable substrate includes almost everything found in living cells, including proteins, lipids, carbohydrates and DNA. The term reactive oxygen species is a collective one that includes not only oxygen centered radicals such as superoxide radical, singlet oxygen and hydroxyl radical, but also some potentially dangerous non-radicals like hydrogen peroxide. The ground state diatomic oxygen molecule (O_2) is itself radical with two unpaired electrons located in a π^* antibonding and is designed as Eg (indicating that ground

state oxygen exists as triplet molecule). The two unpaired electrons have the same spin quantum number (parallel spin), and so if oxygen attempts to oxidize another atom or molecule by accepting electrons from it, both new electrons must be at parallel spin to fit into the vacant spaces of π^* orbital. Most biomolecules are covalently bonded non-radicals and the two electrons forming covalent bond have opposite spin and occupy the same orbital, thereby forcing a spin and orbital restriction for its reaction with oxygen. Transition metals found at the active site of many oxidases and oxygenase, due to their ability to accept and donate single electron can overcome this spin restriction of oxygen.

1.7.2 Oxygen Free Radicals or Reactive Oxygen Species (ROS)

1.7.2.1 Superoxide radical

Arguably the most important free radicals in biological systems are radical derivatives of oxygen. Reduction of oxygen by transfer of a single electron to it will produce the superoxide free radical anion (Saran et al., 1989).

$$O_2 + e^- \longrightarrow O_2^{-\bullet}$$

When a single oxygen is accepted by the ground state O_2 molecule, it enters one of the π^* antibonding orbitals resulting in the formation of superoxide. Superoxide is formed in almost all aerobic cells, a major source being the respiratory burst of phagocytic cells, when they contact foreign particles or immune complexes. Evidence is accumulating that several cell types other than phagocytes, including lymphocyte and fibroblast, also produce superoxide *in vivo*. Superoxide produced by such cells is often thought to be involved in intracellular signaling and growth regulation. Some of the superoxide production in the cells is accidental e.g. the amount of 'leakage' onto oxygen of electrons from various components of the cellular electron transport chains, such as those of mitochondria, chloroplast and the endoplasmic reticulum. The rate of superoxide production rises, as the oxygen concentration increases. There are several reports on the generation of superoxide by vascular endothelial cells, especially so following ischemia-reperfusion. The

univalent reduction of molecular oxygen forming the superoxide anion radical also occurs from other normal biochemical oxidation reactions, both enzymatic (e.g. Xanthin oxidases) and non-enzymatic reactions (such as autoxidation of catecholamines). Superoxide anion radical is metabolized by the metalloenzymes superoxide dismutase (SODs) to form hydrogen peroxide and molecular oxygen (Cheeseman and Slater, 1993).

$$O_2^{-\cdot} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

1.7.2.2 Hydrogen peroxide

A two-electron reduction of oxygen would yield hydrogen peroxide.

$$2 + 2\bar{e} + 2H^+ \longrightarrow H_2O_2$$

Hydrogen peroxide is often generated in biological system *via* the production of superoxide: two superoxide molecules can react together to form hydrogen peroxide and oxygen.

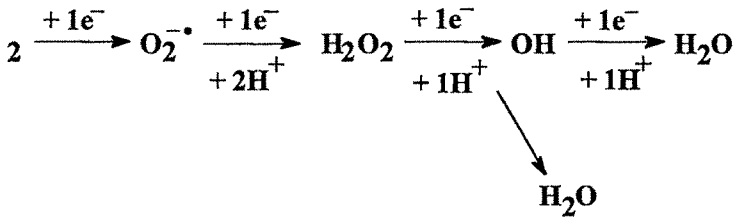
$$O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Because the free radical reactants produce non-radical products this is known as dismutation reaction. It can take place spontaneously (albeit rather slowly) or can be catalyzed by the enzyme superoxide dismutase. Hydrogen peroxide is not a free radical but falls into the category of 'reactive oxygen species' (ROS) that include not only oxygen free radical but also non-radical, oxygen derivatives that are involved in oxygen radical production (Cheeseman and Slater, 1993).

Hydrogen peroxide is very diffusible with and between the cells. Besides arising from superoxide, hydrogen peroxide is produced by the action of several oxidase enzymes (amino acid oxidase, xanthine oxidase) *in vivo*. Oxygen is simultaneously reduced both superoxide and hydrogen peroxide and can find some targets with cell at which they can do direct damage, although on the whole their reactivity is limited. Several metabolic roles of hydrogen peroxide are reported which includes the formation of thyroid hormone, gene expression controlled by NK_kB, induction of genetic expression of the provirus, human immunodeficiency virus-I and activation of human atherosclerotic lesions.

1.7.2.3 Hydroxyl radical

Much of the damage done by superoxide and hydrogen peroxide *in vivo* is thought to be due to their conversion into highly reactive oxidants, the major one being hydroxyl radical (OH). The hydroxyl radical is an extremely reactive oxidizing radical that will react with most biomolecules at diffusion controlled rates. It thereby will not diffuse a significant distance within a cell before reacting and has an extremely short half-life but is capable of causing great damage within a small radius of its site of production.



The above schematic relationship demonstrates the one electron transfer reaction between hydrogen peroxide and the oxy radicals. Hydrogen peroxide is an important compound in free radical biochemistry because it can rather easily breakdown, particularly in presence of transition metal ions, to produce the most reactive and damaging of the oxygen free radicals, the hydroxyl radical (•OH):

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\bullet}OH + OH^{-} + Fe^{3+}$$

The above reaction, probably more complicated than it is written here, is often referred to as the iron-catalyzed Haber-Weiss reaction. The non-catalyzed Haber-Weiss reaction is the reaction of superoxide directly with hydrogen peroxide.

$$2^{-\bullet} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^{-} + O_2$$

The spontaneous reaction is less likely in biological system due to low steady state concentration of reactant. The iron-(or copper) catalyzed reaction can still be considered to be dependent on superoxide as both the source of the hydrogen

peroxide (via dismutation as described above) and as the reductant of the transition metal ion.

$$O_2^{-\cdot} + Fe^{3+} \longrightarrow Fe^{2+} + O_2^{-\cdot}$$

$$O_2^{-\cdot} + Cu^{2+} \longrightarrow Cu^{+} + O_2^{-\cdot}$$

Ferrous (Fe^{2+}) [iron] and cuprous (Cu^{+}) [copper] are much more reactive with hydrogen peroxide than their oxidized counterparts, ferric (Fe^{3+}) and cupric (Cu^{2+}) respectively.

The autoxidation of reduced transition metals can also generate superoxide.

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^{-\cdot}$$

$$Cu^{+} + O_2 \longrightarrow Cu^{2+} + O_2^{-\cdot}$$

Thus the reaction of the transition metal ion with oxygen can be considered reversible redox reaction and are extremely important in the promotion of free radical reactions (Benedetto et al., 1981).

Formation of hydroxyl radical from superoxide requires traces of catalytic transition metal ion of which iron is the most important *in vivo*, although copper ions also plays an important role. In system containing hydrogen peroxide, superoxide and iron ion, reactive species additional to hydroxyl radical are probably also formed including perferryl and ferryl (Rayan and Aust, 1992). The hydroxyl radical is the most reactive of the oxygen radicals. It combines with almost all molecules found in living cells. It is so reactive that no enzyme system involving it as a substrate exists. The cell's efforts are directed at preventing its formation by removing hydrogen peroxide and moving transition metals to inactive sites (such as with the inner core of ferritin protein). Because of its reactivity, the hydroxyl radical does not travel far and has a half-life of a few microseconds. However, hydrogen peroxide can cross cell membranes and lead to hydroxyl radical formation at more distant sites.

The oxidative damage to the membranes of cell and organelles may be a crucial event in toxicity. In addition, there exists a large body of indirect evidence from *in vitro* studies involving isolated organelles (Bacon and Britton, 1990; Rice-Evans et al., 1989; Sevanian et al., 1990; Burkit and Gilbert, 1989), cell (Poli et al., 1987),

and tissue homogenates (Arthur et al., 1988) to suggest that oxidative damage due to hydroxyl radical formation is responsible for the toxic effect of iron.

1.7.2.4 Singlet Oxygen

Apart from superoxide and hydroxyl radicals, there are some other ways by which increased reactivity of oxygen have been noticed. This is by lifting one of the unpaired electrons to an orbital of higher energy with an inversion of spin. There are two forms of singlet oxygen, delta and sigma, corresponding to the first and second excited states respectively. Generally, the terms singlet oxygen ($^1O_2$) denotes the first excited state of singlet oxygen, $^1\Delta_2O_2$ owing to its relatively longer lifetime.

Singlet oxygen ($^1O_2$) is produced under various pathophysiological conditions in mammalian tissues. Excitation of oxygen to singlet state can be achieved when several pigments are illuminated in the presence of oxygen. Singlet oxygen formation is thus likely to be generated in tissues by photochemical reactions (photoexcitation) involving endogenous pigments and xenobiotics. Besides photo excitation reactions, singlet oxygen is also generated in the tissues by dark reactions (chemo excitation) consisting of enzymatic reactions and radical interaction.

