Developmental and hormonal regulation of malate-aspartate shuttle enzymes in chicken

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I, Herbert Goldfield Lyngdoh, hereby declare that the subject matter of thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/ Institute.

This is being submitted to the **North Eastern Hill University** for the degree of **Doctor of Philosophy** in **Biochemistry**.

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(Herbert Goldfield Lyngdoh)

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ABBREVIATIONS

BSA	:	Bovine serum albumin
HC	:	Hydrocortisone
T_3	:	Triiodothyronine
c-MDH	:	Cytosolic malate dehydrogenase
c-AsAT	:	Cytosolic aspartate aminotransferase
m-MDH	:	Mitochondrial malate dehydrogenase
m-AsAT	:	Mitochondrial aspartate aminotransferase
PAGE	:	Polyacrylamide gel electrophoreses
EDTA	:	Ethylene diamine tetra acetic acid
CM	:	Carboxy Methyl
Tris	:	Tris-(hydroxymethyl) aminomethane
TEMED	:	N,N,N',N'-tetramethyl-ethylediamine
NADH	:	Nicotinamide adenine dinucleotide
NaCl	:	Sodium Chloride
HCl	:	Hydrochloric acid
$MgCl_2$	:	Magnesium chloride
KCl	:	Potassium chloride
$(NH_4)_2SO_4$	:	Ammonium sulphate
NaOH	:	Sodium hydroxide
AoAA	:	Amino-oxyacetic acid
α-KG	;	α-ketoglutarate
K_2HPO_4	:	Dipotassium hydrogen orthophosphate
KH_2PO_4	:	Potassium hydrogen orthophosphate

INTRODUCTION

Living organisms have evolved a very complicated regulatory mechanisms for controlling the levels of their enzymes, specially those catalyzing critical reactions involved in adaptation to environmental changes. Enzymes are specific proteins that catalyze chemical reactions in biological systems. The feature, which generally characterizes all multicellular organisms, is the progressive development and gradual impairment in the ability to adapt to the environmental changes. At the biochemical level, adaptation can be expressed as the alterations in the rates of synthesis and degradation of enzymes as well as changes in physiological activities. It is facilitated by regulated rate of synthesis and/or degradation and by post-translational modification of enzymes. Higher organisms, including humans, are like cellular cities in which groups of cells perform specialized functions and cooperate by intricate system of communication. Each specialized group of cells, termed as tissues, requires different rates and patterns of metabolic adjustments during the development of animals. Essentially, all the metabolic reactions are catalysed by enzymes. Therefore, enzymes are one of the prime factors that play pivotal role in regulation of metabolic adjustments as an adaptation to the changing demands made upon developing animals.

Life span is a continuum of development and growth at one end and deterioration of functions or senescence or aging at the other end. In between is the reproductive phase or adulthood. Life span is programmed into the developmental patterns of the organism. The programme onsets as soon as the fertilized egg begins to grow (Hanawalt, 1987). This genetic programme determines the maximum life span for each species, for example, mayflies live only 1 day, houseflies 30 days, rats 3 years, horses 25 years and humans 100 years. Further, all individuals of a species have a more or less similar life span, long-lived individuals have generally long lived progeny, identical twins have almost similar life span. Life span can be defined as the duration of life of an individual or organism in a particular environment. The life span of a multicellular organism may be broadly divided into three phases: development (growth), reproductive period (adulthood) and senescence (aging). The time of onset, duration and rate of each phase (Kanungo,1994) is dependent on the vigour and vitality of the other, and are therefore interrelated. Development normally involves both quantitative and qualitative changes leading to suitable specialization of various cells, tissues and organs of the body, which is usually referred to as differentiation. The development of many animals is divided into prenatal and postnatal periods. For many years, attention was focused on events taking place before birth, when the most striking advances occur in the animals. Prenatal and postnatal stages are characterized by certain distinguishing features, such as morphologic, physiologic, biochemical and psychological features (Timiras, 1994). Accordingly, prenatal developmental studies encompass events that take place right upto birth or hatching, whereas, postnatal developmental studies extend upto at least the attainment of reproductive maturity which include the period of infancy and/or weaning, childhood, adolescence and adulthood. Aging, on the other hand, has been described as a process that causes a gradual decline in the function and adaptability to environmental changes following the onset of reproductive maturity.

The biological nature of aging was postulated to be pleiotropic in nature resulting from the normal by-products of living processes which may be from evolutionary non-selected endogenous properties of an organism. Aging is defined as the time-dependent drifting away of cells, cell organization and homeostatic control from their most optimum state of functions (Cutler, 1984). Two classes of pleiotropic aging processes were postulated to exist. One is associated with energy metabolism and has been called as continuously acting biosenescent processes. The second is associated with developmental processes and has been called developmentally linked biosenescent processes. It is not known which of these two classes might play the most important role in causing aging. Williams (1957), Medawar (1957) and Hamilton (1966) have proposed that a special class of pleiotropic gene whose expression is linked to development causes aging. There are also experimental data indicating that developmentally-linked hormonal processes such as those involved in sexual maturation can act to accelerate the aging process. Thus, aging is the result of pleiotropic effects of development, differentiation and maturation. These processes act to accelerate the destabilization of the differentiated state of a cell, cell organization in tissues and the general homeostatic state of organisms.

One of the basic characteristics of living organisms is their capability to adapt to an ever changing environment. Vertebrates such as fishes and amphibians are capable of an independent existence at relatively immature stages(larvae), with most or much of their development still before them. It is quite otherwise with birds, reptiles and mammals. The newborns are anatomically quite complete, yet utterly dependent on their elders for food and care. Throughout infancy, childhood and adolescence (for humans) come the completion of some organs and a gradual remolding of body shape (Arrey, 1966). These progressive changes continue and stabilize only on reaching adulthood and/or maturity. In higher animals realization of the full genetic potential involve increase in size, changes in structure and function (Kanungo, 1994).

Developmental processes and their regulation by heredity and environment have engaged biologists for many decades. Heredity and environment have been implicated to determine the physiological competence and length of the life span(Timiras, 1994). Longevity is determined by processes governing the time-dependent stability and differentiation. These processes are encoded into longevity-related genes and their levels of expression are governed by other regulatory genes. Environment supplies the external factors that make development possible and allows inherited potentials to find expression.

The transformation from egg to adult is fundamentally important to the study of avian biology. This has long been recognized by developmental biologists and poultry scientists, but only recently by most avian biologists (Ricklefs,1983). Growth of a chick is to traverse the developmental gulf between neonate and adult with a minimum of risks to itself and with efficient utilization of the care provided by its parents. The exact course of development depends to some extent upon the size and body proportions of the neonate and adult and the environment of the chick during its development. A general trend during postnatal development is from greater to lesser dependence upon parental care, and finally to complete independence. Chick development and parental care are complementary in that the behavior of adults is adjusted to the needs of their offspring. Development is gradual, but the progress of the chick can be measured against certain landmarks like hatching, acquisition of flight and independence.

Development begins with a single fertilized egg which in turn gives rise to cells that have different developmental fates. It includes an increase in the number and size of the cells, their differentiation to perform specialized functions and formation of organs. At the molecular level, several genes that play specific roles at specific times have been visualized and that each cell type may be characterized by its pattern of gene expression (Kanungo,1994). The development of an adult organism from a fertilized egg follows a predetermined path in which the principal controlling factor is gene expression at transcriptional level. However, there may be regulation at the processing, transport, and stability of RNA transcript. There also exists post-translational control of gene expression. Gene expression can also be controlled by signaling due to cell-to-cell interaction (Lewin, 2000).

One of the classical examples of sequential activation and repression of genes during development is that of hemoglobin during gestational period in human (Zuckerkandle, 1965). Haemoglobin, a tetrameric metalloprotein, consists of $\alpha_2\varepsilon_2$ chains in the fetus at the age of 1 to 2 months of gestation. In the later phase of gestation, the ε chain is replaced by the γ chain and just before birth, the γ chain is in turn replaced by β chains which together with the α chains comprise the adult haemoglobin characterized by differences in the electrophoretic mobility. The synthesis of these chains is governed by different sets of genes which are sequentially activated and repressed during the development of human fetus.

