Intricate regulatory mechanisms have evolved in living cells to control the concentration of various enzymes that catalyze critical reactions. Enzymes are specific proteins that catalyze chemical reactions in biological systems. Enzymes are the direct phenotypic expression of their genes. The feature which characterizes all developing and aging populations is the progressive development and gradual impairment in the ability to adapt to the environmental changes. Adaptation at the biochemical level can be attributed to the alterations in the rates of synthesis and degradation of enzymes as well as changes in physiological activities. During development and aging, different metabolic adjustments take place as an adaptation in different tissues to the changing demands made upon them.

Several physiological and biochemical changes occur during development, growth, adulthood and senescence of an organism. 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 life span. 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.

Study of all the enzymes of a particular metabolic pathway provides a complete profile of the biological functions as a function of age. Considerable amount of literature has developed concerning changes in enzyme level as a function of age and has been well reviewed (Wilson, 1972; Sharma, 1988, 1994). Keeping in view the importance of studying all the enzymes of a particular metabolic cycle, the work embodies in this thesis was planned to study the developmental and hormonal regulation of malate aspartate shuttle enzymes in different tissues of chicken during postnatal development. As there are set differences in the metabolic requirement of different groups of organisms, it was important to find out the malate aspartate shuttle regulation in chicken in order to compare and contrast with mammalian malate-aspartate shuttle.

The most active NADH shuttle for the movement of reducing equivalents (in the form of NADH) from the cytoplasm to the mitochondria in various tissues is the malate-aspartate
shuttle (McDonald, 1983). The reducing equivalents of cytosolic NADH are first transferred to cytosolic oxalacetate to yield malate by the action of cytosolic malate dehydrogenase (c-MDH). The malate carrying reducing equivalents enters into mitochondrial matrix wherein it transfers them to the matrix NAD⁺ regenerating oxalacetate 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 the mitochondria. Oxalacetate is a physiologically important intermediate of several metabolic pathways. These pathways may either be catabolic (Krebs cycle) or anabolic (gluconeogenesis) in nature. Since oxalacetate is impermeable to mitochondrial membrane, the malate-aspartate shuttle appears to be the primary mode of clearance of NADH reducing equivalents from the cytosol to mitochondria and vice-versa.

The enzymes of malate-aspartate shuttle are the malate dehydrogenase and the aspartate aminotransferase constituting of two homologous and genetically independent isoenzymes localized in the cytosol (c-) and mitosol (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 oxalacetate, 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. Although gluconeogenic reactions are similar in all organisms, the metabolic context and the regulations of the pathway differ from organism to organism, and from tissue to tissue (Lehninger et al., 1993). The major gluconeogenic precursor in chicken liver is lactate instead of pyruvate. The crucial gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK), found predominantly as a cytosolic enzyme in rats and mice, is localized in mitochondria of chicken liver and kidney (Ogata et al., 1982). Oxalacetate produced in mitochondria is converted directly to phosphoenolpyruvate (PEP) by a mitochondrial PEPCK. The PEP is then transported out of mitochondria and serves onto the gluconeogenic pathway. 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 internal milieu within the 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.
Keeping in view the differences in metabolic make up in different groups of organisms, the present thesis describes the regulatory changes in the enzymes of malate-aspartate shuttle during postnatal development to get insight into such metabolic cycle as a function of age. In order to study the regulatory changes in the shuttle, the findings are grouped as:

(i) the endogenous activity levels of shuttle enzymes in different postnatal ages and their tissue-specific patterns.
(ii) Regulation of shuttle enzymes by various hormones such as glucocorticoid and thyroid hormone during postnatal development of chicken
(iii) Reconstitution studies of the malate-aspartate shuttle system to confirm the endogenous level and hormonal regulation of enzymes, and
(iv) Chemical and kinetic properties of one of the shuttle isoenzymes (cytosolic aspartate aminotransferase) from the liver of two different ages (0- and 60-day) to find out change, if any, in such properties as a function of age.

Endogenous activities of malate-aspartate shuttle isoenzymes:

The activity of isoenzymes of malate dehydrogenase and aspartate aminotransferase in the male chicken exhibited tissue specificity as well as age-related changes. The activity (U/mg protein) was observed to be highest in the liver and kidney followed by a lower activity in the heart and brain tissues. The high activity of malate-aspartate shuttle enzymes in the liver and kidney tissue, compared to others may be ascribed to the fact that liver and kidney metabolic functions especially at these ages are not only dictated by local needs but also directed towards the control of other systems, therefore requiring higher inputs. Secondly, development and growth in general will require additional enhanced biosynthetic turnover. In avian system, hatching is followed by a prodigious growth rate (30-50 times their hatching weight) which is not exhibited by any other class of vertebrates and is induced and regulated by growth hormone (Ricklefs, 1983). Growth hormone (GH) level which is highest between 4-8 weeks and decreases by the 12th week has earlier been reported in chickens (Harvey et al., 1977, '79). Fat levels are reported to increase during the growth period in aves, but there are marked differences between species. Greater activity of shuttle enzymes in the liver and kidney of chicken clearly indicate the higher role of malate-aspartate shuttle in these tissues compared to heart and brain tissues (Lyngdoh and Sharma, 2001). As compared to mammalian
shuttle, the chicken shuttle enzymes exhibit lower activity, indicating lower role of this shuttle in chicken for the transfer of reducing equivalents from the cytosol to mitochondria.

The endogenous activities of isoenzymes of malate dehydrogenase and aspartate aminotransferase show a significant change during postnatal development of chicken. The activities of cytosolic malate dehydrogenase (c-MDH) was significantly higher in the liver of day 30 as compared to day 0 and 60. In contrast, mitochondrial malate dehydrogenase (m-MDH) activity was higher at day 0 and 30 in the liver. However, both c- and m- MDH had significantly lower activities at day 0, which increased sharply at day 30 and 60 in the kidney. It however, did not exhibit any significant change in the heart and brain tissues at different postnatal ages studied, except a gradual increase in the cMDH activity in the heart tissue only. On the other hand, activity of both cytosolic and mitochondrial aspartate aminotransferase (c- and m- AsAT) showed a peak value at day 30 in both the liver and kidney. In the heart and brain, however, both the isoenzymes showed a steady decrease during postnatal development except c-AsAT of heart which increased in level at day 30 and 60 as compared to day 0. The higher level of cAsAT in the liver, kidney and heart at day 30 may be correlated with a higher degree of transamination during these phases in the life span of chicken. These findings indicate not much role of this shuttle at day 0 and implicate the involvement of malate-aspartate shuttle at a relatively advance age in the transfer of reducing equivalents to compensate the metabolic demands in these tissues of growing chicken. MDH isoenzymes showed a differential pattern of activity in the kidney as compared to liver at day 30 and 60 of postnatal ages studied. This is in agreement with the previous report that kidney has a unique role in gluconeogenesis in chicken (Watford et al., 1981). The MDH isoenzymes also showed a pattern of activity expression like that of asparate aminotransferase isoenzymes in all tissues studied. Earlier reports have shown that the rates of the mitochondrial and cytoplasmic enzyme must be equal for the steady operation of this shuttle (Wiseman et al., 1991). Sharma et al., (1992) have also reported an early expression of malate aspartate shuttle activity and its enzymes in the liver of mice as compared to kidney. The findings on chicken shuttle enzymes corroborate the metabolic differences in the chicken as compared to rats and mice. It also manifests an age- and tissue-specificity with respect to shuttle activity.
Hormonal regulation of shuttle enzymes:

Alterations in the level of enzymes and their inducibility by certain hormones are age-related phenomenon (Wilson, 1972; Adelman, 1975; Kanungo, 1980; Sharma, 1988). Much interest focusing on the mechanisms of hormonal regulation involving feedback control, synergistic, reciprocal interactions and cross-talk in signal transduction appeared in recent reviews (Wada et al., 1990; Sharma, 1993). It must be emphasized that a change in the activity cannot be directly equated with a change in enzyme concentration and a number of other possible mechanisms exist to affect enzyme induction and/or repression (Walker, 1983; Sharma, 1994). During development, strong influence of growth hormones (GH) in aves has been well established and reviewed (Scannes et al., 1984; Harvey, 1990). Further, in aves, it has been shown that amongst the plethora of GH-responsive system, only changes in endocrine function appear to provide inhibitory feedback in GH regulation. The hormones involved are insulin, glucagon, corticosterone, somatostatin and triiodothyronine, exhibiting either synergistic and/or antagonistic interaction (Wada et al., 1990).

The results on hormonal studies indicated that there exists differential response to these hormones viv-a-vis age and tissue of chicken. At these ages (0-, 30- and 60-day) used in this study, avian tissues undergo marked growth spurt and are under complex hormonal control, with many hormones influencing development of different organs and tissues. The influence of GH, T₃ and steroid hormones in avian development and the morphological changes have been well documented (Freeman and Vince, 1974; Scannes et al., 1990; Harvey, 1990; Kuhn et al., 1991) together with their combinational effects. Further, the role of hormones in controlling growth and development and also in homeostasis has been envisaged (Scannes et al., 1990).

