DISCUSSION

In an organism, several physiological and biochemical changes occur during development, growth, adulthood and senescence. The development includes an increase in the number and size of cells, their differentiation to perform specialized functions and formation of organs. The metabolic events that occur during development might influence the later part of lifespan. During development, several new proteins appear, indicating the expression of their cognate genes. The level of proteins changes as cells differentiate and organs formed, exhibiting changes in the expression of corresponding genes.

Aging is the characteristic of all multicellular organisms. The functional abilities of most organs and the organisms decrease during senescence. The decline becomes perceptible towards the later part of the reproductive phase. Thus, the reproductive phase smoothly merges into the senescence phase, unlike the transition from the developmental to the reproductive phase in which specific genes are expressed, and specific structures and functions appear that confer reproductive ability to the organism. During senescence, adaptability to external and internal stresses decreases and the homeostatic mechanism deteriorates which increases the susceptibility to diseases in old age.

Enzymes are specific proteins that catalyze chemical reactions in biological system. Enzymes are the direct phenotypic expression of their genes. Considerable amount of literature has developed concerning changes in enzyme level as a function of age and has been well reviewed (Wilson, 1981; Sharma, 1988, 94). At present, this collected information is hard to correlate owing to the variability and contradictory nature of the reported results. One of the problems in obtaining consistent results is that the enzymes have been studied individually without relationship to others in a particular metabolic pathway. But very few studies have reported the activities of all the key enzymes of a particular metabolic pathway which provide quantitative knowledge about the directionality of the pathway. Activities of several enzymes of glycolysis, Krebs cycle and pentose-phosphate pathway of several tissues in mature and old rats have been studied individually (Ardawi, 1982, 85; Dealmeida, 1989). Keeping in view the importance of studying all the enzymes of a particular metabolic cycle, the work embodies in this thesis was planned onto study the regulatory changes in enzymes of malate-aspartate shuttle to elucidate the mechanism of regulation of this shuttle during development and aging.
The malate-aspartate shuttle is primarily involved in the transfer of reducing equivalents from cytosolic NADH to the mitochondria in various tissues (McDonald, 1983). The reducing equivalents of cytosolic NADH are first transferred to cytosolic oxaloacetate to yield malate by the action of cytosolic malate dehydrogenase (c-MDH). The malate carrying reducing equivalents, passes through the inner mitochondrial membrane into the matrix, where the reducing equivalents are passed onto the matrix NAD⁺ yielding oxaloacetate by the action of mitochondrial malate dehydrogenase (m-MDH). The shuttle involves an influx of malate and glutamate and an efflux of aspartate and α-ketoglutarate from mitochondria. Oxaloacetate is a physiologically important intermediate of several metabolic pathways. These pathways may either be catabolic (Krebs cycle) or anabolic (gluconeogenesis) in nature. Since oxaloacetate is impermeable to mitochondrial membrane, the malate-aspartate shuttle appears to be the primary mechanism for the transfer of reducing equivalents from cytosol to mitochondria or vice-versa.

Two homologous and genetically independent isoenzymes of aspartate aminotransferase (c- & m-AsAT) and malate dehydrogenase (c- & m-MDH) are localized in the cytosolic (c-) and mitochondrial (m-) fraction of several animal tissues (Boyd, 1961; Braunstein, 1973). The cytosolic isoenzymes of both MDH and AsAT are also implicated in gluconeogenesis, since the former converts malate and the later aspartate to oxaloacetate, which is then converted to phosphoenolpyruvate. The functional significance of malate-aspartate shuttle also unfolds the degree of control points for glycolysis, gluconeogenesis and Krebs cycle. On the other hand, development encompasses programmed processes which occur by sequential activation and repression of genes. The programme of sequential activation and repression of genes, which are responsible for differentiation and development may continue after maturity and regulate the form and function of the organism. Enzymes are known to constitute a regulatory mechanism which is necessary to coordinate a complex series of reactions in the body. The interval milieu within a cell may change during the course of development and aging; contiguous with this change, the activities of several enzymes may also undergo physical or functional alteration. The present thesis describes the regulatory changes in the enzymes of malate-aspartate shuttle during development and aging to get insight into such metabolic cycle as a function of age. In order to study regulatory changes in the shuttle, the findings are grouped as:

i) the endogenous activity levels of shuttle enzymes at different postnatal ages and their tissue-specific patterns.
ii) regulation of shuttle enzymes by various hormones such as glucocorticoid and thyroid hormones during the same postnatal ages of mice.

