CHAPTER I

INTRODUCTION

MITOCHONDRIAL STRUCTURE AND FUNCTION

Mitochondrial respiration and ATP synthesis are two pathways lying at the heart of the metabolism. Respiration, broadly speaking, consists of the oxidation of mitochondrial NADH by O_2, which is coupled by the electron transport chain (ETC) to the pumping of protons out across the mitochondrial inner membrane, generating an electrochemical gradient of protons, consisting of a membrane potential and pH gradient [1]. Electron transfer from respiratory chain substrates to molecular oxygen results in the vectorial translocation of protons and this protonic energy is utilized for various cellular processes such as ATP synthesis, ion translocation, protein import etc. The ATP synthase couples the transport of protons across the membrane to the synthesis of ATP inside the mitochondrial matrix and this transport of electrons is catalyzed by discrete multisubunit enzyme complexes located in the mitochondrial inner membrane [2].

Mitochondria are the second largest organelles in the cell and on an average measure around 0.5 to 1.0 μm in diameter and 1.0 to 10 μm in length. The number of mitochondria per cell varies depending on the cell or tissue type; for example, sperm and yeast cells have only a few large mitochondria, while the egg cells contain many thousands. Liver cells on an average contain about 1000 mitochondria [3].

The mitochondria are enclosed by a bilayer membrane system consisting of an outer and an inner membrane; in between these is an intermembrane space while the inner membrane encloses a region known as the matrix. The inner membrane has numerous infoldings: the cristae; their number varies depending on the type of tissue from which the mitochondria are obtained. Kidneys and heart which carry out a lot of external work (osmotic and mechanical, respectively) have a greater number of cristae as against liver, which does not carry out much external work [4].

The mitochondrial inner membrane is composed of 70% proteins and 30% lipids, mostly phosphatidylcholine (PC)-40%, phosphatidylethanolamine (PE)-35% and diphosphatidylglycerol (DPG)-15%. The enzyme complexes involved in the oxidative phosphorylation comprise the major part of the protein [2,5-7].

Functionally the mitochondrial oxidative phosphorylation system is composed of 5 protein-lipid complexes. They are :

1. NADH: Ubiquinone Oxidoreductase (Complex I)
2. Succinate : Ubiquinone Oxidoreductase (Complex II)
3. Ubiquinol : Ferricytochrome c Oxidoreductase (Complex III)
4. Ferrocytochrome c : Oxygen Oxidoreductase (Complex IV)
5. ATP synthase (Complex V) [2].

These five complexes are made up of about 60 different proteins, of these only 6 are synthesized in the mitochondria [2]. The remainder are cytoribosomal products and are imported.

As shown in Figure 1, these complexes interact with each other and complexes I, II, III and IV plus Ubiquinone (Q) and cytochrome c make up the actual respiratory chain. The electron carriers are quinoid structures (FMN, FAD, Q) and transition metal complexes (FeS clusters, hemes, protein bound copper) [2].

As can be seen from Figure 1 the electron carriers comprise 3 quasi-equipotential regions, within each of which the energy drop is less than 100 mv and while in between each of these it is more than 100 mv. At these 3 stages the oxidative energy is conserved and is used to drive ATP synthesis [2].

The electron transfer in the ETC is reversible up to cytochrome c [8]. However, the final step of electron transfer from a_3 to oxygen is not .

Complex I - NADH : ubiquinone oxidoreductase

This catalyzes rotenone sensitive reduction of Q analogs by NADH and vectorial proton translocation coupled to electron transfer [6,9]. This enzyme can also catalyze electron transfer from NADPH at pH $\leq$ 6, but at a slower rate [9,10].

The complex I has FMN, nonheme iron (Fe), acid labile sulfide (S^{2-}), Q and phospholipids. FMN : Fe : S^{2-} are present in the ratio 1:21-22:22-24 [8]. There are about 25 different polypeptides present in 1:1 molar ratio with FMN in which 4 to 8 FeS clusters are present. The Q content is variable while the phospholipids are in the same relative ratios as that present in the inner membrane [11-13].

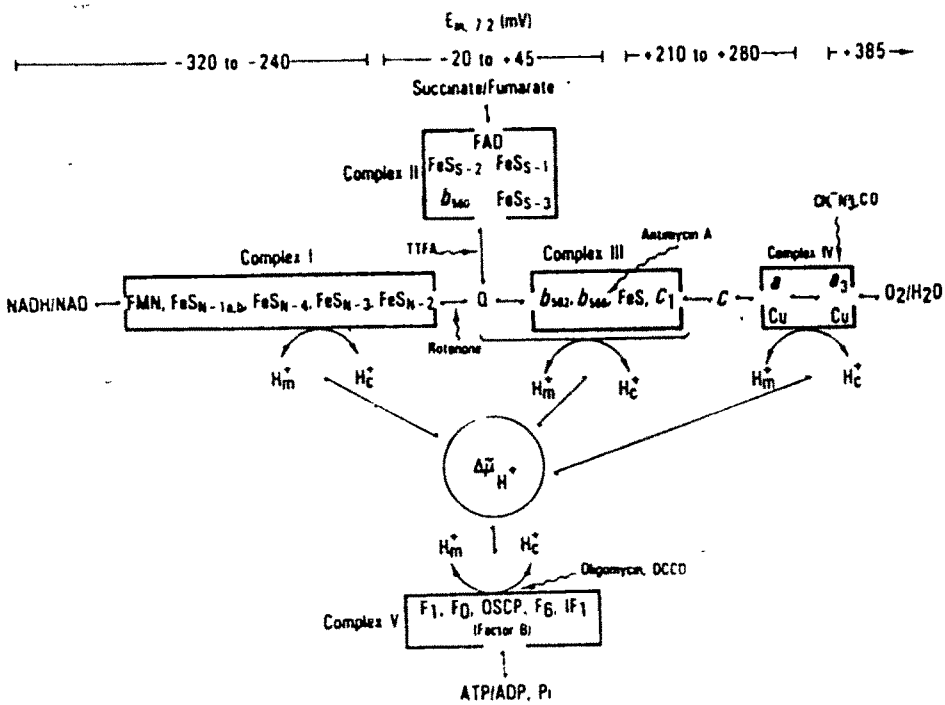

Figure 1

Mitochondrial electron transport chain and oxidative phosphorylation system, showing wel-characterized components of complexes I, II, III, IV and V. Fes, iron sulfur cluster, and cytochromes a, b and c.

The electrons that are channeled through the ETC are pumped in by a group of enzymes acting as feeder pathways i.e. the dehydrogenases. Amongst others, glutamate and malate dehydrogenases are two such enzymes which channel electrons into the complex I and which form a part of this study.

Glutamate dehydrogenase (GDH):

This enzyme catalyzes the interconversion of α - ketoglutarate and L-glutamic acid. The enzyme is important because of the pivotal position in metabolism occupied by glutamate and α - ketoglutarate and the ability of these compounds to enter into different metabolic pathways. GDH provides a means for incorporation of nitrogen into organic compounds, and thus serves as a link between carbohydrate and amino acid metabolism. There are three types of GDH which differ in their coenzyme specificity : those that can utilize either NAD^+ or $NADP^+$ and those that can function with both. The animal cell GDH is a soluble enzyme located in the mitochondrial matrix,which is synthesized in the microsomes and transported to the mitochondria [14].