Administration of hydrocortisone (HC) increases the activity of c-MDH in the liver of chicken at all ages studied and increases the activity of m-MDH only at day 0 compared to other postnatal ages. The magnitude of increase of c-MDH at different postnatal ages is variable indicating that glucocorticoids do play a role in the regulation of this isoenzyme and the variability may be due to the endogenous level of glucocorticoid receptors and/or post-receptor events at different postnatal ages.
(Bohme et al., 1986). It confirms earlier reports that injection of adrenal cortical extracts resulted in retardation of growth in chicken embryo is dependent on the stage of embryonic development. In kidney, however, hydrocortisone increased only the activity of m-MDH at day 30 compared to other postnatal ages, corroborating earlier reports in mammals that the same enzyme in different tissues of developing animals might be regulated differentially by the same physiological stimuli. The differential effect of hydrocortisone also takes place in different tissues of chicken during development. The gene responsible for the synthesis of c- and m-MDH are reported to be different (Whitt, 1970). The variation in inducibility may be due to differential responsiveness of genes of c- and m-MDH isoenzymes towards hydrocortisone. On the other hand, hydrocortisone administration does not seem to be involved in the regulation of AsAT isoenzymes in liver and kidney of chicken at all postnatal ages studied, indicating that AsAT may not be responsive to glucocorticoids in chicken. This is in contrast to the glucocorticoid regulation of c-AsAT in rats and mice (Sharma and Patnaik, 1982). It also reflects that the role of glucocorticoid in avian and mammalian systems may be different due to variability in their metabolic context (Lehninger, 1993). Non-inducibility of cAsAT isoenzyme by hydrocortisone a gluconeogenic inducer, in chicken liver and kidney indicate that this isoenzyme may not be involved in gluconeogenesis so much as in case of mammalian system. In rats and mice, cAsAT is induced at the transcriptional level by glucocorticoids as well as at enzyme level (Santa and Sharma, 1997). It is well documented that major gluconeogenic precursor in chicken is the lactate and not the pyruvate.

Thyroid and pituitary hormones are known to influence growth in chicken (Scanes et al., 1983). However, there are variable reports on attempts to stimulate growth in intact chicks with exogenous T₃ or T₄. For instance, growth of broiler chicken was not affected by T₄ administration in the diet, while growth was decreased by dietary T₃ (May, 1980, 1982; Scanes et al., 1983). In addition, studies made by feeding a dietary supplement of T₃ was observed to interact genotypically and combination of T₃ and growth hormone exerts variable growth promoting effects in different tissues (Marsh et al., 1984 a&b). Studies on growing broilers using long term thyroid hormone supplement resulted in the decrease of both growth and fat deposition, with T₃ being more effective than T₄ (Kuhn et al., 1991).
Results on the hormonal regulation by triiodothyronine (T₃) of shuttle enzymes demonstrate that T₃ increased the activity of both isoenzymes of MDH in both the tissues studied at different postnatal ages of chicken. Our findings also exhibit that the magnitude of increase in the activity of both isoenzymes of MDH is significantly higher at day 30 and 60 as compared to day 0 in both liver and kidney. This is in agreement with the earlier reports that the effects of thyroid hormones at the cellular level are slow to occur in chicken at early stage of life span (Hazelwood, 1986). The effect, however, does not influence isoenzymes of AsAT in liver and kidney except for an increase in m-AsAT level in 0-day and 60-day old chicken liver, corroborating that both the isoenzymes of AsAT are also genetically independent differing from one another even in different tissues for their response towards triiodothyronine. These studies indicate that factors like hormones and their adaptive responses to enzyme exhibit a tissue- and age-related patterns depending on the metabolic state of the concerned tissue at that phase of organism's life span.

Shuttle reconstitution studies:

In order to confirm the differential expression of malate-aspartate shuttle enzymes in the liver and kidney of chicken, the shuttle activity in a reconstituted system was studied. In vitro reconstitution of malate-aspartate shuttle showed a higher activity in the liver of 30-day old chicken compared with that in 0-day chicken. A similar pattern could also be seen in the kidney of 30-day old compared to that in 0-day chicks indicating that the shuttle activity corresponds similarly to the expression of enzymatic activities in the liver and kidney of developing chicken, exhibiting higher level at day 30 in both the tissues of chicken. This also indicates that the role of this shuttle may be at a slower pace in 0-day old chicks and boosts to a higher level during later part of postnatal development of chicken.

The activity of malate-aspartate shuttle in the liver of 0-day and 30-day old chicken also showed a higher activity in T₃-treated chicken as compared with control, untreated chicken. An identical pattern was also seen in the kidney of 0-day and 30-day reconstituted system confirming that the shuttle activity is induced upon T₃ administration in parallel with induction of enzymatic activities by T₃ treatment during postnatal development of chicken. These reconstitution studies confirm a
similar pattern of endogenous and hormone treated activities of malate-aspartate shuttle enzymes in developing chicken in a similar way as seen in mice (Sharma et al., 1992).