iii) one of the shuttle isoenzymes (cytosolic aspartate aminotransferase) was purified from the liver of two different ages (immature and mature) and its chemical and kinetic properties were studied in order to find out change, if any, in such properties as a function of age.

**Endogenous activities of malate-aspartate shuttle isoenzymes:**

The endogenous activities of isoenzymes of aspartate aminotransferase and malate dehydrogenase show a significant change during postnatal development of mice. The activities of both the isoenzymes (cytosolic and mitochondrial) of aspartate aminotransferase and malate dehydrogenase were significantly higher in the liver of mice at day 15, declined at day 30 and remained unchanged thereafter until day 60. In contrast, the activities of these isoenzymes showed lower values at day 15, increases to a peak value at day 30 in the kidney of mice. It indicates an early developmental expression of shuttle enzymes in the liver than in the kidney of mice which may in turn, show an early involvement of malate-aspartate shuttle in the transfer of reducing equivalents to compensate the metabolic demands of this tissue in growing mice. Interestingly, MDH isoenzymes showed a pattern of activity expression like that of aspartate aminotransferase isoenzymes in both the tissues studied. Earlier reports have shown that the rates of the mitochondrial and cytosolic enzymes must be equal for the steady operation of this shuttle (Wiseman et al., 1991). The present findings are in agreement with the previous reports that the AsAT develop differentially in different rat tissues (Herzfeld and Greengard, 1971). Unlike other amino transferases whose levels are insignificant in the fetal liver, the activity of AsAT expresses very early in the fetus about 4-5 days before birth and reaches a peak level by the second week of postnatal life in the rat liver (Herzfeld and Greengard 1971). Our findings of higher level of AsAT isoenzymes in the liver of mice at day 15 of postnatal life corroborate this observation. Using inhibition studies, it has earlier been reported that the malate-aspartate shuttle operates in suckling rat liver (Ferre and Williamson, 1978). In order to confirm the differential expression of malate-aspartate shuttle enzymes in the liver and kidney of mice during postnatal development, the shuttle activity has been studied in an *in vitro* reconstituted system. Reconstituted malate-aspartate shuttle also showed a higher activity (oxidation of NADH as measured by decrease in
absorbance at 340 nm) in the liver of 15-day old mice compared to that of 30-day old animals. Whereas, the shuttle activity was significantly higher in the kidney of 30-day old mice than that of 15-day old ones. Similar to the expression of enzymatic activities, the shuttle activity showed an identical pattern in the liver and kidney of developing mice.

These findings indicate a differential expression of malate-aspartate shuttle in the liver and kidney of mice at different postnatal ages. This may reflect differential metabolic transfer of reducing equivalent to commensurate the specific tissue's requirements at various developmental ages.