Genetic control of development can be best exemplified by studies on the fruitfly, *Drosophila melanogaster*. These studies demonstrate that development involves an orderly pattern of gene expression in which individual genes have both temporal as well as spatial specificity in their expression, and that some important controlling genes apparently maintain this pattern by regulating the action of other genes (Russel, 1987). Similar studies on the soil nematode (*C. elegans*) showed valuable informations relating to switching on and switching off of genes during development and aging process (Edgar and Wood, 1977: Sulstan and White, 1980). These studies indicate that the sequential activation and repression of genes could be a continuing process and may operate throughout the life span of an organism.

During senescence, on the other hand, the functional abilities of most organs and organism decrease. The decline becomes apparent towards the later part of the reproductive phase. Thus, the reproductive phase smoothly merges into senescence phase, unlike the transition from the developmental to the reproductive phase wherein specific genes are expressed and confer reproductive ability to an organism. An important feature of senescence is that reproduction ceases in this phase and no special structure or function appears, rather than those already present undergo change (Kanungo, 1994).

To explain the phenomena of aging, various theories have been proposed. These theories can be broadly divided into molecular, cellular and system level theories (Sharma, 1988;1994).

Molecular theories begin with the following assumptions-

(i) All individuals within a species have an almost similar length of life span.

(ii) Individuals from different species have different life span.

According to these theories, it is presumed that there is some genetic programme which determines the maximum life span for each species. An equally significant contribution to a genetic basis of aging is deduced from the duration of the three phases of life span. Molecular theories of aging include-

(a)*Codon restriction theory* which is based on the assumption that the fidelity or accuracy of translation in a cell depends on its ability to decode the triplet codons in mRNA (Strehler et al., 1971)

(b)*Somatic mutation theory* which states that mutations that occur randomly and spontaneously destroy genes and chromosomes in post-mitotic cells during the life span of an organism. This process causes a gradual increase in the mutation load which in turn decreases the production of functional proteins (Szilard, 1959)

(c)*Error theory* postulated by Orgel (1963) states that error occurring during information transfer, that is, transcription and translation may cause accumulation of defective proteins that may lead to the aging of an organism.

(d)*Gene regulation theory* proposed by Kanungo (1980,'94) states that senescence may occur due to the changes in the expression of genes after the onset of reproductive maturity. It is based on the presumption that senescence would follow a pattern similar to that of differentiation and growth, that is, a sequential activation and repression of certain genes which are unique to these phases.

Cellular theories relate to changes that occur in structural and functional elements of cell with the passage of time. These theories include Wear and tear, Age pigments, and Cross linking theory (Sharma, 1994).

(a) *Wear and tear-* Living organisms may be compared with the machines and as with repeated use, parts of machine wear out and become defective and the machinery finally fails to function (Sacher, 1977). However, the same cannot be applied to organisms since they have a well defined self controlled repair mechanisms which when fail can destabilize the system lending to aging and death.

(b) *Age pigments-* A marked change that occurs in the cell composition during aging is the increasing accumulation of age pigments (lipofuscin) in both the pre- and post-mitotic tissues of animals (Strehler, 1964: Reichel, 1968: Toth, 1968). It is one of the common morphological features associated with aging and has been correlated with the loss of neurons in old age (Brizee et al., 1969, 1975; Miquel et al., 1978).

(c) *Free-radical and cross-linking theory–* Harman (1986) proposed that the decrease in the adaptability of an organism during aging may be partly due to free radical-mediated damages in the body. Both the "Cross linking" theory of Bjorksten (1964) and the free radical ftheory of Harman (1986) are almost similar as both involve the process of inactivation of biomolecules by cross-linking due to free radical damages.

System level theories include neuroendocrine and immunologic theories. The overall performance of an animal is closely related to the effectiveness of a variety of control mechanisms that regulate the interplay between different organs and tissues (Shock, 1979). With aging, the homeostatic adjustments decline with consequent failure of adaptive mechanisms and that aging and death may be viewed as the result of this failure (Frolkis,1982; Timiras, 1994). The efficiency of immune system also decreases as a funtion of age (Walford,1969). The immune system has also been known to be influenced by hormones. Thus, a decline efficiency of endocrine and neural system with age also influence the immune system in several functions.

During the early period of development, the mother bears much of the responsibility for the maintenance of homeostasis, which serves to maintain a normal state in case of imbalance, and is necessary for growth. Homeostatic regulation together with inherited traits, define the competence with which the individual will continue to respond to environmental changes (Timiras, 1994). When the fetus leaves the uterus or when the egg hatches, there is an abrupt change in the manner and form in which the nutrients enter into the body for postnatal development of organisms. Metabolic routes change as an adaptation to the changing demands made upon them. Adaptation to these changes involve changes in the activities of many important enzymes in the body's tissue which in turn play a key role in bringing about developmental changes ensuring better adaptability of growing animals.

Enzymes during development:

Enzymes are specific proteins, baring some RNA, that catalyse chemical reactions in biological systems. Essentially, all biochemical reactions are enzyme catalysed. The most striking features of enzymes are their enormous catalytic power and specificity. They accelerate reactions by factors of 10^6 to 10^{12} as compared to uncatalysed reactions. They catalyse a wide variety of chemical reactions, such as oxidation-reduction, transfer of chemical moiety such as tranferases, hydrolytic reactions and lyases. Each enzyme catalyses only one type of reaction showing a high selectivity for both reactants and products. Within a single species, there may exists different forms of enzyme catalyzing the same reaction. These could differ from one another in terms of amino acid sequences and termed as isoenzymes. Isoenzymes are the enzymes that arise from genetically determined differences in amino acid sequences. For instance, malate dehydrogenase has an isoenzyme present in cytoplasm as well as in mitochondria of many different tissues (Price, 1989). Enzymatic studies were among the first biochemical changes demonstrated to occur during development and aging. Subsequently, a large literature has developed concerning the age-related changes in enzyme levels and has been well reviewed (Kanungo,1980; Walker,1983; Sharma,1988).The rate of synthesis of some enzymes changes in response to changes in the extracellular environment. In bacterial cells, these changes in enzyme synthesis are quite pronounce with many fold increase in the activity level of specific enzymes being observed. In animal cells, however, the situation is relatively different, although whole organism may be exposed to marked changes in the environment, mechanism exists which minimizes the number of cells or tissues to exhibit response (Walker, 1983). The vast majority of enzymes in multicellular organisms are expressed constitutively, differing from adaptive enzymes, a term which was introduced by Karstrom (1936) to describe those enzymes which are induced in a microorganism only when they grow in presence of specific substrates in contrast to constitutive enzymes which are present in a given organism irrespective of the composition of the medium in which the organism grows. Later, the term induction was defined as an increase in enzyme activity caused by a change in substrate concentration or by hormonal action (Cohn et al., 1953), whereas enzyme repression was defined as a relative decrease in the rate of synthesis of a particular apoenzyme resulting from exposure of cell to a given substance (Vogel, 1957). Induction of enzyme is attributed to several inducers, or effectors which may either be a substrate or a hormone or a metabolite or even an exogenous factor (Sharma, 1988). A substantial amount of literature and reviews are available on the relationship of mammalian hormone, enzyme levels and induction (Pitot and Yatvin, 1973; Dickerson and Basu, 1975; Kanungo, 1980; Walker, 1983; Sharma, 1988).

Growth and development proceed with characteristic alterations of the enzymes through which the organism acquires the capability of coping with the demands of altered environmental conditions as imposed by birth and postnatal life (Bohme et al., 1986). A number of studies have reported changes in enzyme activities with development and aging (Benzi et al., 1980; Nordenberg, 1981; Sharma, 1988,1994). Expression of enzymes during development varies with age, sex and strain of an organism. Glucose-6-phosphate dehydrogenase increases in the kidney of aging male, whereas it decreases in the females (Wilson & Franks, 1971) and that of lactate dehydrogenase increases in liver of aging male mice but shows no change in females (Wilson, 1972).The activity of pyruvate kinase does not change in the heart of old male rats but it decreases in the same tissues of old female (Chainy and Kanungo, 1978). Pyruvate kinase (PK II) isoenzyme is present in fetal rat liver but absent in adult tissue (Walker, 1974).