1.7.2.5 Nitric oxide

Recently, the biological importance of nitric oxide (NO) has been appreciated. The radical has a structure similar to that of superoxide, except that it has two electrons less. It is widely thought that the endothelium derived relaxing factor (EDRF) produced by vascular endothelium, which is an important mediator of vascular responses induced by several pharmacological agents is nitric oxide. The free radical nitric oxide is synthesized from the amino acid, L-arginine, in the presence of nitric oxide synthase, by vascular endothelial cells, phagocytes, certain cells in the brain and many other cell types. Nitric oxide is very unstable species; under aerobic conditions it reacts with oxygen to produce intermediates such as NO_2, N_2O_4, N_3O_4, the stable products nitrates and nitrite. Nitric oxide acts as a

neurotransmitter, prevents platelet aggregation, and is a defense molecule of the immune system against tumor cells, parasites and bacteria. Nitric oxide can react with superoxide anion to generate peroxynitrite, which is a damaging species that can get protonated and undergo decomposition to hydroxyl radical, causing additional damage (McCord, 1991).

1.7.2.6 Hypochlorous acid

Although not a free radical, hypochlorous acid (HOCl) is a potent chlorinating and oxidizing agent. HOCl attacks primary anions and sulphydryl groups in proteins and may chlorinate purine bases in DNA. One of the most important targets attacked by HOCl is α-proteinase, which is the major inhibitor in body fluids.

Oxygen free radicals are not the only important free radicals in biochemistry, although they are often the initial species formed. Other free radicals of importance are the wide range of carbon-centered radicals ($R^{\bullet}$) that arise from the attack of an oxidizing radical (e.g. $OH^{\bullet}$) on a biological molecule (RH) such as a lipid, nucleic acid, carbohydrates or proteins. These react very rapidly with oxygen to form the corresponding peroxy radicals ($ROO^{\bullet}$). In turn, these peroxy radicals can participate in reactions that generate alkoxyl radicals ($RO^{\bullet}$). Sulphur atoms can also be the center for free radicals thiyl radicals, ($RS^{\bullet}$) formed, for example, in the oxidation of glutathione. In addition to these, certain foreign compounds can be activated to free radical species.

1.7.3 Production of Free Radicals in Cells

With the exception of unusual circumstances such as the influence of ionizing radiation, free radicals are generally produced in cell by electron transfer reactions. These can be mediated by the action of enzymes or non-enzymatically, often through the redox chemistry of transition metal ions.

Free radical production in animal cells can be either accidental or deliberate. Free radicals are at their active site in the process of catalysis; for e.g. ribonucleotide reductase (Stubbe, 1990; Reichard and Ehrenberg, 1983).

In these cases the free radical is not really 'free' at all and its reactivity is targeted towards a specific reaction. Activated phagocytes also deliberately generate superoxide as part of their bacterial role (Babior, 1978). Although the free radicals are produced only at the interface of the phagocyte plasma membrane and bacterium, some leakage of superoxide, hydrogen peroxide and other reactive oxygen species is inevitable.

Under normal circumstances, the major source of free radicals in cells is electron 'leakage' from electron transport chains, such as those in mitochondria and in the endoplasmic reticulum, to molecular oxygen, generating superoxide. Other enzymes can also produce superoxide or hydrogen peroxide, such as the range of flavin oxidases located in peroxisomes. Another source of superoxide in animal cell is the autoxidation of certain compounds including ascorbic acid (vitamin C), thiols (e.g. glutathione, cysteine), adrenaline and flavin co-enzymes. These autoxidation reactions can be greatly enhanced by the involvement of transition metal ions. This accidental production of free radicals is kept to a minimum by the high efficiency of enzyme-mediated electron transfer and by keeping metal ions tightly sequestered; these are the fundamental means of preventive antioxidant defense. Such precautions cannot be completely efficient and animals have evolved enzymic and non-enzymic antioxidant defenses to deal with the inevitable low-level production of free radicals during normal metabolic activity.

Free radical production in cells can be greatly increased by certain toxic foreign compounds. The classic example is carbon tetrachloride, the first compound to be shown to exert its toxicity through a free radical mechanism, being metabolized to the trichloromethyl free radical by the action of cytochrome P-450 in the liver (Slater, 1966; Cheeseman et al., 1985). The generation of reactive free radicals overwhelms the antioxidant defenses in the liver and results in the oxidative destruction of cellular membranes and serious tissue damage. Other examples of toxic compounds exerting their toxicity via the production of free radicals are now well established. Many of these are 'redox-cycling' compounds that readily accept an electron to form a free radical and then transfer it to oxygen, generating superoxide and thence hydrogen peroxide. The efforts of glutahione peroxidase to remove the continuously generated hydrogen peroxide result in the depletion of glutathione and allow oxidative damage to the cell (Ross, 1988; Ross and Moldeus, 1991). It is possible to postulate the involvement of free radical mechanism in the

toxicity of many compounds but caution must be exercised. In many cases, the free radical production may be secondary to the initial toxic mechanism, a consequence rather than the cause of cell damage.

1.7.4 Lipid peroxidation (LPO)

Lipid peroxidation (LPO) is defined as the 'Oxidative deterioration of polyunsaturated fatty acid (Tappel, 1973). While the chemistry of lipid peroxidation is well known with regard to the potential mechanism for radical formation (Firdovich, 1978; Mason and Chignell, 1982). LPO is thought to be an important biological consequence of oxidative cellular damage (Plaa and Witschi, 1976). Lipid peroxidation is a destructive free radical mediated process for biological membranes, which has been implicated in a variety of disease state. It involves the formation and propagation of lipid radicals, the uptake of oxygen, a re-arrangement of the double bond in unsaturated lipids that results in the variety of degraded products like alkenes, malondialdehyde, lipid hydroperoxide and conjugated diene and eventually destruction of membrane lipids. Biological membranes are often rich in the unsaturated fatty acids and bathed in an oxygen rich, metal containing fluid. Therefore, the membrane lipids are more susceptible to peroxidative attack. It has been associated with several physiological, pathological and toxic processes including prostaglandin biosynthesis (Fridovich, 1978), ageing (Hochstein and Jain, 1981) and many toxic reactions to various organs (Plaa and Witschi, 1976; Smith et al., 1983b).

Lipid peroxidation has been studied in liver, lungs, brain, spleen, kidney, blood, eye, uterus, adrenals, heart, testis and semen by many authors (Kornburst and Maris, 1980; Alvarez and Storey, 1982; Yagi, 1982; Dawra et al., 1983; Younse and Siegers, 1984; Devasagayam, 1986). The two major systems of lipid peroxidation in the liver are enzymatic and non-enzymatic lipid peroxidation. The enzymatic lipid peroxidation is mediated by the NADPH, cytochrome-c-reductase (Pederson and Aust, 1972) and non-enzymatic LPO is mediated by the transition metal ions like iron and copper (Ottolenghi, 1959). LPO in liver has been studied and the mechanism has been explained (Ottolenghi, 1959; Slater and Sawyer, 1971; Wills, 1969; Vladimirov et al., 1980; Younse and Siegers, 1984) and also been studied in mitochondria, lysosomes and microsomes (Tappel, 1973).

LPO has a profound effect on the macromolecules, RNA, DNA and other substances, which possess amino groups and reacts with malondialdehyde (Tappel, 1973). Nucleic acids are also damaged by direct reactions with lipid radical (Nielsen, 1981). The mechanism is thought to occur via single and double strand breaks in DNA, which leads to mutagenesis, carcinogenesis and cell death (Seholes, 1983; Kensler and Trush, 1984). It is thought that hydrogen peroxide interacts with metal ions on DNA causing site-specific damage due to hydroxyl radicals. The mutagenic consequences of ionizing radiations and oxidative DNA damage in bacteria include base substitutions at both A:T and G:C base pairs (Glickman et al., 1981; Levin et al., 1982). Ionizing radiation produces 60-70 percent breakdown of DNA strand, chromosomal aberrations, mutations and cell killing (Okada et al., 1983). Proteins have long been known to be responsive to oxidation by free radicals. Proteins are damaged by peroxidative reactions (Funes and Karel, 1981) or indirectly by secondary peroxidation products (Benedetti et al., 1981). The reaction of free radicals with proteins is the induction of autofluoresence with excitations and emission maxima in the region of 360 and 460 nm, respectively (Lunce et al., 1985; Lunce et al., 1987). The possibility of free radical involvement in the changes in diabetic persons have been studied by Stevens et al., 1978, who described the dependence of browning reaction upon the presence of oxygen and their inhibition by reductants.

Free radical induced oxidation of carbohydrates has been studied to an even lesser extent than free radical reactions with proteins. Sugars such as glucose, mannose and deoxysugars autoxidizes to produce hydrogen peroxide contributing to intracellular levels of 10^{-8} M. Oxaldehydes, which are produced from monosaccharide autoxidation, have been implicated in the pathogenesis of diabetes, cancer and chronic disease associated with the smoking of tobacco. Monosaccharide autoxidation may mediate protein cross linking, causing proteins aggregation and basement membrane thickening, resulting in the development of diabetic cataract and microangiopathy (Thornally, 1985; Von Santag, 1980). Fluorescent IgG complexes and fluorescent albumin complexes are also found in the sera of patients with diabetes mellitus. This fluorescence is generated by oxygen radicals but mediated by the covalent attachment of glucose to these proteins (Lunce et al., 1985). Oxygen radicals also cause fragmentation of carbohydrate polymers such as hyaluronic acid (Greenwald and Moy, 1980).