**Hormonal regulation of shuttle enzymes**:

**Regulation of malate dehydrogenase isoenzymes by hydrocortisone** — It was observed from the result obtained that adrenalectomy decreases and administration of hydrocortisone increases the activity of cytosolic and mitochondrial malate dehydrogenase in the liver of 15-, 30- and 60-day old mice. Per cent decrease following adrenalectomy is almost similar in all the postnatal ages studied. However, the magnitude of increase of cytosolic MDH of 15-day old mice was higher compared to the other two ages studied. This indicates that adrenal steroid do play a role in the regulation of this isoenzyme. The magnitude of increase of cytosolic malate dehydrogenase at 15-day old mice is higher may be because of the endogenous level of glucocorticoid receptor and/or post-receptor events at this postnatal age (Böhme et al., 1986). The increase of NAD⁺-linked isocitrate dehydrogenase by hydrocortisone and estradiol in the liver and brain of rats of various ages has been studied by Yadav and Singh (1980). Adrenalectomy lowers the level of NAD⁺ linked isocitrate dehydrogenase considerably. Administration of hydrocortisone to rats increases the activity significantly in the adult, but not in the old animals. The effects are not so pronounced for NADP⁺-linked isocitrate dehydrogenase. Kanungo and Gandhi (1972) showed that the level of mitochondrial malate dehydrogenase decreases in the liver of young rats after adrenalectomy, but not in old adrenalectomized rats. Sharma and Patnaik (1982) have also reported that the magnitude of induction of liver cytosolic malate dehydrogenase by hydrocortisone decreases as a function of age.

Adrenalectomy decreases and administration of hydrocortisone to adrenalectomized mice increases the activity of kidney cytosolic and mitochondrial malate dehydrogenase only in 30- and 60-day old mice. It does not show any effect on the activity of kidney malate dehydrogenase.
(cytosolic and mitochondrial) in preweaned mice (15-day old). These findings corroborate the observation (Herzfeld and Greengard, 1969) that: a) the same enzyme in different tissues of the developing animals need not be regulated by the same physiological stimuli. b) in the same tissue, the developmental formation of an enzyme may not be regulated by one particular signal but by the interaction of many others. c) the hormonal signals important for the developmental formation of the enzyme may or may not regulate the level of the same enzyme in adult tissues.

The genes responsible for the synthesis of cytosolic and mitochondrial malate dehydrogenase are reported to be different (Whitt, 1971; Basaglia, 1989). The inducibility of mitochondrial malate dehydrogenase by hydrocortisone is significantly lower than that of cytosolic MDH. This may be due to the differential responsiveness of both the genes of cytosolic and mitochondrial malate dehydrogenase isoenzymes towards hydrocortisone such as, their location on the chromosomes, availability for the inducer, nature of the trans-acting factors. Previous studies (Kanungs and Gandhi, 1972; Sharma and Patnaik, 1982) on inducibility of liver cytosolic and mitochondrial malate dehydrogenase isoenzymes by cortisone as well as hydrocortisone in the rats of various ages also indicate that the level of these isoenzymes are regulated by the adrenal corticoids.

**Regulation of aspartate aminotransferase isoenzymes by hydrocortisone** — Results on hormonal regulation of shuttle enzymes demonstrate that adrenalectomy decreases and administration of hydrocortisone increases the activity of cytosolic aspartate aminotransferase significantly in the liver of all the three postnatal ages (15-, 30-, and 60-day old). These observations point out that adrenal steroids also play a role in the regulation of this isoenzyme. However, adrenalectomy and hydrocortisone treatments do not exhibit any significant effect on the activity of m-AsAT of the liver of mice at these postnatal ages studied. The results are in agreement with the earlier studies (Bulankina and Movchan, 1977; Sharma and Patnaik, 1982) wherein, mitochondrial AsAT was shown to be irresponsive to glucocorticoids.

Adrenalectomy decreases and the administration of hydrocortisone increases the activity of kidney cytosolic aspartate aminotransferase in post-weaned mice (30- and 60-day old). Since both the isoenzymes of AsAT are genetically independent (Braunstein, 1973), they differ from one another even in the pre- and post-mitotic tissues for their responses towards hydrocortisone. Although both the isoenzymes of AsAT are involved in gluconeogenesis, it is the cytosolic isoens-
zyme whose activity is regulated by glucocorticoids (Shield and Roth, 1965; Shrago and Lardy, 1966; Sharma and Patnaik, 1982, 84,85).