Most studied example is lactate dehydrogenase (LDH), a tetramer made up of two different subunits called M and H. These two subunits are controlled by two separate genes. Various isoenzymes of lactate dehydrogenase are formed by combination of M and H 'submits (M_4,, M_3H, M_2H_2, MH_3 & H_4). Each isoenzyme is characteristic of individual tissue or cell population and is subjected to different regulatory mechanisms which are primarily concerned with the conversion of lactate to pyruvate. M-type LDH is predominantly found in anaerobic and glycolytic tissues, where it converts pyruvate to lactate (Markert & Ursprung, 1962). LDH isoenzyme composition is not only tissue–specific but also changes in the same tissue during development (Markert & Moller, 1959). The greater proportion of M_4 isoenzyme is present in developing embryos of mammals as their metabolism is mostly anaerobic in nature. A shift towards H_4 occurs as development proceeds. Contrary to this, a greater proportion of H_4 isoenzyme is found in developing chick embryo which grows in aerobic milieu and shows a shift towards M_4–type during later stages of development.

These changes in the composition of isoenzymes are not restricted to development but extent into adulthood and aging (Kanungo, 1980). The proportion of M_4-LDH is considerably lower in the heart, skeletal muscle and brain of older rats, with concomitant increase of H_4-LDH. The shift in isoenzyme of LDH has been correlated with the differing metabolic functions of the organism as a function of age. The lower proportion of M_4-LDH may cause a decrease in the ability of the tissue to cope with anaerobic conditions (Singh and Kanungo, 1968). Studies on cytoplasmic alanine aminotransferase (c-AlAT) of the old rat liver revealed that the phenomena of sequential changes in the expression of proteins do extent to old age (Patnaik and Kanungo, 1976). Young (5 weeks) rat liver has A-type and adult (52 weeks) has both the isotypes (A- & B-) while old (100 weeks) has only the B-isotype. Both subunits are under the control of two separate genes (Chen and Giblett, 1971) and are sequentially activated and repressed at different phases of life span in rats (Patnaik and Kanungo, 1976).

Many studies have been done on changes in the activity of individual enzyme with aging (Benzi et al., 1980; Kanungo, 1980). However, the key enzyme of a particular metabolic pathway might provide a complete profile of their biological functions during development and aging (Sharma,1988). Studies on the developmental expression of arginine synthesizing enzymes during lactation and aging reveal that these enzymes are highly expressed in the small intestine of sucking and weaning rats. The activity of malate-aspartate shuttle enzymes in the liver and kidney of mice was differentially expressed in an age- and tissue-specific manner (Sharma et al.,1992;Santa, 1997). This may reflect differential metabolic transfer of reducing equivalents to commensurate the specific tissue's requirements at various developmental ages. Studies on the activities of key enzymes of glycolysis, krebs cycle and pentose phosphate pathways of several tissues at different ages have shown that these enzymes show different patterns of expression in different tissues at various ages (Keast, 1989).

Enzyme induction during development:

Similar to the cellular requirements for various proteins vary, the mechanism by which their respective genes are regulated also vary. The degree and the type of regulation reflect the function of the protein product of that gene. Some gene products are required all the time and hence they are expressed at a more or less constant level in virtually all the cells of an organism. Many of the genes for enzymes those catalyse reactions to control metabolic pathways, such as the citric acid cycle fall in this category. These genes are often referred to as housekeeping genes. Unregulated expression of such genes is called constitutive gene expression. The amounts of other gene products rise and fall in response to molecular signals. Gene products that increase in concentration under defined molecular circumstances are referred to as an inducible and the process of increasing the expression of such genes is called induction. Expression of many genes encoding DNA repair enzymes, for example, is induced in response to high level of DNA damage. Conversely, gene products that decrease in concentration in response to a molecular signal are referred to as repressible, and the decrease in gene expression is called repression. For example, high level of tryptophan leads to repression of the genes for the enzymes catalyzing tryptophan biosynthesis in bacteria.

A single gene generally contains in an eukaryotic transcription unit and that most of the cellular enzymes and proteins are encoded by solitary non repeated gene. In general, the rate of synthesis of mRNAs from solitary protein coding genes is sufficient to meet the cells requirement of various individual proteins. The rates of their transcription are controlled by several short DNA sequences, called *cis-acting elements*, spread over a region of >100 basepairs either upstream or downstream of the regulated gene. The transcription factors including steroid and thyroid hormone-receptors, termed as *trans-acting factors*, regulate the expression of specific gene in the presence of an enhancer element located at a variable distance from the promoter in either direction and orientation.

The enhancers are tissue-specific and activate the promoter of the gene only in that tissue and not in the other tissues (Gillies et al., 1983).

The promoter region consists of TATA box (centered at –30 basepair), CAAT box (-75 basepair) and GC box (-90 basepair). They are present in most genes and the proteins that bind to them are distributed widely. Therefore, they are not responsible for cell-specific expression of genes and are mainly responsible for generic transcription. Besides these sequences required for both initiation and efficiency of transcription, there are several tissues-specific cis-acting sequences which are involved in regulation of transcription and have specificity for binding to specific trans-acting factors including receptors for steroid and thyroid hormones, retinoic acid and Vit D. Steroid and thyroid hormones as well as retinoic acid receptors belong to a family of receptors that undergo conformational change after binding to their respective ligands. The receptor-ligand complexes then interact with definite cis-acting sequences of genes which are expressed in a tissue-specific manner and are responsive to these hormones (Allan et al., 1991; Wahli and Martinez, 1991; Wiseman et al., 1991).

Impacts of enzyme catalysed reactions may either be (a) by changing the absolute quantity of enzyme present or (b) by altering the pool size of reactants rather than the enzyme, or (c) by altering the catalytic efficiency of the enzyme (Rodwell, 1996). All the three options can be exploited in most organisms with the help of several types of inducers or effectors which may either be the substrate or a hormone or a metabolite or even an exogenous factor.

The expression of age-related adaptive changes in enzyme induction has been categorized into four general patterns of response: (i) the response has an altered adaptive latent period or initiation time following stimulus without affecting the magnitude of induction, (ii) the response shows decrease or increase in the magnitude of induction with no change in the latent periods, (iii) the response alters both the latent period and magnitude of induction and (iv) age-related changes do not occur in the induction pattern (Adelman, 1975; 1981). The patterns of enzyme induction, as in the case for enzyme levels, are also susceptible to considerable variations related to differences in species, strain, sex and conditions of environmental maintenance and the physiologic state of animals (Sharma, 1988).

Age-related changes in the enzyme induction by hormones have been extensively studied. 17β-estradiol was reported to induce acetylcholinesterase and choline acetyltransferase in the cerebral hemisphere of immature and adult ovarectomized rats, but not in old rats. If the level of 17β-estradiol is prevented from alteration, theoretically, it would be possible to prolong the reproductive period and some of the adulthood activities (Kanungo, 1994). The molecular basis for the impairment of induction of acetylcholinesterase by estradiol in old rats appears to be because of a decrease in the estradiol-binding protein (Kanungo et al., 1975). Patnaik and Kanungo (1976) have shown that cytosolic-AIAT is inducible in the liver of immature, adult and old rats by hydrocortisone, whereas mitochondrial-AIAT is inducible only in the older rats. Furthermore, Kanungo and Gandhi (1972) showed that the level of mitochondrial-MDH decreases in the liver of young rats after adrenalectomy, but not in old adrenalectomised rats. Administration of cortisone to these rats causes induction of the enzyme in young rats but not in old rats. In contrast, administration of cortisone in hepatectomized old rats could induce the enzyme.

Homones during development:

Hormones are signal molecules synthesized and secreted in small quantity by specialized group of cells. They act on target cells by interacting with cognate receptors located either on the cell surface (protein and peptide hormones) or within the cytosol/nucleus (steroid and thyroid hormones). Hormones of endocrine, paracrine and autocrine systems control wide variety of cellular and metabolic processes. Adaptation to external and/or internal stresses during development depends on control mechanisms of the combined interplay of nervous and endocrine systems (Timiras, 1994). Neurons in brain of higher animals act as pacemaker, regulating the "biological clock" which govern development and aging (Sharma, 1994). This pacemaker role of neurons coordinates with the hormones resulting dramatic changes in metabolic patterns including altered patterns of gene expression. Changes in the level of hormones and their properties of binding to receptors during development have been extensively studied. Hence, the level of hormones, their receptors and post-receptor events might influence the process of development, growth and reproduction in animals.