1.7.5 Role of Transition Metals in Oxidative Damage

Iron and copper ions in chemical forms that can decompose hydrogen peroxide to hydroxyl radicals are in very short supply *in vivo*. The human body is very careful to ensure that as much iron and copper as possible is kept safely bound to transport or storage proteins (transferrin, hemosiderine, ferritin, ceruloplasmin). This sequestration of metal ions is an important antioxidant defense. However, oxidative stress can provide iron for free radical reactions. Thus, superoxide can mobilize iron from ferritin, although the amount of superoxide releasable iron is small (Simpson et al., 1992). Hydrogen peroxide can degrade heme protein to release iron (Cotran et al., 1989). Cells also appear to contain a small 'low molecular mass pool of iron, which is a putative catalyst for hydroxyl radical formation. This intracellular iron can be released into the surrounding tissues as a result of cell lysis and may accelerate oxidative damage. Hence, a major determinant of the nature of the damage done by excess generation of reactive oxygen species *in vivo* may be the availability and location of metal in catalyzed hydroxyl radical formation. It has been argued that iron and copper have evolved in transport and storage proteins so as to safely sequester the metal ions that they transport to store into forms that are incapable of stimulating free radical reactions (Cheeseman and Slater, 1993).

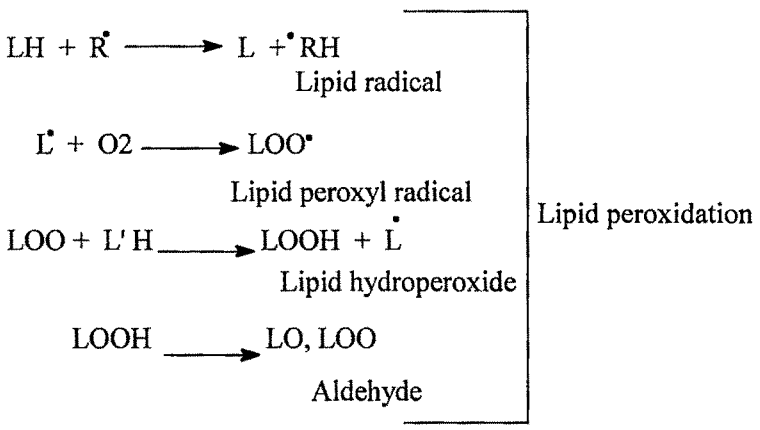
1.7.6 Damaging Reactions of Free Radicals

Oxygen free radicals are capable of reversibly or irreversibly damaging compounds of all biochemical classes, including nucleic acids, proteins, lipids, carbohydrates, and connective tissue micromolecules.

1.7.6.1 Lipid Damage

Lipids are by far the most susceptible targets for free radical attack. Cell membranes are rich source of polyunsaturated fatty acids (PUFAs), which are readily attacked by oxidizing radicals. The oxidative destruction of PUFAs, known as lipid peroxidation, is particularly damaging because it proceeds as a self-perpetuating chain reaction (Cheeseman and Slater, 1993). The general process of

lipid peroxidation can be envisaged as in the scheme below, where LH is the target PUFA and $R^{\bullet}$ the initiating, oxidizing radical. Oxidation of the PUFA generates a fatty acid radical ($L^{\bullet}$) that rapidly adds oxygen to form a fatty acid peroxy radical ($LOO^{\bullet}$). The peroxy radicals are the carriers of the chain reaction, they can oxidize further PUFA molecules and initiate new chains, producing lipid hydroperoxides (LOOH) that can break down to yet more radical species and to wide range of compounds, notably aldehydes (Porter, 1990; Esterbauer et al., 1991b).



The breakdown of lipid hydroperoxides often involves transition metal ion catalysis, in reaction analogous to that with hydrogen peroxide, yielding lipid peroxyl and lipid alkoxyl radicals. Aldehydes are always formed when lipid hydroperoxides break down and many of them are biologically active, particularly a class known as the hydroxynoneal (Esterbauer et al., 1988; 1990b). These compounds can diffuse from the original site of attack and spread the damage to other parts of the cell. In summary, lipid peroxidation is of particular significance as a damaging reaction consequent to free radical production in cell because; first, it is very likely to occur when enhances the availability and susceptibility of PUFA in membranes. Secondly, it is destructive chain-reaction that can directly damage the structure of the membrane and indirectly damage other cell components by the production of reactive aldehydes.

The detection and measurement of lipid peroxidation is most frequently cited to support the involvement of free radical reaction in toxicology and human diseases. Free iron (or certain types of ion chelates) may reduce hydrogen peroxide to the hydroxyl radicals, which initiate oxidation of PUFA by abstraction of a hydrogen

atom from the methylene group of the fatty acid. This abstraction of hydrogen ion can also be brought about by transition metal ions. This ultimately results in the formation of the lipid radical, which tends to stabilize by molecular re-arrangement to form a conjugated diene. The conjugated diene readily combines with oxygen to give peroxy radicals, which are capable of abstracting of hydrogen atoms from adjacent lipid molecule, propagating the chain reaction described above. Many of the products from the original site of attack various lipid molecules and spreads damages to other parts of the cell. Lipid peroxidation has been implicated in a wide range of tissue injuries and diseases (Cheeseman and Slater, 1993).

1.7.6.2 DNA damage

Oxidizing radicals if formed in vicinity readily attacks DNA. Free radical induced damage of the DNA in living organism may be due to breakage of the main strand, degradation of bases, or cleavage of hydrogen bond. All components of nucleic acids may be exposed to free radical damage, which may become permanent, or may be repaired by special mechanisms. Un-repaired damage in the bases may lead to mutation, while damage in the pentose part, to chain breakage. The detection of oxidized nucleic bases in human urine has been taken as an evidence for continual oxidation attack on DNA. When cells are subjected to oxidative stress, for example by generation of superoxide and hydrogen peroxide, by reactions with high external concentrations of hydrogen peroxide or by exposure to cigarette smoke, it usually causes DNA strand breakage. This could occur because the oxidant stress leads to activation of some specific DNA-cleavage mechanism, such as a calcium dependent endonuclease. Hydrogen peroxide can also react with intracellular metal ions to give hydroxyl radical which fragments the DNA by site-specific hydroxyl attack. Oxidant stress can also liberate iron ions from their sites of sequestration within the cell, so that they can bind to DNA. Recent studies show that $^1O_2$ is capable of inducing DNA damage. Its reaction with DNA results in single-strand breaks besides formation of the altered bases. The biological consequences associated with these modifications in DNA are the loss of biological activity as assessed by transforming ability as well as mutagenicity and genotoxicity (Fraga et al., 1990; Kasai and Nishimura et al., 1991).

1.7.6.3 Damage of Enzyme and Proteins

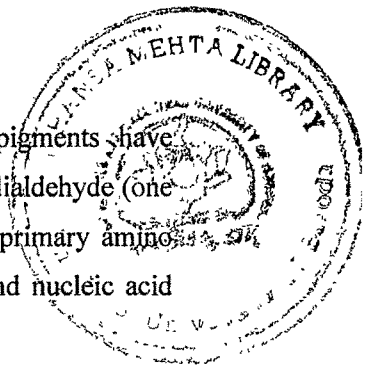
Metal catalyzed oxidation has been identified as a post translational covalent modification of proteins which may be important in several physiological and pathological process which include the aging process, intracellular protein turnover, arthritis and pulmonary disease. Proteins are also membrane constituents. Therefore, their damage explains the membrane damaging effects of free radicals, while the loss of specific activity of enzyme may also have severe consequences. Introduction of carbonyl groups into amino acid residues of proteins also results in loss of catalytic activity; loss of histidine residues of proteins also results in loss of residue and change in surface hydrophobicity. Oxidative damage results in the oxidization of -SH groups of proteins, cross-linking of proteins and peptide fragmentation. Hydroxyl radicals are capable of attacking many amino residues. Proteins often bind transition metal ions, making them a target for attack by site-specific hydroxyl radicals. As yet, convincing evidence of the involvement of free radical induced damage to proteins as an important mechanism in a tissue injury or disease process is lacking though oxidative damage to eye lens has long been suggested to be involved in cataract (Stadtman, 1990; Spector, 1985; Seccia et al., 1991; Simpson et al., 1992).

1.7.6.4 Damage to Carbohydrates

Sugars including glucose, mannitol and deoxysugars react readily with hydroxyl radical. Hyaluronic acid, which forms the central axis of protoglycans and maintains the viscosity of synovial fluid, is fragmented following exposure to free radicals resulting in destabilization of connective tissue and loss of synovial fluid viscosity.

1.7.6.5 Lipofuscin Pigments

Lipofuscin pigments accumulate in human and animal tissues as a result of aging. Recent studies have shown that they are lipid-protein complexes resulting from the peroxidation of PUFA of subcellular membranes and correspond to the so-called



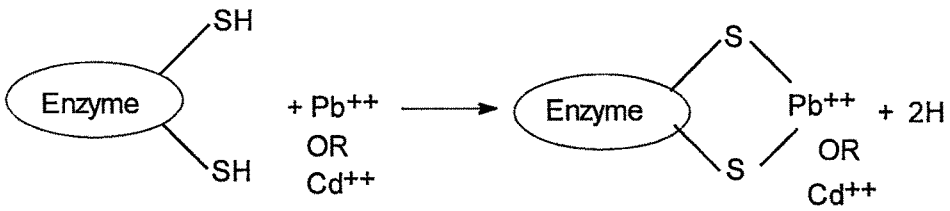
residual body, the end product of lysosomal digestion. The pigments have characteristic fluoroscent spectra. The schiff base product of malondialdehyde (one of the terminal products of lipid peroxidation) cross linking with primary amino groups of proteins, and with the amino groups of nucleic acids and nucleic acid bases, phospholipid has similar fluorescent characteristics.