Herzfeld and Greengard (1971) reported that the amounts of the two forms of aspartate aminotransferase are subject to different physiological controls in different tissues. The response of the liver isoenzyme to hormones depends on the stage of development and after maturity on the sex of the animal. Adrenalectomy and hydrocortisone do not show any effect on kidney aspartate aminotransferase in 15-day-old mice. The similar phenomenon was observed in the case of kidney MDH isoenzymes at this postnatal age of mice. It may be due to the differential level of glucocorticoid receptors and or other trans-acting factors in the liver and kidney of mice during this phase of postnatal development (Kalimi et al., 1988; Ming-Jerand and O'Malley, 1994; Borbhuiya and Sharma, 1995). It has earlier been reported that the degree of induction of cytosolic aspartate aminotransferase by hydrocortisone in the liver and brain decreases with increasing age of the rat. This was attributed to the gradual loss in corticosteroid receptors and/or certain regulatory changes which occur in the genome and decreases the responsiveness towards hormone-receptor complexes (Sharma and patnaik, 1982). These studies have shown that factors like hormones, their receptors, and the tissue-specific trans-acting factors, needed for expression of specific genes are important for the maintenance of the levels and adaptive response of enzymes (Sharma, 1994; Kanungo, 1994).

Regulation of malate-aspartate shuttle enzymes by dibutyryl cyclic AMP (Bt2-cAMP) and Combination of Bt2-cAMP and Hydrocortisone — Past couple of years, group of workers visualized the cross-talk between the steroid and protein/peptide hormone action. The discovery of cross-talk between membrane-associated receptors and intracellular steroid and thyroid hormone receptors has gained much attention in recent years because of its multiple functional implications and biomedical significance (Sharma, 1993).

Various doses of Bt2-cAMP, a membrane permeable analog of cyclic AMP, were administered in different postnatal ages of normal male mice (i.e. 15-, 30- and 60-day old). None of these single doses of Bt2-cAMP were effective on both isoenzymes of malate dehydrogenase and aspartate-aminotransferase in the liver and kidney of mice. These findings indicated that none of the shuttle enzymes of mice liver and kidney are regulated by cAMP at those postnatal ages studied. Hydrocortisone alone on intact mice fails to give any effect on the shuttle enzymes,
when used as a single dose. Hence, in order to find out the synergistic or antagonistic role of cAMP on hydrocortisone action, a combination of Bt2-cAMP with hydrocortisone was injected in the mice of above age groups. It has been seen that only liver mitochondrial malate dehydrogenase and cytosolic aspartate aminotransferase show an increase in the activity. In case of mitochondrial malate dehydrogenase, the increase in activity has been seen only at 30- and 60-day of postnatal age. The activity of both the shuttle enzymes show no change with this combination in the kidney of mice at either of the age groups. This indicates that only liver and not the kidney is possibly equipped with the cross-talk mechanism in regulating the enzyme activity. Aggerbeck and coworkers (1993) reported that cytosolic aspartate aminotransferase (c-AsAT) is a ubiquitous enzyme that displays liver-specific hormonal regulation. Both the activity as well as mRNA level of c-AsAT are increased by glucocorticoids and the effect is potentiated and inhibited by cAMP and insulin, respectively in cultured hepatoma cell lines. The presence of two regulatory regions in the cytosolic AsAT promoter separate the positive cAMP effect from the negative insulin effect. Toussaint et al (1994) studied the expression and regulation of the rat testis cytosolic aspartate aminotransferase gene and showed that the pattern of transcription inhibition and polyadenylation site selection of a housekeeping gene can be tissue-specific. Aruzzese et al (1995) reported that the translation of mRNA for the two isoenzymes of AsAT is subject to tissue-specific regulation in an age-related manner.