This enzyme has a complex hexameric structure with discrete binding sites for substrates, coenzymes and allosteric effectors. The molecular weight of this hexamer is about 330 Kd and this is the smallest enzymatically active unit and this can undergo further aggregation [14,15].

Malate dehydrogenase (MDH):

The oxidation of L-malate in most living organisms is catalyzed in either of two ways. In one case the product is oxaloacetate, i.e. by malate dehydrogenase and in other the products are pyruvate and CO_2 i.e. by malic enzyme.

The malate dehydrogenase is present in two forms identified as the mitochondrial (m-MDH) and soluble or cytoplasmic (s-MDH) depending on their localization in the eucaryotic cells.

The soluble isozyme takes part in the cytoplasmic side of the "malate shuttle", providing a means of transporting NADH equivalents in the form of malate across the mitochondrial membrane. The mitochondrial enzyme in addition to its role as the other half of the malate shuttle, also is a necessary component of the tricarboxylic acid cycle (TCA) [16].

The molecular weight of the enzyme lies between 65 to 75 Kd; the s-MDH has a molecular weight of about 72 Kd and m-MDH about 68 Kd. The enzyme is thought to be composed of two identical subunits having a molecular weight of 33 to 35 Kd. The two isozymes have only small differences in their protein sequences. [16,17].

Complex II - Succinate : ubiquinone oxidoreductase:

The major component of this complex is succinate dehydrogenase (SDH) which is a part of the TCA cycle. This complex catalyzes electron transfer from succinate to Q or to

dyes such as ferricyanide and dichlorophenol-indophenol (DCIP) or to its natural electron acceptor in the ETC [6,9]. SDH can also transfer electrons from a suitable dye to fumarate [2,18]. The electron transfer capacity of the enzyme is stable when bound to the membrane, but in isolated condition there is deterioration of activity [9].

Mammalian complex II is made up of 4 polypeptides, 70, 27, 15.5 and 13.5 Kd [2]. SDH is associated with 1 mol/mol of covalently bound FAD, 7 to 8 mol of Fe and 7 to 8 mol of acid labile sulfide groups (S^{2-}) [2]. SDH has two polypeptides: a 70 Kd ferroflavoprotein (Fp) and a 27 Kd iron protein (Ip) [2]. A type b cytochrome is present in equimolar concentration with FAD which is distinct from the complex III cytochrome b [18]; it is a nuclear DNA product containing one polypeptide and two hemes [2].

Complex III - Ubiquinol : cytochrome c oxidoreductase:

This complex catalyzes the electron transfer from dihydroubiquinone (QH2) to cytochrome c, and this reaction is coupled to transmembrane proton translocation [19]. The complex contains two b type cytochromes, one c type cytochrome and an FeS protein [2].

This complex is composed of 9 to 10 different polypeptides; of these, three are associated with redox centres i.e. b_{562}, b_{566} and c_1 hemes and a 2Fe-2S cluster and

two ubisemiquinone species in two separate domains [20,21]. The two b hemes are associated with a single hydrophobic, mitochondrial-DNA synthesized polypeptide. The heme content within the purified cytochrome b varies between 30 to 37 n mol/mg protein [2,22]. There are two high potential and two low potential redox centres [23], containing FeS, cytochrome c and cytochrome b_{556} and b_{562} respectively [2].

Complex IV - Ferrocytochrome c : oxygen oxidoreductase:

This is a multisubunit metalloprotein enzyme complex which catalyzes a critical reaction in the cellular respiration. The energy released in this exergonic reaction is conserved as a pH gradient and membrane potential across the membrane barrier [24].

The cytochrome c oxidase redox centres contain two heme a moieties (a and a_3) and two copper atoms (Cu_A and Cu_B) [25], along with 1 mol each of Mg and Zn [24].

The subunit composition varies with evolution; bacterial enzyme has 3 subunits while mammalian enzyme has 13 with a combined molecular weight of 204 Kd [2,24,26]. Of these the three largest, i.e. I, II and III, polypeptides are mitochondrial in origin and the rest are nuclear DNA encoded [25,27]. The mitochondrial DNA encoded subunits are catalytic in function and are similar to the bacterial enzyme subunits. The nuclear DNA encoded subunits on the other hand have a regulatory role to play and are thought to be the key

components necessary for the tight regulation of the critical function of cytochrome c oxidase [2,23,28].

This enzyme catalyzes the 4 electron reduction of molecular oxygen by the single electron donor ferrocytochrome c. Subunit III of the enzyme functions as a proton pump [24,29,30] which forms a part of the proton channel and any dysfunction of this leads to impairment in ETC function and coupling between electron transfer and phosphorylation [2,24,31].

Complex V - ATP synthase:

This is involved in synthesizing ATP from ADP and inorganic phosphate (Pi), utilizing the protonic energy derived from the activity of respiratory complexes I, II, and IV. This can also hydrolyze ATP if the protons are translocating in an opposite direction from the matrix to the cytosolic side of the mitochondrial inner membrane [2]. Recent work by Syroeshkin et. al. [32] has shown that these two reactions are not reversal of each other.The enzyme exists in two slowly interconvertible, hydrolase and synthase states and an equilibrium between them depends on ATP/ADP ratio and a number of other ligands.

The mitochondrial ATP synthase is made up of two subparts F_o and F_1 connected by a stalk. F_1 is a highly active ATPase capable of rapid uncoupled ATP hydrolysis when detached from

F_O, a membrane embedded protein, and is involved in proton translocation [32,33].

The F_1 subunit has five different proteins $\alpha, \beta, \gamma, \delta$ and ϵ in the ratio 3:3:1:1:1 [34], F_O has three proteins a,b and c in the ratio 1:2:6 [2]. The c subunit is the DCCD binding proteolipid. The stalk is made up of two proteins,oligomycin sensitivity conferring protein (OSCP) and coupling factor 6 (F_6) required for binding of F_1 to F_O. There are in all 12 subunits; in addition to the above there is the ATPase inhibitor protein (IF_1) required to prevent ATP hydrolysis *in situ*, coupling factor B (F_B) and the mitochondrial DNA encoded subunits 6 and 8 [2].

Lipids of mitochondria:

In addition to the enzymes and proteins, lipids constitute the other important components of the mitochondrial membrane and as such are extremely important for the proper functioning of the mitochondrial ETC.

Mitochondrial inner and outer membrane lipids are very different in function and composition. Even the synthesis of lipids is carried out at different places, the endoplasmic reticulum, plasma membrane and the mitochondria, while some are produced by these systems acting in concert [35].

The lipid composition of different tissues, cells and mitochondria differ. However, the major phospholipids (PL) are phosphatidylcholine (PC) and phosphatidylethanolamine (PE),

which make up about 80% of total phospholipids (TPL). Diphosphatidylglycerol (DPG) is present around 10 to 20% in all the tissues, except the brain, which has only 5%. The content of phosphatidylinositol (PI) and phosphatidylserine (PS) are about 1 to 9 % while phosphatidic acid (PA) and sphingomyelin (SM) are in very low amounts [36-38].