1.7.6.6 Gene Activation

The ability of free radicals to activate the transcription is now recognized. The immediate early genes c-fos, c-myc, c-jun and beta actin are induced rapidly by oxygen free radicals, possibly through the induction of DNA strand breaks. These genes encode transcription factors, which participate in the induction of cell growth, differentiation and development. Free radicals have been also implicated in the activation of transcription factor, NK_kB, which is important for controlling the transcription of a member of cytokine genes.

1.7.7 Metals and Oxidative Stress

Metal ions (mainly copper and iron) are known to catalyze the cleavage of hydrogen peroxide and organic peroxides. Thus they play a major role in propagating free radical reactions and consequently in determining the degree of free radical pathology. Furthermore, metal complexes especially in a non-polar environment are particularly effective in the initiation of lipid peroxidation.
Transition metal ions such as Fe^{3+}, CO^{3+}, Ce^{4+}, Mn^{3+}, Cu^{2+} and Cd^{2+} are able to oxidize various substrates by one electron withdrawal to form cation free radicals. In general, toxic metals attack the active sites of enzymes and inhibiting essential enzyme function. Heavy metal ions, in particular Pb^{2+}, Cd^{2+}, Hg^{2+} and Ar^{2+} acts as an effective enzyme inhibitor. They have affinity for S-containing ligands especially $-SCH_3$ and -SH in methionine and cystein amino acids, which are parts of the enzyme structure.



Metalloenzyme contain metal in their structures. Their action is inhibited when one metal ion of a metalloenzyme is replaced by another metal ion of similar size and charges. Thus, zinc in some metalloenzymes is substituted by cadmium and lead, which leads to toxicity. The enzyme inhibited by cadmium includes adenosine triphosphate, alcohol dehydrogenase, carbonic anhydrase etc. Lead inhibits acetylcholine esterase, alkaline phosphatase, carbonic anhydrase and some of the key enzymes in the synthesis of heme (De, 1997). Recent studies (Stohs and Bagchi, 1995) have reported that the metals, including iron, copper, chromium and vanadium, undergo redox cycling; while cadmium, mercury, nickel as well as lead deplete glutathione, protein-bound sulfhydryl groups resulting in the production of reactive oxygen species as superoxide ion, hydrogen peroxide and hydroxyl radicals. As a consequence, enhanced lipid peroxidation, DNA damage and altered calcium and/or sulfhydryl homeostasis occurs. Fenton like reactions may be commonly associated with most membrane fractions including mitochondria, microsomes and peroxisomes. Phagocytic cell may be another important source of reactive oxygen species in response to metal ions. Thus the ability to generate reactive oxygen species by redox cycling quinones and related compounds may require metal ions. Metal ions may enhance the production of tumor necrosis factor alpha (TNF alpha) and activate protein kinase C as well as induce the production of stress proteins (Kohler and Eckwert, 1997).

1.7.7.1 Lead Induced Oxidative Stress

There are various reports suggesting the involvement of lead in initiation and propagation of free radical reactions. This is probably because of lead-calcium and lead-zinc interactions (Simons, 1993), its effect on secondary messenger system (Nathansan and Bloom, 1975), during autooxidation of delta amino levulinic acid (Monteiro et al., 1989) and its interference with calcium dependent secondary

messenger system that regulate the cell function (Goldstein, 1993), thereby showing the toxicity. Moreover, reports confirming the role of lead in induction of lipid peroxidation by producing various reactive oxygen species in various organs are highlighting another mechanism of lead toxicity. It is well known that lead induces toxicity in cells by accelerating iron dependent lipid peroxidation leading to cell death (Quinlan et al., 1988). These peroxides lead to cellular damage (Monteiro et al., 1989). The peroxides change the membrane structure restricting phospholipid movement and facilitate the propagation of peroxides (Quinlan et al., 1988). Generation of free radicals in organs causes changes in the levels of various enzymes, consequently the functional changes in enzymes. Intramuscular administration of single dose of lead acetate (100 μM/kg) in male rats showed marked increase in lipid peroxidation in liver and kidney. Increased levels of alkaline phosphatase (Alkp), acid phosphatase (Acidp), SGPT, proteins, cholesterol and triglyceride in serum and in organs indicating that lead may exert its toxic effects via peroxidative damage to renal and hepatic cell membrane after 24 hrs (Othmon and El-Missiry, 1998). Vaziri et al. (1999a) reported the induction of free radicals in rats after exposure to lead (100 ppm) through drinking water, increase in the levels of plasma malondialdehyde (MDA) after 12 weeks of exposure leading to oxidative stress. Similarly, Ding et al. (1998) have reported that rats exposed to lead (100 ppm for 3 months, p.o.) induced hypertension. This may be caused by one species of reactive oxygen species (ROS), which enhances vascular reactivity. They also reported marked increase in the levels of MDA in rat serum. Columbano et al. (1983) documented the mechanism of liver damage in male wistar rats with a single dose of lead nitrate; a marked enlargement of liver with cell proliferation characterizes it. This anatomic effect was accompanied by biochemical changes such as an increase in total proteins and DNA content within a maximum of 3 and 4 days respectively. An increase in DNA synthesis was found probably because of mitogenic stimulus, which occurred in parenchymal as well as non-parenchymal cells of liver, along with slight increase in SGPT levels. Gonick et al. (1997) reported elevation in MDA levels in liver and kidney after administration of 0.01 percent lead acetate for 3 months in rats. Endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) scavenge the free radicals, which are formed by oxidative

stress. During the scavenging reactions, the endogenous antioxidants get exhausted and their level starts decreasing. Increase in levels of MDA and decrease in the levels of SOD have been reported by various researchers in rats (He et al., 1998; Mylroie et al., 1986; Chaurasia et al., 2000) as well as in humans (Ito et al., 1985) after exposure to lead. Sakaguchi et al. (1982) studied the effect of lead acetate in rats and reported the stimulation of superoxide free radical release in liver, which led to depletion in the levels of SOD, GSH. This was associated with marked increase in LDH and Acid phosphatase. Similarly, Calderon Salinas et al. (1993) also reports inhibition of G-6-PD with an increase in erythrocytic lead levels. Lead (10 mg/kg) has a potential to decrease the free -SH groups and tryptophan levels in blood (Dudka et al., 1997).

Seven hepatic phosphatase were histochemically investigated in male rats pretreated with chronic sub-toxic doses of lead acetate. Lead increased the activities of Alkp, Acidp, Neutral phosphatase, adenosin monophosphate and G-6-PD activity markedly decreased and the activity of membrane bound sodium potassium ATPase was not altered. This could be an adaptation to the metabolic, structural and functional changes in the organelles of hepatic cells due to lead intoxication (Jarrar and Mohmoud, 2000).

Rats exposed to 100 ppm lead through drinking water for 20 days showed increase in the levels of serum GPT and GOT. Further, it was also observed that there was no significant change in distribution and toxicological symptoms in fasting or fed animals (Hayashi et al., 1993).

Hsu (1981) studied the effect of lead poisoning on glutathione metabolism in rats, receiving 0.5 percent lead acetate. Results showed the body weight gain, decreased hematocrit levels and hemoglobin value and increase in weights of liver, kidney, spleen and brain with significant decrease in protein content. No significant change in glutathione reductase and glutathione peroxidase was observed. Enlargement of liver and kidney with decreased protein content and protein / RNA ratio in rats was also reported by Dhar and Banerjee (1979).

Patra and Swarup (2000) have reported the significant reduction in the erythrocytic SOD, CAT and GSH levels after administration of lead acetate (7.5 mg /kg, b.wt.) in calves with a prominent increase in lipid peroxidation. It is also demonstrated that decrease in erythrocytic -SH content have also been found indicating a possible role of free radicals in pathogenesis of lead toxicity. Similar results have

been reported by El-Missiry (2000) in rats after intramuscular injection of lead acetate (10 mg/kg, b. wt.) daily for 7 days, which significantly abolished heme sysnthesis as evidenced by decreased blood hemoglobin and hepatic iron content. These effects were accompanied with marked elevation in hepatic lipid peroxidation and decreased enzymatic antioxidants such as SOD and CAT as well as non-enzymatic antioxidants such as total sulfhydryl group and glutathione. Furthermore, lead treatment caused hepatic deficiency in copper and zinc accompanied by a significant elevation of lead concentration in both plasma and liver, similar effects were also observed by He et al. (1998). Gurer et al. (1998) reported increase in lipid peroxidation and decrease in SOD levels in rats exposed to 2000 ppm of lead acetate via drinking water. Single intramuscular injection of lead acetate produced significant elevation of lipid peroxidation in liver and kidney. The antioxidant capacity of hepatic and renal cells in terms of the activities of SOD and GSH content was diminished confirming the induction of toxicity by lead via peroxidative damage to renal and hepatic cell membranes (Cothman and Ei-Missiry, 1998). Lead toxicity with single dose injection has been reported to cause oxidative consequences. A single intraperitoneal injection of lead acetate (200 mg/kg, b. wt.) increased the LPO in liver, kidney, heart and lung of Swiss mice (Acharya and Acharya, 1997). Rats exposed to 0.1 percent lead acetate in drinking water for 4 weeks showed prominent increase in lipid peroxidation and decreasing the levels of SOD in liver, kidney and blood of rats have been reported by Flora et al. (1993).