**Chemical and Kinetic properties:**

In order to determine 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 chicken of two selected postnatal ages. One of the age (0-day) was selected as immature and the other as mature (60-day). 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 chicken exhibited the requirement of the same ionic strength (0.11 M) of sodium acetate buffer. This indicates that there is no difference in the charge content of the enzyme molecule between the two different age groups. The degree and the yield of purification achieved are approximately similar for both the ages. It was further confirmed by non-denaturing polyacrylamide gel electrophoresis.

Using non-denaturing (Native) polyacrylamide slab gel of 7.5% cross-linking, the preparation representing the immature (0-day) and mature (60-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 chicken. Further, this band corresponds to the major band obtained after staining for the general proteins. The mobility of this enzyme of both 0- and 60-day old chicken on acrylamide gel is similar. These observations also indicate that the net charges on the enzyme molecule of the liver does not alter as a function of age of chicken. It also corroborates with the earlier reports that the number of bands varied from tissue to tissue but the electrophoretic mobilities of a given form in all tissues were analogous (Imperial et al., 1989).

Kinetic studies on the purified c-AsAT, of the liver of two age groups (0-day and 60-day old) were carried out to elucidate kinetic changes, if any, which occur in the active site of enzyme molecule as a function of age. Both the 0- and 60-day old
chicken liver c-AsAT showed a hyperbolic curve when the velocity of cytosolic aspartate aminotransferase reaction was plotted against varying concentrations of both the substrates, that is, 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 these substrates do not exhibit allosteric effects on the enzyme activity. Analysis of data indicates no significance difference between the \( K_m \), \( V_{max} \) and \( K_{cat} \) values of this enzyme for both the substrates in 0-day and 60-day old chicken liver. Albeit, the \( K_m \) values were higher for α-ketoglutarate than for aspartate indicating that the affinity for L-aspartate is much more than for α-ketoglutarate at either ages. The c-AsAT from the liver of chicken 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 \( K_v \) values of this enzyme for AoAA at 0- and 60-day old chicken are also 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 in various groups of animals (Braunstein, 1973; Rej, 1977; Sharma and Patnaik, 1982). The aminotransferase inhibitor, AoAA, completely inhibited gluconeogenesis from lactate in the perfused rat liver and to a small extend in the perfused chicken liver. In chicken liver, the highest rate of glucose production was seen with lactate, followed by fructose, pyruvate, and glycerol (Sugano et al., 1982). Excess cytosolic reducing equivalents generated by the oxidation of lactate to pyruvate are transferred from the cytosol to the mitochondria by the malate-aspartate shuttle. Amino-oxyacetic acid inhibits the shuttle and, consequently, glucose synthesis for want of pyruvate (Ochs and Harris, 1980).

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 and aldolase of skeletal muscle, cytosolic alanine aminotransferase of liver, 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. Differences in the kinetic parameters of enzymes seen in older animals may be due to post translational modifications. This kinetic difference in the \( V_{max} \) and \( K_{cat} \) has been reported in immature and mature chicken liver inorganic pyrophosphatase (Syiem,
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.

The present findings provide firm support to the view that the enzymes synthesized in 60-day old chicken are structurally similar to those of 0-day old chicken. Hence, the genes coding for these enzymes do not undergo any structural alterations during such period of chicken's life span. Therefore, the alterations in the levels and differential induction of these enzymes that occur as a function of age may be due to the changes in the template activities of the corresponding genes which are brought about by various modulators (Kanungo, 1980, '94).

**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 chicken was performed. c-AsAT from the liver of both the ages depict no differential folded structure since urea requirement for 50% inactivation remained the same. These findings indicate that the molecular structure of this isoenzyme does not change at these two postnatal ages of chicken.

Taken together, it can be concluded that the malate-aspartate shuttle enzymes exhibit age- and tissue-specificity with respect to its activity expression and further the shuttle enzymes are also subjected to hormonal regulation in an age- and tissue-specific manner. Purification and kinetic analyses of cytosolic aspartate aminotransferase of the liver of 0-day and 60-day old chicken reveal that the structure of the enzyme molecule remains unaltered during such phases of chicken's life span. These findings indicate that factors like hormones and their adaptive responses to enzymes depend on the metabolic state of the concerned tissue at that phase of the organism's life span regulated by the level of hormones, their receptor/post-receptor events and tissue-specific factors needed for the expression of cognate genes in an organism-specific manner brought about by various extrinsic or intrinsic factors.