**Regulation of malate-aspartate shuttle enzymes by thyroid hormone** — Thyroid hormones have been implicated in controlling development and differentiation of many animals. Böttger et al (1970) studied many gluconeogenic enzymes (pyruvate carboxylase, phosphoenolpyruvate carboxykinase and pyruvate kinase) under different states of thyroid function and have shown variations in the involvement of these enzymes in controlling the overall rate of hepatic gluconeogenesis. This envisaged the role of thyroid hormones in gluconeogenesis. It has been reported that in thyroidectomized rats, there is a marked decrease in mitochondrial citrate, 2-oxoglutarate and glutamate with a smaller changes in aspartate and malate. These changes are interpreted as providing evidence for the importance of modification in the malate-aspartate shuttle in hypothyroidism, albeit to a moderate degree. In our study, administration of T3 which is a potent thyroid hormone, in normal mice of three different postnatal ages (i.e. 15-, 30- and 60-day) showed no significant change in the activities of cytosolic and mitochondrial malate dehydrogenase as well as aspartate aminotransferase in the liver and kidney of mice. Most likely, this might be due to the tonic regulation of these enzymes by the endogenous circulating level of the
thyroid hormone.

**Purification and properties of cytosolic aspartate aminotransferase:**

To find out the change, if any, as a function of age in the chemical and kinetic properties of one of the shuttle enzymes, cytosolic aspartate aminotransferase was isolated and purified from the liver of mice of two selected ages. One of the age (15-day old) we selected as immature (preweaned) and the other as reproductively mature (180-day old). The enzyme preparations from both the ages were passed through the CM-cellulose column. Elution profile of the specific activities of this isoenzyme from the liver of two ages of mice exhibited the requirement of two different ionic strengths. c-AsAT from immature mice eluted out at the ionic strength of 0.11 M sodium acetate buffer whereas, from mature mice, it eluted out at 0.14 M sodium acetate buffer. This indicates that there might be an overall charge difference on the isoenzyme from two different age groups. It was further confirmed by non-denaturing polyacrylamide gel electrophoresis.

Using non-denaturing polyacrylamide slab gel of 7.5% cross-linking, the preparation representing the immature (15-day) and mature (180-day) cytosolic aspartate aminotransferase showed the presence of one major and one or two minor bands in both the cases, when the gels were stained for general proteins. However, when the gels were stained specifically for this enzyme, they showed the presence of a single band for both the ages of mice. Further, this band corresponds to the major band obtained after staining for the general proteins. The relative mobilities of the isoenzyme from 15-day old mice was more compared to the isoenzyme from 180-day old. This finding supports our earlier observation of ion binding properties of this isoenzyme at two ages onto ion exchange chromatography. These observations entail marked difference in the overall charge of the liver cytosolic aspartate aminotransferase of the two age groups. Changes in the isoenzyme patterns and their electrophoretic mobilities have earlier been reported and reviewed (Sharma, 1988,94). They can arise due to genetic variability or sometimes due to epigenetic events (such as acetylation, phosphorylation and proteolysis). Depending on the metabolic demand isoenzymes control the biochemical pathways to commensurate the requirement at specific stage of development (Coppes, 1984). Patnaik and Kanungo (1975, 76) reported that cytoplasmic alanine-aminotransferase of rat liver shows a phenomenon of sequential changes in the isoenzymes pattern during aging of rats. c-AIAT is a dimer made up of two subunits, A and B and has two active isoenzymes; c-AIAT-A and c-AIAT-B. Polyacrylamide gel electrophoresis of
purified c-AIAT of the liver of 5-, 52-, and 100-week old female rats showed that the liver of the immature rat has only c-AIAT-A and the liver of old rats only c-AIAT-B. The adult rat liver has both the isoenzymes, but the level of c-AIAT-A was lower than c-AIAT-B. The A and B subunits are under the control of two separate genes. Hence, the sequential appearance and disappearance of the two isoenzymes during the lifespan of the rat could be due to the sequential expression and repression of specific genes responsible for the synthesis of their subunits.