Skoczynska and Smolik (1994) reported that the combined exposure of lead (70 mg /kg, b. wt.) and cadmium (20 mg/kg. b. wt.) twice a week for 7 weeks to male Buffalo rats have shown significant increase in LPO and other lipids such as HDL cholesterol and significant decrease in SOD levels.

In vivo experiments stated above gave an idea about induction of oxidative stress, which was confirmed by *in vitro* experiments reporting the induction of oxidative stress by lead and cadmium in cultured cells by some researchers.

Ding et al., (2000) reported that lead induced hypertension is closely related to enhanced activity of reactive oxygen species. Formation of hydroxyl free radical during the experiments was considered to be a prime culprit in lead exposed animals. The experiment was carried out on rat aortic endothelial cells, which were incubated in the presence of 0.0, 0.01, 0.1, 0.5 and 1.0 ppm of lead for 1, 24 and 48

hrs. Lipid peroxidation products were measured as malondialdehyde- thiobarbituric acid and hydroxyl radical was measured as 2, 3-dihydroxybenzoic acid in the cells. There was significant increase in MDA-TBA after exposure, which clearly indicated release of hydroxyl radical and induction of oxidative stress. Another report by Kaji et al. (1992) showed that lead decreases the release of plasminogen activator antigen from cultured endothelial cells of human without non-specific inhibition of protein synthesis. Lead may stimulate the calcium dependent down regulation of endothelial cells; plasminogen activator antigen release by calcium or by mimicking calcium.

Eickhoff et al. (1995) have reported the interactions between lead and cadmium, the putative iron and redox dependent regulatory system. Proteus mirabilus expressed three superoxide dismutase activities, which depends on the levels of soluble iron and dioxygen in the cultured medium. Lead and cadmium decreased production of superoxide dismutase in liquid cultured and on solid medium.

1.7.7.2 Cadmium Induced Oxidative Stress

Chronic exposure to cadmium via food and drinking water is a major human health concern. The mechanism of cadmium-mediated toxicity has been the subject of investigations; although some uncertainties persist, sufficient evidence has emerged to provide a reasonable account of toxic processess. These involve two pathways, one for the initial injury produced by direct effects of cadmium and the other for the subsequent injuries produced by inflammation. Primary injury appears to be caused by the binding of cadmium to sulfhydryl groups as critical molecule in mitochondria. Inactivation of thiol groups' causes oxidative stress, mitochondrial permeability transition and mitochondrial dysfunction (Rikans and Yamano, 2000; Müller, 1986).

Cadmium induced lipid peroxidation and the activity of antioxidant enzyme after the administration of single dose of cadmium chloride (0.4 mg/kg, b.wt., i.p.) was studied in rat erythrocytes by Sarkar et al. (1998). They observed that cadmium intoxication increased the erythrocyte lipid peroxidation with decrease in SOD, CAT and GSH, indicating that cadmium intoxication induces oxidative stress and alters the antioxidant system resulting in oxidative damage to rat erythrocytes. A correlative study by Karmarkar et al. (1998) provides evidence that cadmium

administration to mice (2.5 mg /kg, b.wt.) induces duration dependent (102 percent) increase in lipid peroxidation in liver and bone marrow with decreased GSH levels. The decreased GSH catalyzed detoxification capacity suggests the clastogenic efficacy of this heavy metal. The effect of cadmium intake (100 μgm /kg b.wt./day) for the period of 3 months on the pro-oxidative-antioxidative state of liver was studied in 30 days old weaned rats by Krajcovicova-Kudlackova and Ozdin (1995). A significant increase in lipid peroxidation and decrease in levels of SOD was observed. Under specific conditions, cadmium can induce a pro-oxidant stage in biological systems resulting in the peroxidation of polyunsaturated fatty acids. Chevalier et al. (1994) investigated this phenomenon *in vivo* in major target organs of rats (12 and 36 week old), 24 hrs after being injected i.p. with a range of cadmium chloride doses (0 - 2.5 mg/ kg). The measurement of the thiobarbituric acid reactive substances demonstrated that the lung showed highest LPO in 12-week-old rats though the liver and kidney accumulated the greatest amount of cadmium. Hudecova and Ginter (1992) have also reported increased lipid peroxidation after exposure to cadmium (1 mg/animal/day in drinking water). The vital organs like liver, kidney, brain, lung and heart were found to be more susceptible to lipid peroxidation after exposure to cadmium (Manca et al., 1991; Yaragicoglu et al., 1999). Rats were administered intraperitoneally with various doses of cadmium (25-1250 μgm/kg). Lung and brain were found to be most responsive and liver displayed early responses following cadmium exposure along with decreased SOD, G-6-PD activities. These results suggest that LPO is an early and sensitive consequence of cadmium exposure. Shimizu and Morita (1990) reported that the acute oral toxicity of cadmium was enhanced in rats. On determination it has been found the increased SGPT activity after 24 hr exposure to cadmium along with depletion in glutathione and non-protein sulfhydryl in liver.

Some *in vitro* studies strongly supports the generation of oxygen species and modulation of endogenous antioxidant levels by cadmium. Primary cultures of oligodendrocytes were used to study the toxic effect of cadmium chloride (Almazon et al., 2000). The result showed that cadmium induced toxicity was dependent on time and dose of exposure. Treatment with metal ion caused a more pronounced reduction in intracellular GSH levels and significantly higher free radical accumulation in progenitors. Another study by Traore et al., (2000)

reported that, exposure of Caco-2 cells to cadmium for 24 hr inhibited the protein synthesis by 90 percent, while MDA production was increased 11496-pMol/mg proteins. In addition cadmium induced modified bases in DNA. The results strongly suggested that DNA damage resulting from oxidative stress induced by cadmium might significantly contribute to increasing their carcinogenecity via epigenetic processes. O'Brien and Salacinski (1998) have reported the production of hydroxyl radicals by cadmium, the evidence for release of free radical by cadmium. Manca et al. (1994) evaluated the role of cadmium in pulmonary toxicity after intraperitoneal injection of 50 to 1000 μgm of cadmium/kg. The authors have reported the dose-related non-linear evolution of total lung TBARS and total lung proteins as a function of increasing lung cadmium concentration.

Transition metal ions including selenium, cadmium, mercury, manganese, lead have been thought to disturb metabolism directly. Chae et al., (2000) demonstrated that MC3T3E3 osteoblast exposed to these metals generated massive amount of ROS thereby causing apoptosis. Fariss (1991) and Stacey et al. (1980) demonstrated the generation of free radicals and increase lipid peroxidation in rat hepatocytes. Rat hepatocytes exposed to cadmium in presence and absence of alpha tocopheryl succinate resulted in significant reduction in lipid peroxidation in the cells. Cadmium induced testicular lipid peroxidation is reported by Sugawara and Sugawara (1984).

1.7.8 Defenses Against Free Radicals

All the living cells are inevitably prone to oxygen toxicity. It is perhaps, not surprising that the cells likely to have developed a number of different protective measures to pre-empt or combat any free radical onslaught. These are known as antioxidant defenses. Antioxidants are compounds capable of providing free radicals with the electrons they are missing while remaining stable themselves, thus preventing a cascade of interactions that could create even more free radicals (Cotgreave et al., 1988).

Broadly, the possible mechanisms by which antioxidants may protect against ROS toxicity are: I) by preventing ROS formation, II) by intercepting the ROS attack by scavenging the reactive metabolites and converting them into less reactive

molecules and /or by enhancing the resistance ability of sensitive biological targets to ROS attack III) by facilitating the repair of damage caused by the ROS and IV) by providing (e.g. as a co-factor or by acting to maintain a suitable redox status) a favorable environment for the effective functioning of other antioxidants (Cotgreave et al., 1988). The human body a complex combination of enzymatic and non-enzymatic function to minimize the stress induced by ROS.

These antioxidants may be classified as:

Endogenous antioxidants: Those which are physiological in origin and

Exogenous antioxidants: Those are not be produced by the human body but may protect against pro-oxidant forces when administered as supplement (Sen, 1995).

All these antioxidants are enzymes or non-enzymes which can exists in both the aqueous and membrane compartments of the body.

1.7.8.1 Endogenous Antioxidant Defense

The antioxidant enzymes are major cell defense against acute oxygen toxicity. Their function is to protect membrane and cytosolic components against damage caused by free radicals. Superoxide dismutase, catalase, glutathione peroxidase, glutathione-s-transferase, xanthine oxidase are some of the more important antioxidants.

1.7.8.1.1 Superoxide dismutase

Superoxide dismutase (SOD) is found in all oxygen-consuming organisms (McCord et al., 1971) and in some aero-tolerant anaerobes (Talley et al., 1977). SODs are of three types depending on some obligate anaerobes (Hevitt, 1975). The copper zinc containing superoxide dismutases (CuZnSODs) are highly stable enzymes and thus can be easily isolated. They are found virtually in all eucaryotic cells such as yeasts, plants and animals but not generally in prokaryotic cells such as bacteria or blue green algae (Harries and Steinman., 1977).