The lipids of inner and outer membrane differ in their characteristics and contents; for example, the PL/protein ratio of outer membrane is higher than that of the inner membrane. DPG is synthesized and enriched in the inner membrane, while the outer membrane has higher content of PI and SM. PE is present preferentially in the inner membrane, while sterol levels are low in the inner membrane compared to the outer membrane [35].

The proteins and lipids in the mitochondrial membranes are arranged asymetrically, like in the other cellular membranes. The major amount of DPG (75-90%) and PE (50-60%) is present on the matrix side of the inner membrane while 10 to 30% of PE is buried in the inner membrane [35].

The PLs of inner membrane are oriented according to their charges, thus the negatively charged DPG and PI are localized preferentially on the matrix side. The outer membrane lipids are not as well studied, but their orientation also would be quite important considering the various functional attributes of the outer membrane [35].

The lipids of biological membranes are not only structural elements; they considerably influence the proteins in their membranous environment and thereby participate in the regulation of biochemical processes.

The effects of lipids and their functions in mitochondria can be summarized as follows:

1. To maintain the stability and osmotic behavior of mitochondria.
2. Effects on respiration and energy production.
3. Control of permeability and transport processes across mitochondrial membranes.
4. Influence on mitochondrial protein synthesis, import of proteins into mitochondria, and assembly of mitochondrial membranes.
5. Influence on the membrane structure, phase behavior and lipid-protein interactions and
6. Controlling the activity of mitochondrial membranous enzymes [35].

Control of respiration and ATP synthesis in mitochondria:

The ATP demand of the cellular ATP utilizing reactions was thought to be the major mechanism of control of rspiration [39,40]. However, another mechanism known as the `near equilibrium hypothesis' was put forward by Klingenberg [41]. According to this the rates of respiration and ATP synthesis were controlled by : a) mitochondrial $NADH/NAD^+$ ratio,

b) phosphorylation potential and c) any effectors of cytochrome oxidase (C.O.) such as pH or oxygen concentration. Atkinson [42] suggested that control is due to net adenylate charge i.e. [ATP] + 0.5 [ADP]/[ATP] + [ADP] + [AMP] and that all these act as allosteric regulators of the various glycolytic enzymes, matrix dehydrogenases and various ATP utilizing pathways.

Cellular calcium level was also considered to exert control by acting as an activator of several key matrix dehydrogenases [41,43].

Regulation of ETC and its function is not solely due to the linked pathways of energy metabolism, but the various compartments of the ETC themselves are under tight control. For example, regulation of fatty acyl CoA [44], cytochrome oxidase [24], Ca control of mitochondrial volume, which in turn controlls the respiratory chain [45], regulation of ATP synthesis by inhibitory proteins [1,46] etc.

Groen et. al. [47] have proposed that the control over mitochondrial respiration and ATP synthesis is managed by a number of reactions and pathways and that the distribution of control changes under different metabolic conditions.

The control over energy metabolism becomes more complex since this consists of branching pathways and control of one pathway is linked to, but not equivalent to, that of the other pathway. The energy metabolism is not unidirectional, but consists of chain or chains of moiety conserved cycles and

hence the process can now be controlled from either end or from inbetween. Also, any end can now be considered as a starting point and any of the conserved moieties can exert control over the rate of energy metabolism [1].

Within the cell, respiration and ATP synthesis is controlled by various factors e.g. :

a) Substrates and products:

Substrates such as ADP, Pi, O_2 or respiratory substrates all can control the respiration and ATP synthesis by various mechanisms [48-53], while the products of these reactions themselves can, in turn, exert control over these reactions [49,50].

b) Control by metabolism:

This control is shared by, and the distribution of control changes, depending on the activities of different enzymes and transporters [1,47,48,54-56].

c) Control by calcium (Ca):

Intramitochondrial Ca stimulates matrix dehydrogenases [1,43,45,48,57]. The level of free and not the total Ca in the cytoplasm is important, which is controlled by Ca mobilizing and antagonizing hormones and electrical excitation. The free cytoplasmic Ca controls the mitochondrial matrix free Ca via two mitochondrial transporters; an electrogenic uniporter for

uptake and an electroneutral Ca^{2+}/Na^{+} exchanger for efflux. Ca can also control the mitochondrial functions by controlling the matrix volume [45].

d) Control over ATP synthase by inhibitory proteins:

Two proteins have been found in heart and skeletal muscles of higher mammals which are additively inhibitory to ATP synthase and regulate under different conditions of pH, ATP concentration and redox state [44,46,50,58].

e) Control by moiety pool size:

The conserved moiety cycle characteristic of the energy metabolism ensures control by the moieties involved in the reaction as well as the pathways that produce them [1,59,60].

Mitochondria are the "Power House" of the cell and are involved in a number of vital energy yielding and metabolic functions. Hence, the proper functioning of mitochondrial enzymes and enzyme complexes is of paramount importance for survival and normal functioning of the cell [61]. As described above, mitochondria are under tight control by intrinsic factors [1]. However, there are numerous extrinsic factors which can influence and control mitochondrial metabolism, some of which are mentioned below.

Hormones such as growth hormone [62], noradrenaline [63,64], glucagon, adrenaline [65], insulin [66-68], thyroid

hormones [69-71], steroid hormones [72] are known to affect the mitohcondrial structure and function.

Various diseases and metabolic disorders also affect the functioning of the mitochondria i.e. diabetes [73-75], cirrhosis of liver [76], myopathies [77], achondroplasia [78], mitochondrial encephalopathy [79], mitochondrial cytopathy [80], Leber's hereditery optic neuropathy [81], Alzheimer's disease [82], Parkinson's disease [83,84]. Obesity vis-a-vis mitochondrial functions was studied by Katyare and Howland [85] and Rogers et. al. [86], while hypoxia [87] and osmotic lysis [88] also have been studied for this effect.

Drugs such as imipramine [89], propranlol [90], paracetamol [91], ethanol [92], anesthetics such as procaine and lidocaine [93], hypolipidemic drugs such as clofibrate [94], sedatives such as diazepam [95] all adversely affect mitochondrial functions.

Environmental toxicants: either chemical such as insecticides and pesticides (DDT) [96,97], hexachlorobenzene [98] or heavy metals such as lead [99] and cadmium are also known to cause mitochondrial dysfunction.

The influence exerted by diet and nutrition on mitochondria has been collated by Ferreira and Gill [99], and Aw and Jones [100].

It is a common observation that aging animals tend to be less energetic and alterations in mitochondrial energy

transduction pathways could be the underlying mechanism. A review by Hansford [101] covers this aspect.In addition the mitochondrial DNA damage vis-a-vis the onset of aging has also been reviewed [101,102].

Complex I and cytochrome oxidase (C.O.) activity was shown to be impaired in aged rats [102], similarly a decrease in the content of CoQ, cytochrome c + c_1 and aa_3, respiratory activity and oxygen consumption was reported [103,104]. The mitochondrial membrane composition as well as integrity is seen to alter [105-107] while the adenine nucleotide pool decreased [59] with age.

In addition to all the above mentioned factors, other conditions such as embryogenesis, birth and ontogenic development also have a strong influence on the mitochondria. These are stages of intense synthetic activity and since mitochondria play a central role in cellular energy production [108] they would need to play an important role since, inherent to this developmental period is a transition by the mitochondria from a lower to a higher metabolic rate [109].