**Kinetic studies on purified cytosolic aspartate aminotransferase:**

Kinetic studies on the purified c-AsAT, of the liver of immature and mature mice were carried out to elucidate structural changes, if any, which occur in the active site of enzyme molecule as a function of age. For both the immature and mature mice liver c-AsAT, hyperbolic curve was obtained when the velocity of the enzyme catalyzed reaction was plotted against varying concentrations of both the substrates (i.e. L-aspartate and α-ketoglutarate) by using enzfitter programme (Perella, 1988). The figures were drawn using the Michaelis-Menten equation and the insets of these figures were drawn using the Lineweaver-Burk transformation. The plots indicate that none of the two substrates exhibit allosteric effect on the enzyme activity. Analysis of data indicates no significance difference between the $K_m$ values of this enzyme for both the substrates in immature and mature mice. However, the enzyme from the mature mice showed higher $V_{max}$ and $K_{cat}$, indicating higher turnover compare to the immature one. This indicates that the c-AsAT from mature mice catalyses the reaction at a faster rate than that of c-AsAT from immature mice, although the binding affinities for substrates remained the same. It may be plausible that the substrate binding sites of the enzyme are not affected for by the charge difference between the enzymes from the two ages. However, the charge difference in the c-AsAT at two ages might contribute to the catalytic turnover of the enzyme at respective ages. The higher catalytic rate of mature enzyme might extend an adaptation to control the homeostatic function of the metabolic demands of the mature mice since malate aspartate shuttle is one of the major control points for glycolysis, Krebs cycle and gluconeogenesis. The c-AsAT from the liver of mice of both the ages are competitively inhibited by amino-oxyacetic acid (AoAA) with respect to L-aspartate and non-competitively with respect to α-ketoglutarate. The $K_i$ values of this enzyme for AoAA at immature and mature ages are similar. It is known that AoAA inhibits c-AsAT competitively with respect to its amino acid substrate and noncompetitively with respect to its keto acid substrate (Braunstein, 1973; Rej, 1976; Sharma and Patnaik, 1982).
The kinetic parameters of a number of enzymes have been measured as a function of age. Studies on pyruvate kinase (Chainy and Kanungo, 1978) of the brain, myosin ATPase (Koldor and Min, 1975; Srivastava and Kanungo, 1979) and aldolase (Gershon and Gershon, 1973) of skeletal muscle, cytosolic alanine-aminotransferase of liver (Patnaik and Kanungo, 1976) cytosolic aspartate aminotransferase of rat liver (Sharma and Patnaik, 1982) showed that, in general, there is no significant difference between $K_m$, $K_i$, and molecular weight from young and old rats. Reiss and Gershon (1976) and Gupta and Rothstein (1976) have proposed that the differences in the kinetic parameters of enzymes seen in old animals may be due to post translational modifications. Recently, a similar kinetic difference in the $V_{\text{max}}$ and $K_{\text{cat}}$ of inorganic pyrophosphatase in immature and mature chicken liver has been reported (Syiem, 1996). The kinetic differences in the catalytic efficiency of enzyme without affecting the affinities for substrate have been attributed to adaptational significance depending on the age-specific metabolic demand in animal's tissues.

**Unfolding and inactivation of c-AsAT:**

A comparison of the result of unfolding and inactivation studies using different concentrations of urea on the purified liver c-AsAT of immature and mature ages of mice was performed. c-AsAT from the liver of mature mice required higher concentration of urea to attain 50% inactivation than that of the enzyme from immature mice. It indicates that the immature c-AsAT is more susceptible compared to mature one as for urea denaturation is concerned. This in turn point out that the enzymes are depicting differential folded structure which in corroborates with our earlier assumption of differences in the overall charge of the enzymes at two ages.

It may be concluded that the enzymes of malate-aspartate shuttle as well as the shuttle activity expressed differentially in different tissues of mice as a function of postnatal development. And the shuttle enzymes are also regulated differentially by glucocorticoid, whereas they do not exhibit any change with the exogenously added cyclic AMP as well as thyroid hormones. However, a combination of cAMP and glucocorticoid regulates the shuttle enzymes differentially in a tissue- and age- specific manner. Purification and kinetic analyses show a definite charge difference in c-AsAT at two different ages i.e. immature and mature. The $K_m$ remains the same while catalytic efficiency is higher in mature as compared to immature, owing to greater adaptation in mature animals.