A considerable importance to the understanding of ways in which gene expression in animal cells operate *per se*, is to establish whether the activation of genes is under the control of specific developmental factor(s), or whether same hormones that control the enzyme syntheses in the adult are also responsible for switching these genes on, during development. Since hormones are known to play definite roles in the regulation of the appearance of enzymes, therefore, changes in hormone levels together with the hormone-receptor concentration are critically important. Changes in secretion of hormones during development play a major role in the regulation of enzyme synthesis both during development and in adulthood. Some of the studies on the major metabolic hormones in mammals during early period of postnatal development are reported. However, given the distinct differences that exist in hormonal regulation of metabolism between species, references, wherever available have been cited with emphasis on chickens.

In aves, as in mammals, growth and development appear to be under an intricate but judicious control mechanism, which involves endocrine, paracrine and autocrine factors. A number of hormones have long been recognized as playing major roles in the control of growth. These include growth hormone, thyroxine (T_4) and the active triiodothyronine (T_3) and the sex steroids (Scanes et al., 1990). In addition, other growth factors such as epidermal growth factor (EGF), insulin like growth factor (IGF) and nerve growth factor (NGF) have also been found to influence growth (O'Keef et al., 1988).

Knowledge of avian growth hormones have made considerable strides (Harvey and Scanes, 1977; Scanes et al., 1990). In aves, growth hormones (GH) is required for the normal rate of growth as in the case of mammals. It was shown that hypophysectomy reduces growth while replacement therapy with mammalian GH partially restores growth rates (King and Scanes, 1986). However, administration of GH to intact young chickens does not stimulate growth (Libby et al., 1955; Scanes et al., 1984). The absence of major effects of chicken GH in intact young chicks was speculated to be due to the maximal effect exerted by endogenous GH at these ages studied (Scanes et al., 1990). However, repeated administration of biosynthetic chicken GH stimulated growth in the older (12-weeks) chicks but not in the younger (8-weeks) chicks (Scanes et al., 1990). In chickens, plasma GH rises gradually, with larger increase occurring during the week following hatching (Harvey et al., 1979). Plasma GH also decreases considerably before puberty/mid-postnatal period (Scanes and Johnson, 1984). Growth hormone shows similar developmental patterns in chicken as that of T_3 and a casual relationship has been described to exists between T_3 and GH during the late embryonic and early post hatch development in chickens (Darras et al., 1992).

Adrenal hormones also play an important role in the development, growth and homeostasis. It was shown in the rat that glucocorticoids are present four days before birth, increase and decline after birth and the neonatal period is characterized by relatively low glucocorticoid concentration until 12-14 days of age, thereafter a second surge of glucocorticoid is released by the adrenals between day 12 and 16 (Cohen, 1973; Martin et al., 1977).

In the avian system, the synthesis and secretion of adrenal hormones are also initiated during embryogenesis and has been suspected to be involved in development (Freeman and Vince, 1974). The principal glucocorticoid, corticosterone (Lauber et al., 1987; Bisbis et al., 1994a), is known to increase during the early stages of development with a marked rise at about the 15th day (Wise and Frye, 1973). It was also reported that exogenous hydrocortisone induces several enzymes (Cohen et al., 1972). These were further reviewed by Freeman and Vince (1974). Subsequent studies have shown that corticosterone treatment in chickens impairs growth and induces fattening and insulin resistance (Simon, 1984; Taouis et al., 1993).

The carbohydrate metabolism of birds reportedly differs from that of mammals in many aspects (Pearce, 1977; Hazelwood, 1986). In rats, the late-fetal period is characterized by high concentration of insulin which drops precipitously at birth (Blazguez et al., 1970). This low level persists throughout the neonatal period. After weaning, when the intake of carbohydrates increases, the steady-state level of the hormone increases again. A pronounced hypoglycaemia accompanies birth and the concentration of glucagon increases markedly during the first hour after birth in contrast to that of insulin, producing, reportedly a 20-30 fold decrease in the insulin:glucagon ratio (Girard et al., 1973; DiMarco and Oliver, 1978), which has been correlated to the appearance of gluconeogenic enzymes such as PEPCK (Yeung et al., 1968) and TAT (Ghisalberti et al., 1980) in the new born.

In aves , the role of insulin in development is well acknowledged (Freeman and Vince, 1974). These early work have established the fact that insulin is present in chicks as early as the 4th day of incubation and known to increase glycogen, affect maturation of hepatocytes (Benzo and De La Haba, 1972), enhance lipogenesis (Foa et al., 1965), and uptake of neutral amino acids (Levi and Lattes, 1969). The role of insulin during embryonic development of chicken has been also established (Pablo et al., 1985; Wada et al.,1990).

Aves differ from rodents in certain metabolic and endocrinological aspects (Pearce, 1977). The relative importance of insulin and glucagon on the regulation of hepatic metabolism are dramatically different between the two groups of animals. In chicken development, carbohydrate accounts for less than 1% of available nutrients and this is primarily utilized within the first 7 days of the 21-day incubation period (Yarnell et al., 1966). Throughout the remainder of the incubation, proteins and lipids serve as the major fuel source, for both energy provisions and for production of intermediates for biosynthetic reactions (Freeman and Vince, 1974).

Thyroid hormones also affect various processes by interacting with their intracellular receptors located primarily in the nucleus of target cells. Thyroid hormone receptors are a member of a class of steroid receptors superfamily. These receptors upon interaction with hormone bind to specific DNA sequences, termed as thyroid response elements (TREs), mostly located upstream of the regulated gene. This leads to a modulation of gene expression and consequently alterations in the metabolic activity of cells.

Thyroid hormones have been implicated in controlling development and differentiation of many animals. They are present in low levels in foetal liver of rat and implicated in the appearance of glucokinase and malic enzyme during the late suckling period (Heide and Ende-Visser, 1980). In addition, thyroid releasing hormone (TRH) and thyroid stimulating hormone (TSH) which regulate the activity of the thyroid gland are reported to increase in concentration in the blood preceding the increase in the concentration of thyroid hormone in the neonate (Pease et al., 1980). It has been pointed out that the increase also coincides with the maturation of the hypothalamic-pituitary axis as a functional regulatory element in the brain.

It has been well established that growth in poultry as well as in other species is regulated by thyroidal and pituitary hormones (Freeman and Vince, 1974; Scanes et al., 1984; Harvey, 1990). Evidence for the role of thyroid hormones comes from the reduction in growth of chicks following chemical ablation of the thyroid and the return to normal growth rate following thyroxine replacement therapy (King and King, 1973). Paradoxically, attempts to stimulate growth in intact chicks with exogenous T_3 or T_4 resulted in reduced growth (May, 1980), whereas growth of sex-linked dwarf chicks was stimulated upon such treatments (Marsh et al., 1984a &b; Scanes et al., 1983). In chickens, the biologically active form is triiodothyronine (T_3) which is however predominantly produced by monodeiodination of T_4 (McNabb, 1988). The plasma level of T_3 and T_4 was reported to have an abrupt increase in T_3 between 19 day of embryonic period to day 1 post-hatch, whereas, T_4 shows a gradual increase during the embryogenesis (Darras et al., 1992).

Hormones, even if present, can only function and interact with tissues that possess appropriate receptors with fully functional signal transduction and second messenger systems. As with enzymes and hormones, changes in the development of receptors and post-receptor events have also been advanced. Insulin being a water soluble, protein/peptide hormone binds to specific receptors located on the membrane of target cells and influences a wide variety of cellular and metabolic processes. Its receptor consists of two α-subunits located on the outer face of the plasma membrane and two β-subunits that traverse the membrane and protrude on the cytosolic face. Binding of insulin to the α-subunit triggers autophosphorylation of a tyrosine residue residing in the carboxyl terminal domain of the β-subunit, which allows the tyrosine kinase domain to catalyse phosphorylation of specific target proteins giving rise ultimately to biological response. Insulin receptor concentration also increases markedly in rat paralleling the increase of insulin in blood just before birth (Caliendo, 1981). At birth, the liver cells possess adult levels of insulin receptors which are maintained throughout the neonatal period (Walker, 1983). In avian species, insulin receptors have been characterised from liver, brain (Simon and Leroith, 1986), skeletal muscle (Adamo, 1987) and kidney (Bisbis et al., 1994b). Insulin receptors in chicken have been detected in brain, liver, heart, limb buds and muscles of 18 hour embryo (Serano et al., 1990). They are shown to be regulated in a tissue-specific manner. In addition, levels of receptor measured by northern blot exhibited a 30-fold increase in post-hatch chicks compared to the embryonic stage.