The activities of NADH oxidase, cytochrome oxidase, SDH and ATPase are all found to increase during embryogenesis between day 10 to 14 of gestation. This increase is paralleled by striking changes in the appearance and number of mitochondria and the cristae [110].

After birth many physiological and biochemical changes take place in a newborn animal, enabling it to adapt to its new environment. Many of the regulatory functions previously carried out by the placenta and other maternal organs need to be assumed by the lungs, liver, kidneys and other organs of the neonate [65].

It is known that in rat tissues the activities of some oxidative enzymes usually considered to reflect mitochondrial function are low in fetus and newborn as compared to adults. The organs in which circulatory changes occur at birth, namely, heart and liver, show relatively rapid increases in the content of different tissue cytochromes and activities of C.O., SDH and succinate oxidase as compared to the more gradual changes in the brain and the kidneys [111,112].

The acquisition of fully developed mitochondria after birth is an important homeostatic mechanism that enables newborn mammals to successfully adapt to extrauterine life. During fetal life, the energy needs of the liver are provided by anaerobic glycolysis since its respiratory activity is low [113,114]. These circumstances trigger a profound modification of the metabolic pathways relevant to energy production that switch from glycolysis to respiration and rely on the maturation of mitochondria [65]. The proliferation of the mitochondria is a continuous process during the entire period of neonatal rat liver development [115]. In contrast maturation or differentiation of pre-existing mitochondria

occurs very rapidly after birth in the first postnatal hour [113]. The enzymes that attain full functional competence within this period are known as the `neonatal cluster of enzymes' [65].

The biochemical changes at birth in the mammalian liver are associated with the change over from a constant source of maternal nutrients to an independent existence. There is also a change over from a relatively anoxic state to the highly oxygenated condition in the new born. The mitochondria during the perinatal period undergo many morphological and metabolic changes [116]. The energy conservation systems in fetal and neonatal rat liver mitochondria are different and it becomes truly operative only in immediate postnatal period. Even the adenylate nucleotide concentration, relative [ATP]/[ADP] ratio is found to be higher in the neonates as compared to the fetus [117-119].

The respiratory chain proteins were found to increase in the liver and heart in the perinatal period compared to the levels in the fetus [112]. The mitochondrial membrane integrity is seen to increase after birth. In the fetus there is a virtual absence of osmotic activity and free permeability to low molecular weight solutes such as sucrose [120]. In addition, the fetal mitochondria are found to have no respiratory control ratio which also improved after birth [112,114,119].

Postnatal mitochondrial development has been studied by examining different mitochondrial functions and an increase in specific activities of Kreb's cycle enzymes and content of certain electron transport chain components has been reported [114]. The enzyme activities gradually increased after birth. The state 3 respiration rate and membrane integrity also improved [114].

In some cases this increase was a continuation of a pattern established before birth, for others this was triggered by parturition. Thus it has been shown that mitochondrial proliferation, including inner mitochondrial membrane enzyme synthesis is regulated so as to be a key regulatory factor in the development of the increased cellular respiratory capacity. Jani et.al. [120] have reported an age-dependent increase in the respiratory activity in the rat liver mitochondria with different substrates.

The rat brain at birth is an immature organ and during second and third weeks of postnatal life it goes through a period of rapid growth and maturation during which the brain develops mature anatomical and biochemical properties [121]. Once fully developed the brain is concerned with providing the appropriate physiological response to stimuli relevant to life processes. Studies on the electrical activity of the rat brain from 0 to 7 days of age have shown an irregular and low amplitude which by day 10 reaches the adult level [122].

The development and maturation of the brain after birth has an inherent transition from the low metabolic rate to a higher one [110,123] as judged by the increased oxygen consumption. Due to this increased dependence on oxygen the newborn and then the adult looses the resistance to anoxia that the fetus is capable of [123].

During development brain is able to utilize ketone bodies and the enzymes involved in their utilization increase during this period [124]. The pentose phosphate pathway and fatty acid and myelin synthesis rates are higher during suckling period than in the fetus and the adults [110,125]. The glucose utilizing and energy metabolism enzymes as well as the neurotransmitter synthesizing enzymes have a regional distribution and the time at which they attain 50% of the adult levels during development varies [126-130] and these parameters are complementary to each other. With regards to the oxidative metabolism of the mitochondria it is seen that during development the activity of the enzymes involved in this function gradually in creased from the fetal stage onwards [131-134].

As seen earlier the lipids of the mitochondrial membrane also play an important role in controlling the mitochondrial functions. In turn they themselves are influenced by various factors similar to the mitochondrial proteins and enzymes.

Thyroid hormones [135-137], steroid hormones [138],

insulin [139], certain drugs and chemicals such as galactosamine [140] and propranolol [141,142], variations in temperature [143,144], ischaemia [145], diet and nutrition [35,100,135,146] are all known to affect the mitochondrial membrane lipids.

In addition to these, as seen with mitochondrial enzymes, aging, embryogenesis, birth and ontogenic development also affect the content and characteristics of mitochondrial membrane lipids.

Studies have shown increased PL content and changes in % contribution by differnt PL subclasses in early newborn and adult rat liver mitochondria [109,115,116,147]. Negatively charged PLs decreased during development [115]

Changes in the fatty acid composition as well as catabolism of PLs were studied in different age groups during development in liver mitochondria and it was found that the catabolism did not change although the fatty acid composition changed [35,115,116,148].

In case of brain the studies have concentrated on the developmental alterations taking place in myelin and other brain specific lipids such as cerebrosides and gangliosides [149].

During development large amount of lipid is accumulated in the white matter of the central nervous system (CNS). Myelin lipid content studied during development showed

presence of more PL and less cerebrosides in the fetal brain compared to the adult [150] while cholesterol (CHL), total phospholipids (TPL) and cerbrosides were also found to be different in the 10-day postnatal and adult rats [149]; the brain cerebrosides and gangliosides were reported to increase with age [150 - 152]

During aging the lipid-protein interaction in the mitochondrial membrane changes and this is thought to play an important role in the impaired functioning of the mitochondria with age [150].

Studies on TPL,CHL and PL show decreased PL content and an increased CHL content which leads to decreased fluidity [106,153,154]. In the whole brain the deposition of dolichol-p and lipofuchsin is seen to increase accompanied by decreased TPL and CHL content and altered fatty acid composition [154-156].

CADMIUM TOXICITY:

General Aspects of Metal Toxicity:

Metals are natural components of the lithosphere and are as such found throughout all ecosystems. The flow of any trace metal through these systems is a dynamic event brought about by both natural and artificial processes. The naturally occuring processes such as volcanic activity are beyond human control, however their contribution is minimal. More

important and significant is the influence of activities and interference of humans on the environment. Increased industrialization and increased usage of various metals has promoted a dramatic increase in their mining and processing. These lead to the increased release of these trace metals in the environment which causes their accumulation and makes them potentially and actually more hazardous [157].

Although the potential hazards of these trace metals have been known from Roman times, when lead poisioning was suspected, in recent times due to increased cases of exposure due to occupational proximity to the metals as well as due to sporadic incidents of severe environmental contamination eg.such as with cadmium (Cd): Itai-Itai disease in Japan (vide infra) [158]; mercury: Minamata disease in Iraq [159]; arsenic, molybdenum and lead [160,161].

