REGULATORY CHANGES IN ENZYMES OF MALATE-ASPARTATE SHUTTLE DURING DEVELOPMENT AND AGING OF MICE

BY
SANTA DEY

ABSTRACT

THESIS
SUBMITTED IN FULFILMENT OF THE REQUIREMENTS
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ABSTRACT

Enzymes are specific proteins that catalyze chemical reactions in biological systems. Living cells have evolved a complex regulatory mechanism to control the concentrations of their enzymes, particularly those catalyzing critical metabolic reactions. In an organism, several physiological and biochemical changes occur during development, growth, adulthood and senescence. The developmental phase includes an increase in the number and size of cells, and their differentiation to perform specialized functions. The metabolic events that occur during development might influence the later part of lifespan. Aging is the characteristic of all multicellular organisms. The functional abilities of most organs and the organisms decrease during senescence. The decline becomes perceptible towards the later part of the reproductive phase. Thus, the reproductive phase smoothly merges into the senescence phase, unlike the transition from the developmental to the reproductive phase in which specific genes are expressed and specific structures and functions appear that confer reproductive ability to the organism. During senescence, adaptability to external and internal stresses decreases and the homeostatic mechanisms deteriorate and that increase the susceptibility in old age.

During development and aging, different metabolic adjustments take place as an adaptation to the changing demand made upon them. Study of all the enzymes of a particular metabolic pathway provides a complete profile of their biological functions. 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 regulatory changes in enzymes of malate-aspartate shuttle to elucidate the mechanism of regulation of this shuttle during development and aging.

The malate-aspartate shuttle appears to be the primary mechanism for the transfer of reducing equivalent from the cytosolic NADH to the mitochondria in many animal tissues. It has been seen that inner mitochondrial membrane is impermeable to NADH. The NADH formed during glycolysis in the cytoplasm by the oxidation of glyceraldehyde-3-phosphate must be regenerated to NAD^+ for glycolysis to operate. The shuttle involves an influx of malate and glutamate and efflux of aspartate and ketoglutarate from the mitochondria. The main enzymes of the shuttle are malate dehydrogenase and aspartate aminotransferase. Both these enzyme have two homologous and genetically independent isoenzymes. One in the cytosolic and the other in the

mitochondrial fraction.The present study aims :

(a) to assess the endogenous activity level of shuttle enzymes in a postnatal age - and tissue - specific manner

b) regulation of enzymes of shuttle by various hormones such as glucocorticoid and thyroid hormone during development of mice

c) lastly, to purify one of the shuttle enzymes that is cytosolic aspartate aminotransferase and to study its chemical and kinetic properties in order to find out changes, if any, in properties as a function of age.

Endogenous level of shuttle enzymes :

The endogenous activities of isoenzymes of malate dehydrogenase and aspartate aminotransferase show a significant change during postnatal development of mice. The activities of both the isoenzymes (cytosolic and mitochondrial) of malate dehydrogenase (MDH) and aspartate aminotransferase (AsAT) were significantly higher in the liver of mice at day 15, declined at day 30 and remained unchanged thereafter until day 60. In contrast, the activities of these isoenzymes showed a lower value at day 15, increased to a peak value at day 30 in the kidney of mice. It indicates an early developmental expression of shuttle enzymes in the liver than in the kidney of mice which may in turn exhibit an early involvement of malate-aspartate shuttle in the transfer of reducing equivalents to compensate the metabolic demands of this tissue in growing mice. Reconstitution studies confirmed the observation of malate- aspartate shuttle enzymes in liver and kidney during postnatal development of mice.

Hormonal regulation of shuttle enzymes :

It was observed that adrenalectomy decreases and administration of hydrocortisone to adrenalectomized mice increases the activity of cytosolic and mitochondrial malate dehydrogenase and cytosolic aspartate aminotransferase in the liver of 15-, 30-, and 60 -day old mice. Per cent decrease following adrenalectomy is almost similar in all the postnatal ages studied. This indicates that adrenal steroid do play a role in the regulation of the shuttle enzymes.