Other two classes of enzymes are iron and manganese containing SODs. They are characteristic of prokaryotic and show a high degree of sequence homology (Steinman and Hill, 1973; Harris and Stainman, 1977). *In vivo* and *in vitro*

superoxide is cytotoxic. Superoxide dismutase catalyzes the superoxide radical by dismutation reaction.

1.7.8.1.2 Catalase

Catalase, a heme containing protein located in the peroxisomes of most tissues, removes hydrogen peroxide within the cells by catalyzing the breakdown of hydrogen peroxide. In animals catalase is present in all major body organs, being especially concentrated in liver and erythrocytes. Catalase consists of four protein subunits, each of which contains one molecule of NADPH bound to it, which helps to stabilize the enzyme (Kirkman and Gaetani, 1984). In erythrocytes it exists in a soluble state. It plays important dual role: firstly, a true catalytic role in the decomposition of hydrogen peroxide to water and oxygen and secondly, a peroxidic role in which the peroxide is utilized to oxidize a range of H donors (AH_2) such as methanol, ethanol and formate. In each case, an active enzyme-hydrogen peroxide complex is formed initially followed by an exceedingly rapid second state in which a second molecule of hydrogen peroxide serves as a H donor for the enzyme-hydrogen peroxide complex. The enzyme is mostly localized in peroxisomes (microbodies) of liver and kidney, and found in other cells in much smaller aggregates (microperoxisomes) (Sen, 1995).

1.7.8.1.3 Other Antioxidant Enzymes

Glutathione peroxidase (GPx) and Glutathione-s-transferase (GST) are amongst the remaining enzymes. GPx is tetramers with selenium (generally known as se-GPx) per molecule. GPx catalyses the reaction of reduced glutathione (GSH) to GSSG (oxidized form of glutathione) at the expense of hydrogen peroxides. GPx is present in all animal tissues, especially very much active in liver and moderately active in heart, lung and brain and very low activity in muscle. Non-se-GPx are generally present in cytosol has been demonstrated in mitochondria (Wahllander et al., 1979). GST is thought to play a physiological role in initiating the detoxification of potential alkylating agents (Booth et al., 1961; Wood, 1970). These compounds catalyze the reaction of such compounds with the –SH group of glutathione, thereby neutralizing their electrophillic sites and rendering the

products more water soluble. The cytosolic GSTs are mainly divided into four subunits: α, μ, π and θ. Within each class, similarities exist on the basis of structural and physical aspects and substrate selectivity (Meyer et al., 1991). Seven different GST have been isolated from the cytosol rat liver. The alpha class of GST must be important for the detoxification of many compounds in human liver. Along with these xanthine oxidase etc. may act as important parameters.

1.7.8.1.4 Glutathione (GSH)

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) is involved in many important cellular functions, ranging from the control of physicochemical properties of cellular proteins and peptides to the detoxification of xenobiotics and free radicals (Meister and Anderson, 1983; Tanigouchi et al., 1989). It also protects cells against the toxic effects of oxygen, by reacting directly or enzymatically with reactive oxygen species (intermediates), and less directly, by maintaining other compounds which have antioxidant activity, such as ascorbate and α-tocopherol, in reduced form (Meister, 1992).

The oxidation-reduction state of the reduced glutathione oxidized glutathione couple (GSH/GSSG) is of major importance in cellular metabolism since it is the largest mobile thiol redox system of the cell (Arias and Jacoby, 1976; Sies and Wendel, 1978). Cellular trans hydrogenase serves to maintain NADH and NADPH in equilibrium (Chance et al., 1965). The NADPH generating systems regulate the cellular concentration of GSH.

1.7.8.2 Exogenous Antioxidants

These are not produced by the body, but supplied externally and remains in body for much longer time and show antioxidant action.

1.7.8.2.1 Vitamin E (α-Tocopherol)

A wide range of literature suggests the usefulness of vitamin E. Amongst variety of usage, its antioxidant use is very important because vitamin E travels through the

body in molecules called lipoproteins and protects them from oxidation. It breaks the chain reactions of lipid peroxidation. This is important because lipid material plays a critical role in membranes, low-density lipoproteins, hormones and many tissues including nerves. Lipids are very susceptible to oxidation from free radical attack (Sherman, 2000). Vitamin E refers to a group of eight naturally occurring tocopherols: alpha, beta, gamma and delta and four tocotrienols with the same first Greek names (Packer and Landvik, 1990). Alpha tocopherol is the most abundant of the eight, and it is by far the most effective in supporting reproduction in its antioxidant effects. The other members of the family are also important. Tocotrienols retard the activity of liver enzymes that play a key role in synthesis of cholesterol. Gamma tocopherol is effective in fighting nitrogen-based free radicals (Sherman, 1997). Vitamin E is well accepted as the first line of defense against lipid peroxidation, protecting polyunsaturated fatty acids in cell membranes through its free radical quenching activity in biomembranes at an early state of free radical attack (Horwitt, 1986; Van Gossum et al., 1988).

1.7.8.2.2 Vitamin C (Ascorbic acid)

The hydrophilic ascorbic acid located in the aqueous phase cannot scavenge lipophilic radicals within the lipid reagents of the membranes and lipoproteins. Ascorbic acid reacts rapidly with O_2^-, $HO_2^\bullet$ and even more rapidly with $OH^{-\bullet}$ to give semidehydrous ascorbate (Nishikimi, 1975; Anbar and Neta, 1965).

Ascorbic acid acts as a synergist with tocopherol and functions as an antioxidant even when the oxidation proceeds (Niki, 1991). Tappel (1969) has also proposed that ascorbic acid plays a key role in restoring 'vitamin E pool' of the system by reacting with tocopheroyl radical. Vitamin E and vitamin C reacts rapidly with free radicals, and it is widely accepted that the antioxidant properties of these compounds are responsible in part of their biological activity (Packer et al., 1979). Vitamin E is considerably lipophilic than vitamin C and in biomembranes has been found to be the more potent antioxidants, particularly with respect to lipid peroxidation; penetration to a precise site in the membrane may be an important feature of the protection against highly reactive radicals. It is known these two vitamins acts synergistically, vitamin E reacting as the primary antioxidant and the

resulting vitamin E radicals then reacting with vitamin C to regenerate vitamin E. Enzymatic systems exist *in vivo* to reduces the NADP dehydro ascorbate to ascorbate which was in agreement with findings by Packer et al. (1979). In certain circumstances ascorbic acid acts as a prooxidant rather than as an antioxidant (Bendich et al., 1986; Halliwell, 1990). In fact, the iron ascorbate mixture has been used as an initiating system in *in vitro* experiments (Niki, 1991). Halliwell (1999) summarized the antioxidant roles of vitamin E and vitamin C as given in Table-1.2.

Table- 1.2: Actions of vitamin E and vitamin C as an antioxidant

ACTION	DETAILS
Scavenges radicals	Scavenges superoxide, hydroxyl, peroxy ($RO_2^{\bullet}$), thiyl ($RS^{\bullet}$), oxysulphur ($RSO^{\bullet}$, $RSO_2^{\bullet}$) and nitroxide ($RNO^{\bullet}$) radicals, drug derived radicals (from phenylbutazone), uric acid derived radicals, a nitrogen dioxide radical ($NO_2^{\bullet}$).
Scavenges non-radical reactive species	Scavenges singlet oxygen, hypochlorous acid, peroxynitrous acid (ONOOH), ozone (O_3), and nitrosating agents.
Inhibits lipid peroxidation	Inhibits lipid peroxidation in plasma / lipoproteins, activated neutrophils or cigarette smoke. Inhibits lipid peroxidation induced by haemoglobin, hydrogen peroxide or myoglobin hydrogen peroxide mixtures; ascorbate reduces the pro-oxidant haem ferryl (Fe[IV]) species (II) and also scavenges free radicals on the protein. Ascorbic acid also co-operates vitamin E: regenerates tocopherol from tocopheryl radicals in membranes and lipoproteins.

1.7.8.2.3 β- Carotene

Carotenoid pigments are widely distributed in nature where they play an important role in protecting cell and organisms. Vitamin A or β- Carotene quenches singlet

oxygen. It may act as an unusual kind of chain breaking antioxidant (Burton and Ingold, 1984).

1.7.8.3 *Spirulina* fusiformis

The blue green alga (Cynobacterium) *spirulina* is an oxygenic phototropic species. *Spirulina* (Oscillatoriaceae) appears as blue green filaments characterized by spiral shaped chains of cells (trichomes) enclosed in a thin sheath. The helical parameters (cell dimension, degree of ceiling and length of filaments) vary with the species and even within the species. The filaments are motile, gliding along their axis. The chemical composition of the algal biomass is the most important factor to evaluate its potential for utilization as feed or food. *Spirulina* has a long history of use as food. There are reports of its use by traditional people for over 1000 years. Recently, *spirulina* has attained the status of a health or given as food in many countries in view of its nutritional excellence. A better knowledge of this organism may encourage its exploitation as a source of food or feed and as a medicine (Kumar and Singh, 1993). Its safety as human food was established by toxicological studies sponsored by the UN Industrial Development Organization. In the past 15 years *spirulina* has gained worldwide acceptance. Studies have shown therapeutic effects of very low concentrations in the diet, suggesting factors other than nutritional ones (Belay and Yoshimichi, 1993). The chemical composition of *spirulina* is given in Table-1.2.

Table-1.2: *Spirulina* powder typical analysis (Dry powder).