Depending on the inherent characteristics any metal on reaching its target organ can exert some biological response, this could be either essential, for example Zn which is required in various metalloproteins, or non-essential or toxic. Cd which has no known function within the body and even in small concentrations can cause deleterious effects falls in the toxic category [157].

Toxicity is defined as a chemically-induced alteration in structure and/or function, resulting in an adverse or malfunctional response [157].

Toxicity of a metal is influenced by number of factors such as :

a) Compound related factors i.e. 1. Dose and duration of exposure, 2. Route of exposure e.g. inhalation, ingestion etc. 3. Physical form of the metal e.g. dust, fume, size of particles etc. and 4. Chemical form of the metal e.g. ionic, organometallic etc.

b) Characteristics of the exposed organism i.e. 1. Genetic factors, 2. Age, 3. Sex, 4. Species, 5. Physiological state e.g. diseases which reduce clearance, pregnancy etc.

c) Other factors such as diet, low intake of essential metals, organic compounds with chelating properties etc. and

d) environmental conditions such as presence of other substances which may compete or synergize etc. [157].

Toxic response may be acute or chronic. An acute toxicity is a single or multiple exposure occuring within a short time (24 hours or less). Chronic exposure, on the other hand, pertains to an intake of small quantities of the toxic agents over a long period of time, often years. The biological effects in acute toxicity develop rapidly and are quite different from those occuring in the chronic type, which are manifested after considerable period of time; this intervening period is termed as a latent period [162].

Another aspect of the metal toxicity is the very low

destructibility of the metals. Organic compounds are broken down within the body and are converted to other intermediates which are either non-toxic or less so. Whereas these trace metals can not be degraded or destroyed, hence they accumulate in various tissues where they are able to reside for long periods of time and are thus said to have a longer half-life, for example, half-life of methyl-mercury is approximately 60 days [163], whereas Cd has a half-life of 15-30 years [164].

Biochemical mechanisms underlying metal toxicity:

The metal toxicity lacks specific, characteristic, pathological legions when studied in vivo. This could be due to the fact that metals on entry into the body undergo distribution phase. The systemic toxicities thus require the proper distribution of the metal to take place after uptake. Figure 2 illustrates the phases during development of metal toxicity.

Basis of the toxic reaction of metals:

Figure 3 shows the various steps involved in toxic reaction. The elemental or organometallic form of a metal normally penetrates cells much more readily than the equivalent inorganic cationic species. However, the intrinsic toxicity of inorganic or organic cationic species is usually considerably greater than that of the elemental form. Thus the ability of cells to oxidize the elemental form may influence significantly its vulnerability towards toxicity. A pre-