Protein and peptide hormones produce varying metabolic responses upon interacting with cell surface receptors. The binding of these hormones activates an amplifier enzyme through G-protein that generates a short-lived in the concentration of an intracellular signaling molecule termed as second messenger. These second messengers may be cAMP, cGMP, 1,2-diacyglycerol (DAG), inositol triphosphate (IP_3) and calcium (Ca^{2+}). The elevated intracellular concentration of one or more such second messengers triggers a rapid change in the activity of specific enzymes or nonenzymatic proteins which then produce the biological response. Changes in the components of the second messenger cascade, such as adenylate cyclase, phosphodiesterase and cyclic-AMP, have been detected 5 days before birth, increase to reach a maximum 5 days after birth before declining to adult levels. In aves, during incubation period, the adenylate cyclase and cyclic-AMP cascade is relatively immature (Freeman and Manning, 1971), maturing only during and after hatching, so that glucagon can stimulate lipolysis and glycogenolysis.

Glucocorticoid receptors are a group of trans-acting factors present predominantly in the cytosolic fraction of many cell types. Upon interaction with glucocorticoids, they translocate into the nucleus and interact with specific DNA sequences located generally 100-200 basepairs upstream of the regulated gene(s). These DNA sequences are termed as glucocorticoid response elements (GRE's). Binding of cognate hormone-receptor complexed to GRE's modulates the expression of gene which in turn influence the metabolic response (Yamamoto, 1985; Beato, 1989). High affinity glucocorticoid binding is detected in fetal rat liver 6 days before birth. With the onset of birth, there is a marked increase in the concentration of glucocorticoid receptors (Feldman, 1974; Giannopoulos, 1975). Hepatocytes isolated from fetus 3-4 days before birth are reported to be stimulated to synthesize tyrosine aminotransferase (TAT) when incubated with glucocorticoids (Cake et al., 1979). TAT is normally synthesized just before birth. These findings alongwith the interpretations have been quite extensively reviewed (Walker, 1983). The changes in the level of glucocorticoid receptors as well as in the physicochemical properties have been well documented (Kalimi, 1984; Sharma, 1988). Glucocorticoid effect on various processes depends on the level as well as on the physicochemical properties of its receptor. Age-dependent changes in the inducibility of enzymes by glucocorticoids have been reported to be influenced by the level of receptors and also by the post-receptor events (Sharma, 1994). It has also been reported that the concentration of glucocorticoid receptor increases during the prenatal as well as postnatal development (Singer and

Litwack, 1971; Feldman, 1974). Receptors also exert a certain degree of control over the responses that are dependent upon the amount of complexes generated between receptors and hormones (Roth, 1988). An age-related reduced entry of activated steroid-receptor complexes in the nuclei has been observed in rat prostate. In rats, pancreatic glucocorticoid receptor and total plasma corticosterone level change during postnatal development and the peak value of binding corresponds to an increase in the plasma cortisone level during weaning. Hence, there is a close relationship between plasma corticosterone level and pancreatic glucocorticoid receptors and both may play a vital role in pancreatic development in rats (Lu et al., 1987). Glucocorticoid receptor concentration as well as its actvation and nuclear binding has been earlier studied in immature and mature Long-Evans rats (Sharma and Timiras, 1987). A higher amount of receptor with greater actvation characteristics was obtained, which was well correlated with the weaning period in rats. An elevated level of glucocorticoid receptor protein has also been reported in the liver and kidney of 30- and 45-day old mice, respectively (Borbhuiya and Sharma, 1995 a,b).

In recent years, it has been realized that steroid hormone and protein/peptide hormone actions are not solely isolated. They do 'cross-talk' each other and show an inter-dependence (Sharma, 1993). Activators and inhibitors of protein kinases have been reported to modulate accordingly the steroid hormone influence on the induction of TAT and TO (Sharma, 1991, '93). Dopamine and Bt_2-cAMP have been shown to modulate the effect of progesterone via progesterone receptors. Biomedical potential in getting desired effect of steroids in presence of protein kinase activators will avoid toxic effect of higher steroid doses used alone. Even, it might provide a prospective field for study of development and aging processes.

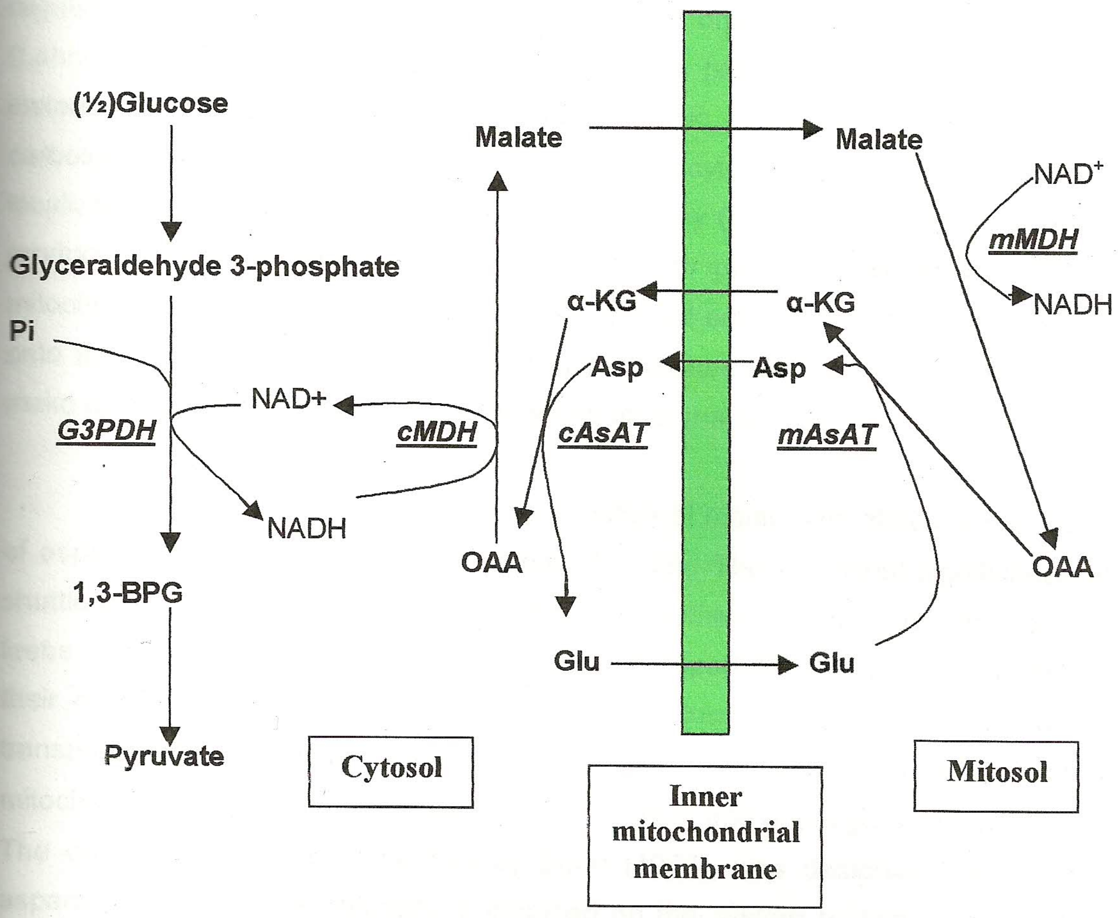
Malate-aspartate shuttle:

Most of the NADH used in electron transport is produced in the mitochondrial matrix space (Garreth and Grisham, 1999) because NADH is oxidized by Complex I on the matrix side of the inner mitochondrial membrane. Lehninger(1951) showed that intact mitochondria are impermeable to externally added NADH. Subsequently it was confirmed that the inner mitochondrial membrane is impermeable to NADH. The mitochondrial inner membrane does not contain a transport system for NAD^+

or NADH. In animal cells, most of the NADH oxidized by the respiratory chain is generated in the mitochondrial matrix by the tricarboxylic acid cycle or by the oxidation of fatty acids. However, a considerable fraction of the total NADH is generated in the cytoplasm during glycolysis, and the transport of these reducing equivalents across mitochondrial membrane is required for respiration in a variety of metabolic processes (Krebs, 1967). The NADH formed in the cytosol during glycolysis must be reoxidized to NAD^+ in order to operate the glycolysis under aerobic condition. It is advantageous to reoxidise the NADH by the respiratory chain rather than by the formation of lactate or ethanol. Approximately, three molecules of ATP are formed for each NADH oxidized in the mitochondria, whereas no ATP is made when NADH is oxidized by the cytoplasmic lactate dehydrogenase or alcohol dehydrogenase. Therefore, eukaryotic cells have a number of *shuttle systems* that harvest the electrons of cytosolic NADH for delivery to mitochondria without actually transporting NADH across the inner membrane. Various mechanisms have been proposed for this transfer, of which the *glycerophosphate shuttle* and the *malate-aspartate shuttle* are the most important (Meijer and VanDam, 1974; Christen, 1985).