GENERAL ANALYSIS					
Content			**Concentration**		
Protein			55-70 %		
Carbohydrate			15-25 %		
Fats (Lipids)			06-08 %		
Minerals (Ash)			07-13 %		
Moisture			03-07 %		
Fibre			08-10 %		
VITAMINS (per 10 gm)					
Vitamin A	23000 IU	Vitamin K	200 µg	B_3 Niacin	1.4 mg
Beta Carotene	14 mg	Biotin	0.5 µg	B_6 Pyridoxine	80 µg
Vitamin C	0 mg	Inositol	6.4 mg	Folate	1 µg
Vitamin D	1200 IU	B1 Thiamine	0.35 mg	B_{12} Cobolamine	20 µg
Vitamin E	1.0 mg	B2 Riboflavin	0.40 mg	Pantothenic cid	10 µg
MINERALS (per 10 gm)					
Calcium	70 mg	Zinc	0.3 mg	Chromium	25 µg
Iron	15 mg	Selenium	10 µg	Sodium	90 mg
Phosphorous	80 mg	Copper	120 µg	Potassium	140 mg
Magnesium	40 mg	Manganese	0.5 mg	Germanium	60 µg
NATURAL CAROTENOIDS (per 10 gm)					
Beta carotene	21 mg	Cryptoxanthin	01 mg		
Other carotene	04 mg	Echinenone	01 mg		
Myxoxanthophyll	09 mg	Other Xanthophylls	03 mg		
Zeaxanthin	08 mg		**Cntd...**		

AMINO ACIDS (per 10 gm)			
ESSENTIAL AMINOS			
Isoleucine	350 mg	Phenyl alanine	280 mg
Leucine	540 mg	Threonine	320 mg
Lysine	290 mg	Tryptophan	90 mg
Methionine	140 mg	Valine	400 mg
NON-ESSENTIAL AMINOS			
Alanine	470 mg	Glycine	320 mg
Arginine	430 mg	Histidine	100 mg
Aspartic acid	610 mg	Proline	270 mg
Cystine	60 mg	Serine	320 mg
Glutamic acid	910 mg	Tyrosine	300 mg

NATURAL PHYTONUTRIENTS (per 10 gm)		
Gamma linolenic acid	(Essential fatty acid)	130 mg
Glycolipids	(Lipid)	200 mg
Sulfolipids	(Glycolipid)	10 mg
Polysaccharide	(Carbohydrate and Sugar)	460 mg

NATURAL PIGMENT POLYNUTRIENTS (per 10 gm)		
Phycocyanin	Blue colored pigment	1400 mg
Chlorophyll	Green colored pigment	100 mg
Carotenoids	Orange colored pigment	47 mg

1.7.8.3.1 Nutritional Studies of *Spirulina*

From the nutritional point of view, although somewhat inferior to the protein of animal origin, *spirulina* protein is one of the best protein sources of plant origin (Becker, 1978; Venkataraman and Becker, 1982). Becker (1978) has summarized the standard parameters used to evaluate the nutritional quality of protein source. Thus there is an even possibility of *spirulina* being used as a food, material by the people of those countries who suffer from protein malnutrition. Different physical and chemical treatments did not affect digestibility of *spirulina*, which ranged between 73 and 78 percent (DeHernandez and Shimada, 1978). *Spirulina* fed rats showed a three-fold increase in lactobacillus content and 43 percent in vitamin B_1

level in the caecum. Protein regeneration studies with algal proteins at 10 to 15 percent levels showed increased body weight (Anusuya Devi et al., 1979). Rats fed on *spirulina fusiformis* supplemented diet showed a significant increase in hemoglobin levels and serum iron levels compared to control (Kapoor and Mehta, 1991). Nutritive supplementation with *spirulina fusiformis* at 1.27 percent level for vitamin A and at 2.74 percent level for riboflavin have shown considerably increased liver stores of vitamin A in the treated rats. National Institute of Nutrition studied (wistar rats) on the efficacy of *spirulina,* as a source of vitamin A and riboflavin in showed is to be superior to the pure substance vitamin A (Annapurna et al., 1991a). *Spirulina* feeding through tubes to malnourished adults showed positive nitrogen balance with no harmful effects (Santier, 1978). Positive results were reported in studies on feeding malnourished children with *Spirulina* supplemented diets for different periods (Santier and Tremolieres. 1976; Fox, 1986). *Spirulina fusiformis* was given as a source of vitamin A over a period of thirty days at 2 g/day level. The study concluded that the bioavailability of carotenes from *spirulina* was comparable to other sources (Annapurna et al., 1991b).

The vitamin content of *spirulina* especially beta-carotene, acts as a potent antioxidant and the presence of the enzyme superoxide dismutase enriches the antioxidative property as well.

1.7.2.3.1 Pharmacological Applications of *Spirulina fusiformis*

Spirulina fed rats showed an improved (60 percent) absorption of iron as compared to those fed with iron supplements alone (Johnson and Shubert, 1986). Areas of improvement with *Spirulina* in humans include obesity (Becker et al., 1986); pancreatitis, hepatitis, constipation etc (Jassby, 1988) and as an antioxidant (Shastri et al., 1999; Murugan, 1995). Nakaya (1986) and Nakaya et al. (1988) showed cholesterol lowering effect of *spirulina* (4.2 gm/day) in eight week time in atherosclerosis. Yang et al. (1997) investigated the effect of the powder of *Spirulina* (0.5 to 1.0 mg/kg) on compound 48/80 induced anaphylactic reactions. *Spirulina* reduced the serum histamine levels by 68.7 percent as well as passive cutaneous anaphylaxis activated by anti-dinitrophenyl IgE. *Spirulina* had a significant effect on anti dinitrophenyl IgE induced histamine release or tumor

necrosis factor alpha production. This implies that *Spirulina* may contain some compounds with mast cell degranulation action in rats. In another study, *Spirulina* (1 gm/day for 12 months) showed the chemopreventive action in patients suffering from Pan and Tobacco induced oral cancers (Mathew et al., 1995), especially in homogenous leukoplakia, erythroplakia, verrucuous leukoplakia etc. Kato and Takemoto (1987) reported the protective action of *Spirulina* in rats. Rats were fed basal diets containing 1 percent cholesterol and 16 percent *Spirulina.* Their results indicate that *spirulina* might prevent dietary hypercholesterolemia and arteriosclerosis. Fatty liver caused by high fat and high cholesterol diet was also reduced rapidly by feeding *Spirulina.*

Phycocyanin from *Spirulina* has been used as a superior source of natural colorent (Jassby, 1988) and *Spirulina* based (5 percent) ointment brought about quick wound healing. *Spirulina* has found wide application in the cosmetic industry in Japan as a base in ointments, creams, lotions etc (Venkataraman, 1989). Moreover, *Spirulina* is devoid of any toxicity in any animals and in humans (Shastri et al., 1999; Becker and Venkataraman, 1982).

1.7.9 Exogenous Antioxidants in Metal Induced Oxidative Stress

The primary site for the generation and propagation of oxygen derived free radical reactions is the lipoidic layer of the cells.

For an effective protection against oxidative abuse that we encounter in our daily lives, regular consumption of at least some antioxidants in the diet or as supplement appear to be very crucial. Among the exogenous antioxidants, vitamin E and vitamin C have been recognized to be especially important and deficiency of these may lead to number of pathological consequences. The term vitamin E is a generic description for all tocopherol and tocotrienol derivatives that qualitatively exhibit the biological activity of α-tocopherol.

1.7.9.1 Vitamin E

α-tocopherol is a lipid soluble molecule, which functions as the most important lipid peroxidation chain breaking stabilizer. α-tocopherol is believed to act as an

antioxidant, protecting membrane-bound polyunsaturated fatty acids (PUFA) and other oxygen sensitive substances, such as vitamin A and vitamin C, from oxidation. It is thought that polyunsaturated fatty acids can be damaged by free radicals (Cheeseman and Slater, 1993), which are generated by many metabolic processes. Free radicals are known to initiate peroxidative chain reactions of unsaturated cell membrane lipids. This reaction between the membrane bound lipid and free radical disrupts cell membrane integrity. Vitamin E is thought to prevent this damage by reacting with free radicals before they bind with cell membrane lipids. Peroxyl and alkoxyl radicals formed during lipid peroxidation preferentially combine with an -OH groups (containing an easily replacable hydrogen atom) attached to hydrophobic structure of the α-tocopherol molecule. In this way, the chain reaction of lipid peroxidation is terminated and vitamin E circumvents the oxyradicals dependent cascade destruction of membrane (Packer and Landvik, 1990).