Adrenalectomy also decreases and administration of hydrocortisone to adrenalectomized

mice increases the activity of kidney cytosolic and mitochondrial malate dehydrogenase and cytosolic aspartate aminotransferase only in 30- and 60- day old mice. It has not shown any effect on the activity of these enzymes in preweaned mice (15-day old). It may be due to the differential level of glucocorticoid receptors and other trans-acting factors in the liver and kidney of mice during this phase of postnatal development. These findings corroborate the observations that the same enzyme in different tissues of the developing animals might be regulated differentially by the same physiological stimuli. The hormonal signals important in the developmental formation of the enzymes may or may not regulate the level of the same enzyme in adult tissues. Differential hormonal regulation of AsAT isoenzymes indicate that they are subject to different physiological controls in different tissues.

Various doses of Bt_2-cAMP, a membrane permeable analog of cyclic AMP, were administered in different postnatal ages of normal male mice. None of these single doses of Bt_2-cAMP were effective on both isoenzymes of MDH and AsAT in the liver and kidney of mice. The finding indicated that none of the shuttle enzyme of mice liver and kidney are regulated by cAMP at those postnatal ages studied. Last couple of years, group of workers visualized the cross-talk between steroid and protein/ peptide hormone action. Hence, in order to find out the synergistic or antagonistic role of cyclic AMP on hydrocortisone action, a combination of Bt_2-cAMP with hydrocortisone was injected in the 15-, 30- and 60- day old mice. It has been observed that only liver mitochondrial malate dehydrogenase and cytosolic aspartate aminotransferase show an increase in the activity. In case of mitochondrial malate dehydrogenase, the increase in activity has been seen only at 30- and 60- day of postnatal age. The activity of both the shuttle enzymes show no effect of this combination in the kidney of mice at either of the age groups. This indicates that only liver and not the kidney is possibly equipped with the cross-talk mechanism in regulating the enzyme activities.

It is seen that administration of T_3 which is a potent thyroid hormone on normal mice of three different postnatal ages (i.e. 15-, 30- and 60- day) showed no significant change in the activities of cytosolic and mitochondrial malate dehydrogenase as well as aspartate aminotransferase in liver and kidney. Most likely this might be due to the tonic regulation of the enzymes by the endogenously circulating level of the thyroid hormones.

Chemical and kinetic properties of c-AsAT :

To find out the change, if any as a function of age, in the chemical and kinetic properties, one of the shuttle enzyme i.e. c-AsAT was isolated and purified from the mice liver of two selected ages (i.e. 15- and 180- day) using similar experimental conditions. The enzyme preparations from both the ages were passed through the CM-cellulose column and the elution profile of the specific activities of this isoenzyme from the liver of two ages of mice exhibited the requirement of two different ionic strength. This indicates that there might be an overall charge difference on the isoenzyme from two different age groups. It was further confirmed by running enzyme preparations onto polyacrylamide gel electrophoresis and staining the gels with general and specific stains. The isoenzyme from immature and mature ages migrated at two different levels, confirming the change difference onto C-AsAT from two ages. Changes in the isoenzyme patterns and their electrophoretic mobilities have earlier been reported and reviewed. They can arise due to genetic variability or sometimes due to epigenic events (such as acetylation, phosphorylation and proteolysis) depending on the metabolic demand to commensurate the requirement at specific stage of development.