The most active NADH shuttle for the movement of reducing equivalents (in the form of NADH) from the cytoplasm to the mitochondria is the malate-aspartate shuttle (Williamson et al., 1971; Cederbaum et al., 1973; Ross et al., 1977; McDonald, 1983). The main enzymes of malate-aspartate shuttle are malate dehydrogenase (MDH; EC 1.1.1.37) (Grimm and Doherty, 1961; Whitt, 1970) and aspartate aminotransferase (AsAT; EC 2.6.1.1) (Whitt, 1970; Braunstein, 1973). The kinetic differences between these two enzymes may be important to the functioning of the shuttle (Majee and Phillips, 1971). Both these enzymes have two homologous and genetically independent isoenzymes; one in the cytosol and the other in the mitosol (Davidson, 1967; Bailey, 1969).

In the malate-aspartate shuttle, 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 formed, which carries the reducing equivalents donated by cytosolic NADH passes through the inner mitochondrial membrane into the matrix (mitosol) by a dicarboxylate (malate-α-ketoglutarate) transport system. Once inside the mitochondria, the reducing equivalents carried by malate are transferred to matrix NAD^+ by the action of mitochondrial malate dehydrogenase (m-MDH), reducing it to NADH. This NADH then passes its electrons directly to the respiratory chain in the inner



Schematic representation of malate-aspartate shuttle

mitochondrial membrane. Three molecules of ATP are generated as this pair of electron passes to molecular oxygen. The rest of the shuttle is concerned with the regeneration of cytosolic oxaloacetate to start another round of the shuttle and the details of schematic representation of malate-aspartate shuttle is shown above. The malate-aspartate shuttle is also reported to be a primary mode of transfer of NADH reducing equivalents from the cytoplasm in vascular smooth muscle. Glucose oxidation and lactate production are influenced by the activity of the shuttle (Barron et al., 1998). Regeneration of NADH in the cytosol is limited in chicken liver, and that gluconeogenesis is regulated, in part, by alterations in the redox states of mitochondria and cytosol (Sugano et al., 1982). 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 phosphoenol pyruvate 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). Oxaloacetate produced in mitochondria is converted directly to phosphoenolpyruvate (PEP) by a mitochondrial PEPCK. The PEP is then transported out of the mitochondria and serves onto the gluconeogenic pathway. It clearly establishes distinct differences in metabolic make up of aves as compared to rodents and other mammalian species.

Malate-aspartate shuttle involves an influx of malate and glutamate and an efflux of aspartate and α-ketoglutarate from mitochondria. The functional significance of this shuttle is to unfold the degree of control points for the glycolysis, gluconeogenesis and krebs cycle (Borst, 1963). The enzymes of malate-aspartate shuttle are freely reversible in their catalysis and generate malate, aspartate, glutamate, α-ketoglutarate which are transported through distinct carrier systems. These carrier systems are located on the mitochondrial membrane (aspartate-glutamate carrier and α-ketoglutarate-malate carrier). The carrier system, first described by Borst (1963), was designated as the malate-aspartate shuttle. A directionality is imposed on the system by two carrier proteins. In chicken, excess cytosolic reducing equivalents generated by the oxidation of lactate to pyruvate are transferred from the cytosol to the mitosol by the malate-aspartate shuttle (Ochs and Harris, 1980).

The malate-aspartate shuttle can be reconstituted, using isolated mitochondria and the extra mitochondrial components of the shuttle (Cederbaum et al., 1973). Dawson (1982) reported that the rate of oxidation of NADH was directly related to the amount of mitochondrial proteins present, while extra mitochondrial reactions become limiting only when the soluble mitochondrial protein ratio fell below 0.8. It has also been observed that a high fat diet increases both the endogenous and total rates of shuttle activity compared to the rates obtained with mitochondria from low fat fed rats (Cederbaum et al., 1973).

Kauppinen (1983) reported aminooxyacetate as a nonspecific inhibitor of pyridoxal phosphate-dependent enzymes. Aminooxyacetate inhibits aspartate aminotransferase, a pyridoxal phosphate-dependent enzyme, both in the cytosolic and mitochondrial compartments. Aspartate aminotransferase is an essential component of the malate-aspartate shuttle which predominantly transports electrons from cytosolic NADH into the mitochondria. Application of aminooxyacetate demonstrated that the NADH shuttle system was essential for coupling glycolysis with activation of mitochondrial ATP generation to trigger glucose-induced insulin secretion (Eto et al., 1999). In chicken, although the pathway for glucose synthesis from lactate is not thought to involve transamination steps, inhibitors of transamination like aminooxyacetate blocks lactate gluconeogenesis. Thus, aminoxyacetate inhibits the malate-aspartate shuttle and, consequently, glucose synthesis for want of pyruvate (Ochs and Harris, 1980). It has also been reported that β-methyleneaspartate inhibits both the cytosolic and mitochondrial aspartate aminotransferase of synaptosomes and also impairs NADH oxidation in a reconstituted malate-aspartate shuttle (Cheeseman, 1988). Malate dehydrogenase and aspartate aminotransferase are also inhibited at relatively low concentration of bilirubin which has been shown to inhibit the malate-aspartate shuttle (McLoughlin et al., 1987). In the perfused rat liver, the redistribution of cellular Ca^{2+} may activate the efflux of aspartate from mitochondria resulting in an increase in the capacity of the malate-aspartate shuttle (Sugano et al., 1988). Alanine metabolism has been shown to increase the capacity of the malate-aspartate shuttle in perfused liver of cold-exposed and steroid replaced rats (Sugano et al., 1986).

Malate dehydrogenase:

Malate dehydrogenase (MDH ; EC 1.1.1.37) is widely distributed in animal tissues and plays an important role in carbohydrate metabolism. It catalyses the oxidation of malate to oxalacetate with a ΔG^0 of +7 Kcal/mole. MDH has been purified from wide variety of sources ranging from *E. coli* to humans. It catalyses the same reaction, albeit differs in physical and chemical properties. It is one of the several enzymes known to exists in two distinct forms: one in the cytosol (c-MDH) and the other in the mitosol (m-MDH). The two forms have different chemical, physical and kinetic properties and each has a characteristic electrophoretic pattern. The two forms also show a dissimilar developmental pattern and may be regulated differently by endogenous effectors (Malik et al., 1993).

The mechanism of action of MDH is closely related to that of lactate dehydrogenase (Larry and Johannes, 1978). m-MDH plays a crucial role in the reversible conversion of malate to oxalacetate in the Krebs cycle. c-MDH, on the other hand, is critically involved in gluconeogenesis (synthesis of glucose from non carbohydrate sources). The initial step in gluconeogenesis in the carboxylation of pyruvate to oxalacetate which is catalyzed by pyruvate carboxylase, a mitochondrial enzyme. Oxalacetate thus formed in the mitochondria cannot cross the membrane. However, malate diffuses from the mitochondria to the cytoplasm where it is oxidized by c-MDH to oxalacetate. Further, oxalacetate is converted to phosphoenlpyruvate by phosphoenolpyruvate carboxykinase (PEPCK). Thus, c- and m-MDH perform distinct metabolic functions in the different compartments (Lardy et al., 1965). The cytosolic enzyme differs from that of mitochondrial in its catalytic and physicochemical properties. Both the isoenzymes are NAD^+-dependent. However, m-MDH but not c-MDH is inhibited by high concentration of NADH. c- and m-MDH are also under the control of two separate genes (Davidson and Cartner, 1967). It was also reported that nutrition and hormone also regulate the gene for avian malic enzyme (Goodridge et al., 1989). MDH isoenzymes genes were also isolated from mouse and reported to have the DNA sequences required for the promoter activity of these genes. Nuclear binding to the promoter regions was also determined and that a transcription factor, CTF/NFI may contribute to the regulation of these isoenzymes.