Animal data have shown that vitamin E is important in not only reproduction, but also for muscle integrity and to protect the liver from injury. Vitamin E may decrease the incidence of red blood cell hemolysis in patients with G-6-PD deficiency. Since vitamin E is structurally similar to one form of coenzyme Q, these compounds share the biologic activity in several enzyme systems. Duval and Poelman (1995) studied free radical effect of vitamin E (0.1 percent) using an original *in vitro* method consisting of free radical production by photoradiation of pheomalanin. Their results showed a significant decrease in the free radicals production. Patra et al., (2001) have reported the beneficial role of vitamin E and vitamin C on lead induced (1 mg/kg, i.p.) oxidative changes in rats. Vitamin E and vitamin C administration (100mg/kg, p.o.) to lead exposed rats for 5 weeks resulted in significant decrease in lipid peroxide levels and non-protein bound thiol contents in liver and brain. Similarly, vitamin E has been reported to reduce the cadmium induced oxidative stress in liver of rats (Krajcovicova-Kudlackova and Ozdin, 1995). An *in vitro* study (Waren et al., 2000) reported numerous mechanisms for cadmium toxicity, one of which is oxidative stress. Vitamin E has been found to have antioxidant and cytoprotective properties in cultured epithelial cells. Vaziri et al. (1999a) reported an increase in lipid peroxidation in aorta and kidney of rats exposed to lead (100 ppm) via drinking water. Vitamin E given as

fortified chow for 12 weeks significantly protected the aorta and kidney from lead induced lipid peroxidation. In another experiment Vaziri et al. (1999b) reported the protective role of vitamin E against lead induced oxidative stress in kidney, heart, liver and brain of rats. Further, they also reported the beneficial effects of high dose of vitamin E on blood pressure, tissue nitrotyrosine burden and urinary nitric oxide metabolites. Lead is reported to cause thyroid dysfunction by inhibiting the type-I iodithyronine 5' monodeidodenase activity in liver of mice (Chaurasia and Kar, 1997). In this report it is further reported that lead significantly increased the lipid peroxidative reactions and decreased the levels of SOD and CAT activity. Simultaneous administration of vitamin E (5 mg/kg) restored the SOD and CAT activities to normal with significant decrease in lipid peroxidation, a univocal cytoprotective property of α-tocopherol succinate in hepatocyte exposed to cadmium. Rat hepatocytes suspension was exposed to toxic concentrations of cadmium in presence and absence of vitamin E. The exogenous administration of vitamin E completely protected the hepatocytes from toxic manifestation of cadmium. Levander et al. (1977) explained the necessity of vitamin E treatment in lead exposed rats. The observations show that lead poisoning via drinking water (250 ppm) increased osmotic and peroxidative fragility of RBCs and vitamin E administration protects the RBCs.

1.7.9.2 Vitamin C

Ascorbate is an essential enzyme cofactor but is often regarded as an important antioxidant *in vivo*, protecting against cancer by scavenging DNA damaging ROS. Vitamin C has an important role in metabolic activities of body. It acts by reducing transition metal ions (iron and copper), which can be present, either at the active sites of enzymes or as free ions. Ascorbic acid is a good reducing agent. The initial products of ascorbate oxidation by many of these species is the semidehydroascorbate radical, a poorly reactive radical that can either be reconverted to ascorbate by NADH dependent enzymes or undergo disproportionation to form dehydroascorbate. This dehydroascorbate either decompose irreversibly or is recycled to ascorbate by glutathione dependent enzymes. Thus ascorbate converts ROS into dependent reactive ascorbate derived

products and thereby acts as an antioxidant that can protect biomolecules against damage by such species, *in vivo*.

In recent years, more attention has been paid to the antioxidant properties of ascorbate than its co-factor roles. Many articles in the literature have suggested that ascorbate intake higher than the RDA (recommended daily allowance) are necessary for optimization of this antioxidant role and maximum protection against the development of some chronic diseases.

Berger et al. (1997) summarized a detail report that vitamin C acts as an antioxidant or a pro-oxidant *in vitro* depending on the absence or presence, respectively, of redox active metal ions. Some others have reported that vitamin C acts as an antioxidant, not as pro-oxidant in plasma containing excess of metals (iron). It has also been reported that vitamin C is very effective in mobilizing the metals (lead) from blood, liver and kidney into urine and/or feces (Tondon and Singh, 2000). Vitamin C decreases the deposition of lead and cadmium in liver, kidney and bone (femur) (Dalley et al., 1989) and decreases the retention and toxicity of lead and cadmium (Flanagan et al., 1982). Hudecova and Ginter (1992) have reported the protection by vitamin C (100 mg/animal/day) against cadmium (2 mg/animal/day) induced lipid peroxidation in liver, kidney and serum of guinea pig. Hill (1980) suggested that ascorbic acid interacts with several elements in such a manner as to render them less available for animals.

It was Retsky et al. (1993) who documented that vitamin C gets oxidized when incubated with metals (copper) leading to formation of dehydro-L-ascorbic acid. Remarkably, dehydro-L-ascorbic acid protects the biomolecules like LDL more effectively against metal induced lipid peroxidation. Two mechanisms were elucidated from the vitamin C protection action on LDL against atherogenic modification these are: Firstly by scavening free radical to prevent the aqueous oxidants to attack and oxidize the LDL and secondly form a stable modification of LDL by dehydro-L-ascorbic acid or decomposition products, thereof imparts increased resistance to metal ion-dependent oxidation.

1.7.9.3 *Spirulina*

Spirulina, a member of the family Oscillatoriaceae, is multi-cellular, filamentous and non-heterocytous blue green algae. Since the isolation in 1827 of the first

species, *Spirulina Oscillarioides*, from fresh water stream (Turpin, 1827), species of *spirulina* have been reported from diverse environments such as brackish water, Tidal pools, saline ponds, subartic water, tropical lagoons and hot springs. *Spirulina* appears to be ubiquitous in distribution and capable of adapting to extreme environmental conditions. It has a long history of human usage. Two independent reports which appeared in France (Dangeared, 1940) and in Belgium (Leonard and Compare, 1967) indicate that dried *S. pletensis* was consumed by the native populations of Kanem tribe, living in the vicinity of Lake Chad, in Central Africa. Even today the algae, in the Kanem region in Chad is collected, sun dried and sold as a vegetable in the market under the name 'dihe'. The same alga also formed part of the diet of the Aztecs in Mexico.

1.7.9.3.1 Antioxidant Effects of *Spirulina fusiformis*

A wide range of pharmacological and therapeutic action of *spirulina* is reported in literature. It has been reported that alcohol and water extracts of *spirulina* effectively inhibit lipid peroxidation induced by ferrous sulphate and ascorbic acid in erythrocyte membranes (Manoj et al., 1992). Lumsden and Hall (1974) reported that *spirulina* contains one major and two distinct minor soluble SOD activities (Cyanide-insensitive). The major isoenzyme was purified and shown to contain iron. These SODs activity was found to bind to particles with chlorophyll, which might be responsible for the antioxidant action. Protective effect of *spirulina* on carbon tetra chloride and d-galactosamine induced oxidative stress which was characterized by an increase in lipid peroxidation and decrease in endogenous antioxidants, membrane bound enzymes in liver and kidney (Murgan, 1995). *Spirulina* is naturally a rich source of SOD (8.1 units/mg protein wet material) (Venkataraman, 1992).

The modulatory effects on lead toxicity by *spirulina* fusiformis were observed on the testes of Swiss albino mice at a dose of 800-mg/kg body weights. The *spirulina* was non-toxic at the dose given. A significant enhancement in the survival time was observed in the pre- and post-treated *spirulina* group as compared to lead treated group. Lead induced toxicity was also reduced in terms of testes weight, animal weight, and tubular diameter in the pre-*spirulina* treated group. These modulatory effects of *spirulina* may be attributed to the presence of the

antioxidants, β-carotene and SOD enzyme (Shastri et al., 1999). It is well known that heavy metals decrease DNA synthesis (Kacew and Singhal, 1980). *Spirulina* has been found to increase the rate of DNA synthesis (Berthold et al., 1995). The antioxidant property of *spirulina* is very well documented (Shastri et al., 1999; Henrikson, 1989).

1.7.10 Free Radicals in Human Disease

With the increasing acceptance of free radicals as commonplace and important biochemical intermediates, they have been implicated in a very large number of human diseases. There is a real danger, however, that the pendulum has swung too far and that free radicals are now sometimes being implicated without sufficient reason except that no other mechanism of pathogenesis can be elucidated. This situation has arisen, as a consequence of the difficulties of detecting and measuring free radicals. Their life is for microseconds and is extremely difficult to measure *per se*, not least in the clinical situation. Consequently, free radical ablation offers a substantial potential for the treatment of human diseases. This is because many constituents of the cell are potentially subjected to free radical attack (Mead, 1976). A frequent cellular target is the lipid component of membrane, resulting in lipid peroxidation. Proteins are also subjected to free radical mediated denaturation, which may lead to structural loss or enzymatic deactivation. Nucleic acids are prone to base hydroxylation, cross-linking or strand scission, which may result in mutation or even cell death. Extracellular tissue components, including hyaluronic acid and collagen, are also vulnerable to injury by toxic oxidants. This may compromise the architectural integrity of the tissue such as basement membrane of blood vessels and epithelia. The ubiquity of damage associated with toxic oxidants illustrates the vulnerable nature of cells and tissue injury yields many levels for potential intervention. They can be classified into five areas: a) blockade of the initial generation of toxic oxidants, b) scavenging oxidants after their generation, c) blocking the chain propagation of secondary oxidants, d) enhancing the endogenous antioxidant capability of the target and e) blocking the secondary generation of toxic metabolites and/or inflammatory mediators.

In recent years, reports confirming the damaging role of the reactive oxygen species in various diseases to human beings and animals, inflammation (Winrow et al., 1993), cancer (Guyton and Kensler, 1993), aging (Nohl, 1983), ischemia (Flitter, 1993), diseases of central nervous system (Evans, 1993), diabetes (Wolff, 1993), liver damage (Poli, 1993), atherosclerosis (Esterbauer et al., 1993), modification of LDL (Steinbrecher, 1987) and many more have been published.