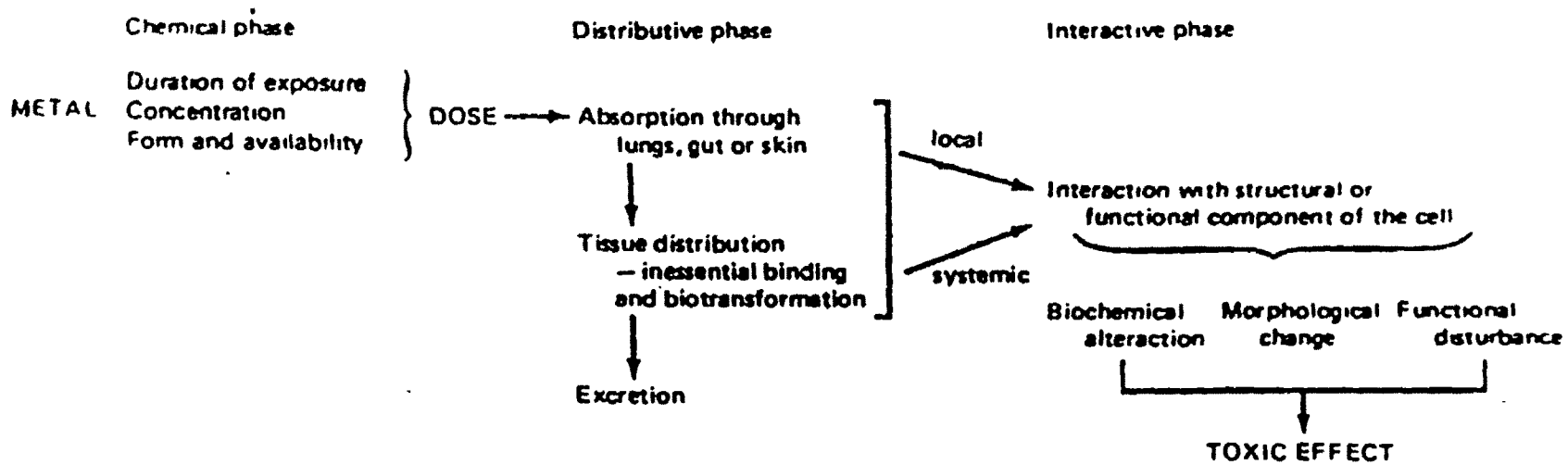

Figure 2

Phases during the development of metal-induced toxicity

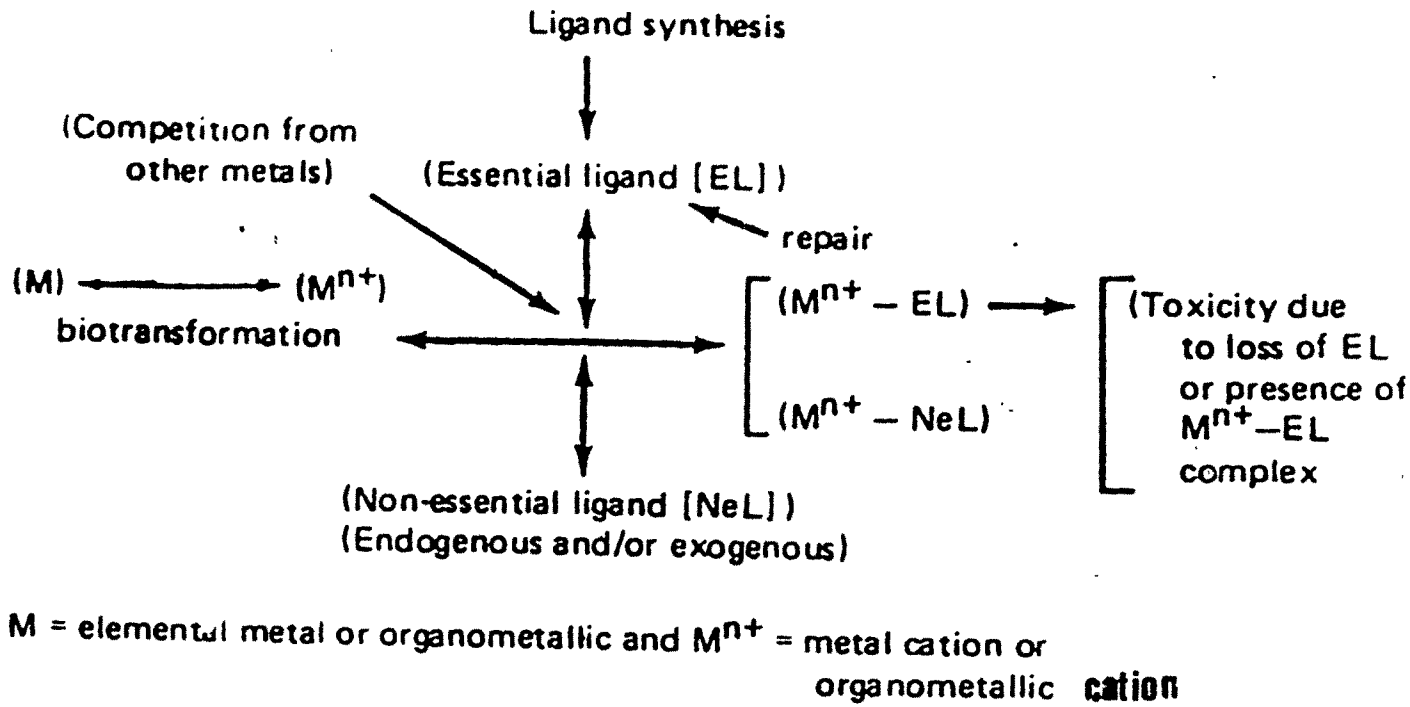

Figure 3

Basis of the toxic reaction of metals

requisite for metal toxicity is its liganding to an endogenous molecule, usually a macromolecule, to produce a covalent or coordination complex. Usually molecules containing electron donating groups such as -SH, $-NH_2$ and -OH are the main targets. This binding may lead to changes in the hydrogen bonding, tertiary structure or binding of essential metal ions. The extent of toxicity depends on the functional and/or structural changes occuring in the ligand due to its binding to the metal, its importance to the normal functioning of the cells or tissues, the percentage of such molecules affected and their rate of repair or replacement, which can lead to reduced toxicity or tolerence to the metal [165].

Following this general overview of the metal toxicity mechanism, an account of the specific heavy trace metal of interest for the present study i.e. cadmium (Cd) is given below:

Cd is a metal (Mol. Wt. 112) that occurs at low concentration in all natural materials. It is used as an anti-corrosive agent in electroplating of steel or galvanizing, as a color pigment, as a stabilizer in PVC plastic and as a cathode material in Ni-Cd batteries. Smaller amounts are utilized in Cu-Cd alloys for automobile radiators, in Ag-Cd alloys for silver solders, in surface treatment of cast iron products and in fission controlling rods of nuclear reactors [166].

Exposure to Cd in humans is via either respiratory or gastrointestinal tract (GIT), while the entry through skin is very low. The main sources are by inhalation of ambient or occupational air, tobacco smoke, food and drinking water [167].

The entry via respiratory tract depends on the size of the Cd particle. A particle less than 5 μm is 'respirable'. The percent of this form and the total concentration of Cd in the environment are the important factors for Cd entry. 10 to 50% of the respired amount is absorbed [168-170]. Tobacco smoke is another major source of Cd exposure, one cigarette contains 1 to 2 μg Cd and a pack of 20 contributes 1 to 2 μg per day at 50% absorption of the 10% inhaled as main stream smoke [171,172].

Cd in soil enters as pollutant and impurity in fertilizers, agricultural chemicals and atmospheric pollution [173] and the concentration varies between 0.06 to 1.0 μg per gram of soil [174]. This accumulated Cd enters the food chain via plants which take it up from the soil [175].

In the aquasphere and fish Cd occurs between 0.03 to 1106 ppm dry weight of fish and the highest concentration is in the shell fish [172,176,177].

From such contaminated plants or fish via the food chain, Cd enters the humans and this entry is unavoidable. The Cd intake via food is 20 to 50 μg per day. Food rather than water is the main source of Cd intake [172].

The provisional tolerable weekly intake for humans is 400 to 500 μg [178] and 50% of this is provided by food stuff. Contamination to food consumed can be via food or beverages contaminated with Cd, from Cd plated utensils and storage of acidic juices in Cd containing ceramics. Another means of entry of Cd is via water from soldered pipes, taps or refrigerating devices [179].

In general Cd is toxic to virtually every system in the animal body and the organ which ultimately gets affected is decided by the route of administration, dose and for what length of time Cd stays in the body, for example i.v. administration leads to accumulation of about 90% in the liver within half an hour while inhalation leads to accumulation in lungs [180]. The higher the Cd exposure and longer the duration the larger is the accumulation in the liver as compared to the kidneys [181].

The daily intake of Cd is about 30 to 60 μg of which only 1 to 4% is absorbed in the GIT and of this only 6% is retained and the rest is excreted slowly [172,182]. Of the Cd present as aerosol 50% gets absorbed in the lungs [183].

The excretion of Cd depends on the total body burden; half the amount is excreted in urine and the other half in feces. Of the fecal excretion large part comes from bile and is from the unabsorbed fraction [184,185].

Fatal dose of Cd is between 350 to 8900 mg [186].

Effect of cadmium on various tissues:

Gastrointestinal tract:

Cd causes damage to the duodenum or jejunum and the ultrastructure of the villi and epithelial cells is severely altered, damaged, and degenerated [187].

Blood:

Anemia is one of the most sensitive parameters of Cd toxicity [188] and in rats this can develop with 31 ppm Cd in diet. The anemia is hypochromic and microcytic and is due to decreased Fe absorption because of Cd competition [187]. Klaassen and Kotsonis [188] reported that initially Cd is higher in content in the plasma although the blood concentration increases by about 150% after some time has elapsed. Friberg [189] showed binding of Cd to RBCs and its interaction with WBCs.

Cardiovascular system:

Cd is implicated as a possible hypertension and arteriosclerotic heart disease causing factor [187]. Cd-induced chronic arterial hypertension once set in does not regress with age or absence of new exposure. Cd-induced alterations and destruction of renal vasculature leads to loss of Zn, Ca and Na and this is thought to lead to hypertension. Cd gets deposited in to the cardiovascular tissue such as

myocardium and leads to myocardial hypertrophy and vacualization of bundle of His conduction cells. Cd also causes alterations in the electrical impulse generation, conduction and proper rythmic pulsation of the heart valves and chambers [190,191].

Lungs:

Cd exposure leads to pulmonary edema and sub-acute interstitial pneumonia [187] along with chronic bronchopneumonia, emphysema and permanent lung damage due to perivascular and peribronchial fibrosis. The bronchiols, alveolar ducts, mucus membrane of the nose and trachea are also affected [192]. The release of the lysosomal enzymes, mainly proteases,is the main mechanism behind lung damage [182]. Death due to Cd inhalation is due to pulmonary edema and a lethal dose is about 5 mg per m^3 for 8 hours [172].

Gonads:

A single parental injection of a soluble Cd salt (0.02 to 0.04 m mol/kg) causes haemorrhagic necrosis followed by atrophy of the testes and permanent sterility [193] in scrotal mammals. However,doses which affect adults do not affect new-born rats till a critical age, though the newborn rat testes seem to have 5 times higher concentration of Cd than adults [194].