Malate dehydrogenase is a dimeric enzyme consisting of identical subunits (M_r= 35,000) (Banaszak and Bradshaw, 1975). Its dissociation and reassociation have been the subject of many studies involving pig heart mitochondrial isoenzyme (m-MDH) (Wood et al., 1978; Muller et al., 1984; Gerl et al., 1985). Malate dehydrogenase from the cytosolic fraction of rat liver has been purified and its molecular weight (71 kDa), isoelectric point (pI 5.1), and amino acid composition were determined. The V_{max} was reported to be 53.3 µmol/min/g wet wt. for oxalacetate and 48 µmol/min/g wet wt for malate oxidation at pH 7.4 (Crow et al., 1982). In chicken, the purified preparations of cytosolic malate dehydrogenase resolved show three electrophoretically distinct species with identical amino-acid compositions and physico-chemical properties very similar to those of the cytosolic isoenzyme of other sources (Domenech et al., 1986). Mitochondrial malate dehydrogenase has been isolated and characterized from a large number of sources (Fahein and Strnecki, 1969; Glatthaar et al., 1974; Hagele et al., 1978; Kuan et al., 1979;

Okayabashi and Nakano, 1984; Cremel et al., 1985). In rat liver, mMDH has a V_{max} of 38 μmol/min/g wet wt. for oxalacetate reduction and 39 μmol/min/g wet wt. for malate oxidation at pH 7.4. Rates of the reaction catalysed by mitochondrial malate dehydrogenase under condition similar to those *in vivo* were calculated and found much lower than the maximum velocity of the enzyme (Wiseman, 1991). Mitochondrial malate dehydrogenase isolated from pig and chicken also known to contain one tyrosine residue which is important for its structure and function, probably involved in a hydrogen bond formation with a carboxylate group (Muller et al., 1983). The polypeptide chain conformation and coenzyme binding site of the crystalline porcine heart mitochondrial malate dehydrogenase by X-ray diffraction studies was determined (Roderick and Banaszak, 1986), indicating that the location of the coenzyme binding site and the catalytically important amino acid residues remain similar in mitochondrial malate dehydrogenase to that of cytosolic MDH and LDH isoenzymes.

The mechanism of substrate inhibition that occurs with pig heart cytosolic and mitochondrial malate dehydrogenase is different from the lactate dehydrogenase from chicken heart (Berstein et al., 1978). The inhibitor constant for oxalacetate is 2.0 mM with mitochondrial isoenzyme and 4.5 mM with the cytoplasmic isoenzyme. *In vivo* concentration of oxalacetate is 10 μM and so the substrate inhibition may not be significant under these conditions. m-MDH exhibits a complex regulatory pattern by citrate, which activates and inhibits mitochondrial malate dehydrogenase in the same direction ($NAD^+ \longrightarrow NADH$), and in the same reaction medium, depending on malate concentration (Gelpi et al., 1992). It was also reported that pig heart citrate synthase and mitochondrial malate dehydrogenase do exhibit electrostatic interactions, whereas no interaction is observed between citrate synthase and cytosolic malate dehydrogenase from these sources (Morgunov & Srere, 1998).

Aspartate amonotransferase:

Aspartate aminotransferase (AsAT; EC 2.6.1.1) is the most extensively studied vit B_6- dependent enzyme (Snell and DiMari, 1970; Braunstein, 1973). It is widely distributed and represents the most abundant of the transaminases in mammalian tissues. The enzyme is implicated in maintaining the balance of the four metabolites which are its substrates. It catalyses the transamination reaction representing key steps at the interconnection between the metabolic pathways of amino acids and dicarboxylic acids. AsAT effects the reversible transfer of an amino group from L-aspartate or L-glutamate to α-ketoglutarate and oxalacetate (Metzler and Snell, 1952). In double displacement reaction, the coenzyme shuttles between the pyridoxal and pyridoxamine p-forms (Gehring and Christen, 1978).

Two homologous genetically independent isoenzymes of AsAT have been found in tissues of higher organisms in two distinct forms, one in the cytosol (c-AsAT) and the other in the mitosol (m-AsAT). The isoenzyme of aspartate aminotransferase differ from one another in chemical, physical, catalytic and immunologic properties (Fleisher et al., 1960; Boyd, 1961; Jaussi et al., 1987; Nagashima et al., 1989). The existence of aspartate aminotransferase isoenzymes has been confirmed in a wide variety of eukaryotic organisms. Several authors have demonstrated (Fleisher et al., 1960; Borst and Poters, 1961; Martinez-Carrion et al., 1967; Banks et al., 1968; Bertland and Kaplan, 1970; Magee and Phillips, 1971; Di Cola et al., 1976) the presence of two main classes of AsAT in animal tissues, located in the mitochondria (m-AsAT) and in the soluble cell fraction (c-AsAT), respectively, with different kinetic and structural properties. Furthermore, several forms have been described for each AsAT isoenzyme and reported that chicken liver contained only the cytoplasmic enzyme (Decker and Rau, 1963;Gil et al., 1987; Morino et al., 1964; Yagi et al., 1979). However, other authors described later the presence of a mitochondrial fraction containing AsAT activity in that tissue (Shrawder and Martinez-Carrion, 1973; Elduque et al., 1982). Some attempts have been made to establish the submitochondrial location of m-AsAT. According to several reports (Landriscina et al., 1970) the enzyme is located exclusively in the mitochondrial matrix (mitosol), while other authors suggest that it is partially bound to the inner side of the inner mitochondrial membrane (Boyd, 1961; Eichel and Bukovsky, 1961; Bhargava and Sreenivasan, 1966; Elduque et al., 1982). This is further supported by the finding that the disruption of mitochondria by freeze-thawing or by treatment with high ionic strength buffers does not result in the complete release of m-AsAT activity, which could be fully achieved only by Triton X-100 treatment (Baumber and Doonan, 1976; Elduque et al., 1982). The mitosolic location of chicken liver AsAT was also ascertained(Gil et al., 1987).

Aspartate aminotransferase isoezymes are well characterized from chicken and pig heart. Borisov et al (1978) have studied the three dimensional structure of c-AsAT from chicken heart at 5 A^0 resolution and have reported that the enzyme molecule appears to consists of two dense and closely packed subunits with a dimension of 40 A^0 x 48 A^0 x 60

A^0 each. Eichele et al (1979) have studied the crystal stucture of m-AsAT from the chicken heart. In the electron density map, the enzyme is clearly seen as anisologous α_2 dimers (105 A^0 x 60 A^0 x 50 A^0). The active sites are located in opposite sides of the dimer, about 30 A^0 apart and close to the inter-subunit boundary so that both the subunits can probably contribute to each active site. X-ray diffraction studies at 2.8 A^0 resolution yielded the 3-D structure of chicken m-AsAT. The subunits are rich in secondary structure and contain two domains, one of which anchors the coenzyme pyridoxal-5'-phosphate (Ford et al., 1980). The cytosolic aspartate aminotransferase from chicken liver also shows molecular heterogeneity in polyacrylamide gel electrophoresis. The enzyme exists as various molecular forms of increasing anodic mobilities with the α-form predominates and its specific activity is the highest (Imperial et al., 1991). These isolated native molecular forms of chicken liver c-AsAT give rise to two kinds of generation processes.

The crystal structure of the stable, closed complexes of chicken mitochondrial aspartate amnotransferase with the natural substrate L-aspartate and L-glutamate have been resolved at 2.4 and 2.3 A^0 resolution, respectively (Malashkevich and Toney, 1993). In both cases, clear electron density at the substrate-coenzyme binding site unequivocally indicates the presence of a covalent intermediate. Crystalline enzyme has a much higher affinity for keto-acid substrates compared to enzyme in solution. The increased affinity is interpreted in terms of perturbation of the open/closed conformational equilibrium by the crystal lattice with the closed form having greater affinity for substrates. Catalytic efficiency of AsAT has been studied by Kohler et al (1994) in different mutants who have shown that improved catalytic efficiency of c-AsAT V_3 92 appears due to closure of the active site upon substrate binding. The primary structure of cytosolic and mitochondrial AsAT of pig heart has also been elucidated. The polypeptide chain of mitochondrial isoenzyme contains 403 amino acid residues, which is 9 residues less than cytosolic form.

The mitochondrial isoenzyme is apparently a matrix bound and is encoded by nuclear DNA (Van Heyningen et al.,1974). Mitochondrial and cytosolic AsAT are two genetically independent isoenzymes that have been found in animal tissues and possibly occur in all eukaryotic cells. Both isoenzymes are composed of two identical polypeptide chains of about 400 amino acid residues and are encoded by nuclear DNA. The mitochondrial isoenzyme is synthesized on free polysomes in the cytosol as a precursor of higher molecular weight (Sonderegger et al., 1980) which is translocated posttranslationally into the mitochondrial matrix. Organ- or tissue-specific forms have not been detected for either isoenzyme (Wada and Morino, 1964; Shrawder and Martinez-Carrion, 1973; Behra et al., 1981).The essential features of catalytic mechanism of both the isoenzymes are identical (Braunstein, 1973). A comparison of the amino acid sequence data of the cytosolic and mitochondrial aspartate aminotransferase from pig heart clearly indicates that these heterotropic isoenzymes are homologous proteins. A comparison of the mitochondrial isoenzyme from two different species, that is, from chicken and pig revealed a close interspecies homology, which appears to exceed the interspecies homology, between the cytosolic and the mitochondrial isoenzymes. Only two amino acid substitutions were found in the sequence of 40-NH_2 terminal amino acid residues.

The genes of mitochondrial and cyosolic aspartate aminotransferase of chicken were cloned and sequenced. In both genes (Juretic et al., 1990), nine exons encode the mature enzyme. The additional exon for the N-terminal presequence that directs mitochondrial aspartate aminotransferase into the mitochondria is separated by the largest intron from the rest of the gene. A comparison of the two genes of chicken with aspartate aminotransferase genes of mouse entails closely similar structures; in the gene of both the mitochondrial and the cytosolic isoenzyme all but one intron positions are conserved in the two species and five introns out of nine are placed at the same positions in all four genes indicating that the introns were in place before the genes of the two isoenzymes diverged. It is proposed that the aspartate aminotransferase genes follow a course of evolution, originating from a common ancestor which probably led to the conservation of certain splicing factors specific for nuclear-encoded mitochondrial proteins.

The degree of structural similarity between the heart mitochondrial isoenzyme of AsAT from pig and chicken was determined by means of their immunological cross-reactivity and compared with the degree of similarity in the cytosolic isoenzyme from the same species. Quantitative micro complement fixation revealed a remarkable similarity of the two mitochondrial isoenzymes corresponding to an immunological distance of 104. The structure of the two cytosolic isoenzymes, on the other hand, diverge with an immunological distance of 203. Thus, a significant structural divergence of the cytosolic isoenzymes appeared to contrast the conspicuous similarity between the mitochondrial isoenzymes (Sonderegger et al., 1977).

Cytosolic and mitochondrial AsAT were purified to homogeneity from human heart and shown to differ distinctly from each other in their structural, immunochemical and kinetic properties. The human isoenzymes were very similar to the corresponding isoenzymes from pig heart in their molecular weight, Michaelis constant (K_m), NH_2- and COOH- terminal amino acid sequences. In electophoretic mobility, the human cytosolic isoenzyme differs from the corresponding isoenzyme from the pig heart while no significant difference was observed between the mitochondrial isoenzymes from the two species (Boyd, 1961; Kagamiyama and Wada, 1975). Cytosolic and mitochondrial isoenzymes of aspartate aminotransferase were also purified to homogeneity from rabbit liver. The rabbit liver isoenzymes were closely similar to the corresponding isoenzymes from other sources, including human heart, pig heart, chicken heart, and rat liver, in their molecular weights, absorption spectra, amino acid compositions, isoelectric points, and Michaelis constants for the substrates. The NH_2-terminal amino acid sequences of rabbit liver isoenzymes were identified up to 30 residues, and showed some differences from those of the corresponding isoenzymes obtained from other animals studied (Kuramitsu et al., 1985). Interspecies comparisons of aspartate aminotransferase was also determined based on the amino acid composition. Subunit molecular weights of various aspartate aminotransferase examined were found to be 46,000 with the exception to that of the *E. coli* enzyme which is 42,700 (Doonan et al., 1981). Compositional difference indices were calculated for pairs of AsAT where indices provide reliable estimates of amino acid sequence differences for those pairs of isoenzymes whose primary structures are known. Comparisons of compositional difference indices also suggested a similar rate of evolution of cytosolic and mitochondrial AsAT throughout the history of the vertebrates examined.

Several workers (Fleisher et al., 1960; Boyd, 1961; Wada and Morino, 1964; Barra et al., 1976) have purified both the cytosolic and mitochondrial isoenzymes of AsAT from various tissues of mammals and have shown that, at a fixed concentration of L-aspartate, the K_m for α-ketoglutarate is much higher for mitochondrial than for the cytosolic enzyme. The reverse applies to the K_m for L-aspartate at a fixed concentration of α-ketoglutarate. The mitochondrial isoenzyme is more susceptible to the inhibition of DL-glyceraldehyde-3-phosphate than that of the cytosolic enzyme (Kopelovich et al., 1970). The inhibition of the mitochondrial isoenzyme is competitive with respect to α-ketoglutarate and noncompetitive with respect to L-aspartate. Aminooxyacetic acid and other aminooxyacetates are effective inhibitors of both c- and m-AsAT (Braunstein, 1973; Rej, 1977). The inhibitory effect of aminooxyacetic acid is shown to be more pronounced for c-AsAT.

Liver AsAT is responsible for the synthesis of glucose from non-carbohydrate precursors (Lardy et al., 1965). Both the isoenzymes of AsAT are involved in the process of gluconeogenesis. The specific activity of c-AsAT was also reported to be higher in the adult as compared to that of fetal liver (Nakata et al., 1964). Ferre and Williamson (1978) observed that the inhibition of the activity of AsAT in the suckling new born rat causes a decrease in the level of all the gluconeogenic precursors and accumulation of lactate but not pyruvate. On the basis of these observations, it has been suggested that the malate-aspartate shuttle is fully operative in suckling rats during development. Although both the isoenzymes of AsAT are involved in the process of gluconeogenesis, it is the cytosolic isoenzyme which is regulated by dietary and hormonal treatments (Sheid and Roth, 1965).

Herzfeld and Greengard (1971) reported that the expression of AsAT during normal development does not follow the same pattern in all the tissues of rat. In liver, the beginning of rapid enzyme accumulation occurs shortly before birth and just after birth there is a transient rise in the level of AsAT. The developmental formation of AsAT in heart and kidney is gradual over the first few weeks of postnatal age. The beginning of rapid AsAT accumulation in fetal liver coincides with the onset of thyroid and adrenocortical secretions. They demonstrated that the hormones which modify the developmental formation of an enzyme in one organ may have no effect or the opposite effect, on the same enzyme in other tissues of the same animal. Even in the same tissue, the response of the enzyme depends on the age, sex and physiological state of the animal.

Several physiological and biochemical changes occur during development, growth, adulthood and senescence of an organism. Different metabolic events that occur during early life of an organism might influence the later part of life span. To study the activities of all the key enzymes of a particular metabolic pathway would provide a complete profile of their biological function during development and aging. Earlier work on the malate-aspartate shuttle has been done mostly on the mammalian system. However, informations on the developmental and hormonal regulation in enzymes of avian malate-aspartate shuttle are scanty. Keeping in view the importance of studying all the enzymes of a particular metabolic pathway and with emphasis on avian system, the work embodies in this thesis is directed towards the following:-

1. Assessment of endogenous activity levels of malate-aspartate shuttle enzymes in different tissues of chicken at various postnatal ages (day 0, 30 and 60) in order to find out the changes, if any, in the activity which may predict tissue- and age-specific involvement of malate-aspartate shuttle enzymes in the early postnatal development of chicken.

2. Effects of various hormones such as glucocorticoid and thyroid hormones on the activity of shuttle enzymes in liver and kidney tissues of chicken at various postnatal ages (day 0, 30 and 60) to find out the role of these hormones, if any, in regulating the shuttle activity in a tissue- and age-specific manner.

3. Purification and characterization of one of the shuttle enzymes, that is, cytosolic aspartate aminotransferase from two different ages (day 0 and 60) to determine alterations if any in the physico-chemical and/or kinetic properties accompany this enzyme during the course of development.