Acute effects of Cd toxicity on testes leads to lesions such as cavities in the arterioles followed by necrosis of endothelial cells of small vessels and still later after about 18 to 24 hours the testes are completely necrosed. Interstitial haemorrhage, eosinophilia and parenchymal cell swelling are also seen [191]. The testicular damage also leads to reduction in androgen concentration [182].

1 mg Cd/100 gm body weight injected subcutaneously to female rats produces marked hyperaemia of ovary and atresia of follicles in 6 hours, while by 48 hours the ovaries are a total haemorrhagic mass and follicular atresia is complete, granular cells and ovum are atrophic and the nuclei are fragmented. However, about 6 to 7 days later the ovary reverts to normal condition [195].

Kidneys :

Although after a single acute dose liver accumulates the maximum amount of Cd in the initial periods, after about 20 days the concentration of Cd in liver falls, and in 5 months kidney cortex has about 2.5 times as much Cd as liver [182].

LD_{50} dose given i.v. causes renal tubular dilatation and generalized congestion of kidney at 8 hours. The proximal tubules are the first to be damaged. The maximum accumulation of Cd is seen in these tubules,followed by vacuolation and congestion with casts leading to complete degeneration of these proximal tubules [196,197].

Renal damage by Cd leads to proteinuria, glycosuria, amino aciduria, polyuria, aciduria and hypercalciuria. Loss of PO_4 and about 300 mg Ca per day in urine is also seen [198,199]. 200 µg Cd per gram wet weight was thought to be a critical Cd concentration sufficient to cause proteinuria [172,200] which is the first sign of kidney damage.

Kidney tubular cell destruction prevents conversion of 25 (OH) D_3 to 1,25 $(OH)_2$ D_3 and this leads to effects of vitamin D deficiency on the bones thus leading to osteomalacia [201].

Bones:

In Japan the Itai- Itai disease was evident in post menopausal women of over 60 years. A direct correlation between Cd exposure and accumulation in bone, kidney dysfunction and osteomalasia was drawn and this gave direct evidence of the role played by Cd in bone malformation [159,200].

Chronic Cd injection decreases mineral content of bone [172], inhibits collagen production by inhibiting proline oxidase activity and lysyl oxidase, an enzyme important for formation of bonds between collagen fibers and all this combined could lead to the bone malformation and abnormalities observed in Cd toxicity [202].

Liver:

Liver has been shown to be the most susceptible organ to toxic effects after an acute exposure [172,203] and extensive hepatic necrosis could be the cause of hepatic failure and hence Cd-induced lethality. Roughly 60% of the parenterally administered Cd accumulates in the liver [204].

An i.v. injection of Cd acetate produces scattered single parenchymal cell necrosis, congestion and mild vacuolation of Kupfer cells after 8 hours. The hepatic necrosis leads to moderate or severe hepatic injury, cell swelling, cytoplasmic eosinophilia, pycnosis, necrosis, hepatic swelling and increased numbers of mitotic figures [191,205].

The Cd uptake by hepatocytes is rapid, biphasic, energy-dependent and involves membrane -SH groups [206]. An acute dose of Cd causes drastic alterations in the various subcellular organelles such as, damage to rough endoplasmic reticulum, mitochondria and nuclei. Cd inhibits hepatic protein synthesis which could be the cause of observed degeneration of the hepatocytes [191,207].

CNS and brain:

Cd is termed as a neurotoxic trace metal. Cd-insult to the brain manifests itself first by abolishing the reflexes followed by the sensation of pain and finally could aid fatality by causig asphyxia. Prostration, flacidity of muscles and respiratory paralysis are also evident [208].

The lesions occuring in the CNS vary with the time of the exposure and the species, although, the main lesions are on the trigeminal and spinal ganglia [72,182].

The amount of Cd in the newborn rat brain is high,since the blood-brain-barrier is not well developed initially. However, the levels decrease gradually after injection to 4-day-old rats and by the 7th day the levels are similar to those in adults [194].

The distribution of Cd into brain has been studied along with liver and kidneys [205] and it was found that not more than 0.5% of the concentration in liver and kidneys ever accumulated in the brain.

CNS has been shown to be sensitive to disturbance in trace metal concentration [209]. Studies on neurotransmitter precursor uptake, synthesis, release, metabolism etc. in the nervous system has been shown to be affected adversely by Cd [210,211]. Sato et.al. [212] and Hamada et.al. [213] reported that Cd, on chronic exposure, may cause peripheral neuropathy with myelin degeneration. Bourre et.al. [214] and Sugawara and Chen [215] reported presence of Cd in myelin at about 0.52 mM concentration. Monoamine oxidase type A and B (MAO A and B) are seen to be differentially affected by Cd. MAO A was inhibited at lower concentrations while MAO B required higher concentration [216].

Carcinogenic effects of Cd:

Cd in the form of $CdSO_4$, $CdCl_2$, CdO or CdS given subcutaneously or intramuscularly to rats causes tumor formation [217]. Workers exposed to the metal have been shown to develop cancer and smoking by these high risk workers increases the incidence 4 fold [218,219].

Mutagenic effects have been reported to occur in terms of chromosomal aberations such as chromosomal breaks, aneuploid cells, chromatid exchange and structural aberations [182]. The mechanism is thought to be due to competetition of Cd with Zn in the Zn containing DNA synthesizing enzymes [157]. Cd may associate with DNA and cause cross-linking between adjacent strands or it could bind to phosphate and nitrogen bases and alter the stability of the DNA [218].

Effects at cellular levels:

Cd interacts with various cellular organelles and their constituents in a deleterious manner [220]. Cd has a high affinity for nucleic acid bases, resulting in aberrant nucleic acid metabolism [205], inhibits DNA polymerase and induces changes in the DNA synthesis by causing base mispairing [205]. Hoffman et.al. [203] observed parenchymal cell mitosis after Cd exposure, while Cd accumulation in hepatocyte nuclei after both in vivo and in vitro Cd exposure has been reported [221]. Cd interactions with DNA in rat liver may stimulate

hepatocyte cell division [222]. Direct interaction of Cd with DNA has been related to its carcinogenic effect just as other metal ions affect DNA polymerase giving rise to abnormal DNA [220]. Cd inhibits thymidine incorporation into DNA by preventing induction of thymidylate kinase [223]. Protein synthesis by ribosomes and the RNA polymerase activity is also seen to be inhibited by Cd [223-226].

Biotransformation of a number of xenobiotics is inhibited concomitant with a decrease in microsomal cytochrome P_{450} content by 50 to 70% and the inhibition of microsomal monoxygenase activity [223-226].

Ultrastructural damage to the various organelles is evidenced by varying degrees of damage in smooth and rough endoplasmic reticulum, lysosomes etc. [203,226,227]. Application of Cd to the external surface of frog skin increases electrical potential difference, short circuit current (SCC) and other such electrical properties of the membrane. This effect was irreversible and wascaused by stimulation of active Na^+ transport. Thus Cd can also influence active ion transport and permeability of epithelial membranes [228].

Another mode of proposed mechanism of Cd toxicity is by causing specific membrane lesions which could be due to lipid peroxidation since Cd -induced membrane alterations are known [229,230]. Lipid peroxidation in the kidneys and testes and damage to membrane intergrity and leakage of intracellular K^+

has been reported [231,232]. The effect of lipid-peroxidation inhibitors on alleviating the toxicity of Cd is, however, contradictory and, although, one report [233] states that such inhibitors do not ameliorate the toxicity, in another report [234] lipid peroxidation was seen to decrease by addition of anti-oxidants and anti-lipid peroxidants.

Mechanism of tolerance and interactions with other metals:

Goering and Klaassen [205] reported that after Cd induces metallothionein (MT) this protein acts as an integral part of detoxification system by sequestering Cd. The highest levels were in liver and kidney [235,236].

MT is induced by Zn and it regulates Zn homeostasis [237,238]. However, the function of MT in Cd transport is important since MT has 300 times more affinity towards Cd as compared to Zn [239]. Liver is the major site of MT synthesis and the induction is seen to increase by pre-treatment with Cd [236,240]. MT is found to vary in concentration with age with the newborn having 20 times more MT than the adults [241].

Cd gets accumulated in liver thus decreasing its concentration in other organs such as kidneys. Even within liver the maximum accumulation is in the cytoplasm and lesser amounts in the other suscpetible organelles [205,242] such as nuclei, microsomes, lysosomes and mitochondria. Pre-treatment with Cd is seen to induce larger tolerence to subsequent doses

of typically lethal concentrations due to production of larger amounts of MT [205,236,243]. Another means of reducing Cd toxicity is by injecting chelators immediately after Cd injection [206,244,245].

The interaction of Cd with other metals has been studied by many workers [206,246]. Cd decreases Fe levels in tissue in vivo [247] and animals with Fe deficiency accumulate more Cd [248]. Cd does not inhibit transferrin-bound Fe uptake [249] but transferrin-independent Fe uptake is inhibited [250].

The Cd uptake is by binding to plasma membrane ligands, by carrier mediated mechanism, ion pores, receptor operated channels or by simple diffusion [206].

Cd competes with Ca, Cu and Zn for common uptake pathways, binding sites, carriers and ion channels [206]. Uptake of Cd is dependent on membrane fluidity and is hence temperature dependent [251].

Cd and Cu compete for -SH containing ligands associated with Cu uptake. On the other hand Cu, Fe, Zn and Hg inhibit Cd uptake in a competitive manner and also by increasing the efflux of Cd [206,252].

Mitochondria as prime targets of Cd-insult:

Accumulation of Cd within the subcellular organelles and differential distribution to these has shown that if the rats are pre-treated with Cd the subcellular distribution is different from that in unpretreated animals [205,252]. In the

un-treated rats about 8 μg/g of liver or about 15% of the total Cd injected is present in the mitochondria. In pre-treated rats this amount is very low and major accumulation is in the cytoplasm [205,221,222]. Mitochondria have been shown to be major targets of Cd-induced hepatotoxicity [253] and the attempt to protect mitochondria as seen above further proves this point [254,255]. Mitochondrial dysfunction is seen to be readily induced by low levels of Cd both in vivo and in vitro [256].

Mitochondria suffer ultrastructural damage due to Cd exposure [203,227] such as swelling and/or presence of inclusion bodies, electron dense structures that were both morphologically and positionally distinct from matrix granules and absence of mitochondrial cristae [207].

Cd accumulates in mitochondria and this leads to inhibition of oxidative phosphorylation [257,258]. This may hamper cellular energy production and cause a further impairment of homeostatic mechanism. Mitochondrial matrix proteins are seen to get denatured and form the inclusion bodies [259]. Regression of mitochondrial cristae, destruction of mitochondrial membrane, swelling with voids in the matrix, clumping of the mitochondria have been reported at different time points after Cd injection [260,261].

Large oral doses of Cd were shown to uncouple oxidative phosphorylation [242] and inhibited succinate and pyruvate + malate stimulated respiration [254]. Liu and Liun [262]

reported decrease in ATP content and found the site of Cd inhibition on the ETC to be between flavoprotein and cytochrome b. Miccadei and Floridi [255] reported the inhibition to be at two sites: one present between ubisemiquinone and cytochrome b_1 in the ubisemiquinone-b-cytochrome c_1 complex and another site in complex I at the site of entry of electrons from the NAD^+ - linked substrate into the respiratory chain. Cd inhibits uncoupler stimulated oxidation of various NADH - linked substrates [255] and this inhibition was thought to be related to the increased acidity induced by Cd in the hepatocytes [263]. The membrane permeability and fluidity of mitochondria is also altered. Uncoupling of oxidative phosphorylation by Cd depended on Pi transport and Cd caused acceleration of proton influx through Pi/proton symporter [264].

Cd was found to inhibit membrane-bound succinate dehydrogenase (SDH) by modification of some amino acid at the active site having a pKa of 7.23 [265]. Swarnalatha et.al. [266] reported inhibition of SDH, NAD^+ - dependent isocitrate dehydrogenase (ICDH) and cytochrome c oxidase while the activity of glucose-6-phosphate dehydrogenas (G6PDH) and $NADP^+$ - dependent ICDH was stimulated.

From the study of literature cited above it is clear that most of the studies on the developmental pattern of mitochondria have concentrated on the structural as well as quantitative development of the mitochondria and on the oxidative phosphorylation and respiratory activity of the ETC.

However, the pattern for other ancillary enzymes related to the ETC, such as the dehydrogenaes as well as the ATPase are not as well studied. In view of this, it was of interest to study the developmental changes occuring in the characteristics of mitochondrial enzymes, such as the dehydrogenases, ATPase and cytochrome oxidase. Since different tissues attain functional and structural maturity after birth at different time periods, the mitochondria from these tissues also attain maturity at dfferent stages. Hence it was decided to study the developmental pattern of the mitochondrial enzymes from two tissues known to mature at widely different periods after birth, i.e. liver and brain. Rats of different age groups from the early postnatal to the adult stage were used.

In addition to the enzymes, the lipids are important constituents of the mitochondria. The earlier work on the developmental profile of the phospholipids of liver mitochondria is quite contradictory. besides the major studies are either carried out on the fetuses or early neonates. In case of brain the mitochondrial phospholipids have received scant attention and most of the work pertains to the brain specific lipids. Thus it was of interest to study this very important component and observe what changes take place during development, more so, since it has been shown that aging brings about changes in the rat liver and brain phospholipids.

The second part of the thesis concentrates on ellucidating the effects of exposure to cadmium (Cd) on energy metabolism in rat liver and brain mitochondria. The mitochondria are a prime target of Cd-insult. However, the studies carried out so far mostly concentrated on the structural damage to the mitochondria. On the other hand, functional derangement has not been studied in any detail. In addition,no such work has been carried out on brain, an organ which is known to be the maximally protected; Cd is claimed to be a neurotoxic trace metal. The exposure to Cd is not age- dependent nor is it age-restricted and could occur at any stage in life. Yet, studies on the young animals are scarce.

In view of this, in continuation with the studies on the developmental pattern, the effects of Cd exposure on the mitochondrial oxidative energy metabolism from liver and brain in the young as well as the adult were studied. In order to check what effect the passage of time has on the mitochondrial functions as affected by Cd exposure, the experiments were carried out at different times after the Cd exposure.

The thesis collates the data obtained from the different experiments mentioned above and presents them in the form of separate Chapters.

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