Kinetic analysis of data indicates no significant difference between the K_m values of this enzyme for both the substrates in immature and mature mice. However, the enzyme from the mature mice showed higher V_{max} and K_{cat}, indicating higher turnover compared to the immature one. This indicates that the c-AsAT from mature mice catalyses the reaction at a faster rate than that of c-AsAT from immature mice, albeit the binding affinities for substrates remained the same. It may reflect that the substrate binding site of the enzyme is not affected for by the charge difference between the enzymes from the two ages. However, the charge difference in the c-AsAT of two ages might contribute to the catalytic turnover of the enzyme at respective ages. The higher catalytic rate of mature enzyme might extend an adaptation to control the metabolic demands of the mature mice since malate-aspartate shuttle is one of the major control point for glycolysis, Krebs cycle and gluconeogenesis. Inactivation studies of the enzymes from both the ages depict differential folded structure as envisaged by the different requirement of urea for their 50% inactivation. It further corroborates our earlier assumption that there is a difference in the overall charge of the enzyme at two ages.

It may be concluded that the enzymes of malate-aspartate shuttle as well as the shuttle activity expressed differentially in different tissues of mice as a function of postnatal development.

And the shuttle enzymes are also regulated differentially by glucocorticoid where as they do not exhibit any change in intact mice with the exogenously added cAMP as well as thyroid hormones. However, a combination of cAMP and glucocorticoid regulates the shuttle enzymes differentially ia tissue- and age- specific manner. Purification and kinetic analyses show a definite charge difference in C-AsAT isoenzyme at two different ages i.e. immature and mature. The K_m remains the same while catalytic efficiency is higher in mature as compared to immature owing to greater adaptation in mature animals.

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Feb. 24, 1997

CERTIFICATE

I certify that the thesis entitled **"Regulatory Changes in Enzymes of Malate-Aspartate Shuttle During Development and Aging of Mice,"** submitted by **Ms Santa Dey** for the degree of **Doctor of Philosophy** of the **North Eastern Hill University**, Shillong, embodies the record of original investigations carried out by her under my supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of Ph.D. degree. This work has not been submitted for any degree of any other University.

Forwarded

Head,
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Shillong, Meghalaya

Ramesh Sharma
Supervisor

Dedicated to my parents

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LIST OF ABBREVIATION

ADP	:	Adenosine diphosphate
ADA	:	Adenosine deaminase
BSA	:	Bovine serum albumin,
Bt_2-cAMP	:	Dibutyrylated cyclic adenosine monophosphate
HC	:	Hydrocortisone
A/d	:	Adrenalectomy
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 electrophoresis
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
AlAT	:	Alanine aminotransferase
K_2HPO_4	:	Dipotassium hydrogen orthophosphate
KH_2PO_4	:	Potassium hydrogen orthophosphate

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INTRODUCTION

Living cells have evolved complex regulatory mechanisms to control the levels of their enzymes, particularly, those catalyzing critical reactions. The feature, which generally characterizes all developing and aging populations, is the progressive development and impairment in the ability to adapt to the environmental changes. Adaptation may be expressed at a biochemical level by alteration in the rates of synthesis and degradation of enzymes, as well as by changes in other physiological parameters. In developing animals, metabolic adjustments take place in different tissues as an adaptation to the changing demands made upon them.

Lifespan can be defined as the duration of the life of an individual/organism in a particular environment. It may be divided into three main phases a) development b) reproduction (c) senescence and each phase has a characteristic duration, rate, sequential events and regulatory mechanisms (Kanungo, 1994). Early period of lifespan of a particular species is called developing period. The term development has been defined by various ways in biology which in a broad sense, encompasses the complex changes an individual/organism undergoes in its lifespan from the fertilization to reproductive maturity (Timiras, 1988). 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, however, can be separated into two main periods:, prenatal and postnatal.

Many animal species are capable of an independent existence at relatively immature stages, others including mammals are utterly dependent on adults for food and care during that stage. Both prenatal and postnatal stages can be distinguished by morphological, physiological and biochemical features. The prenatal period includes three main stages : ovum, embryonic and fetal stages. The postnatal period commences with birth and continues to stages of neonatal, infancy, childhood, adolescence, adulthood and old age (Timiras, 1994).

The biological nature of aging was postulated to be pleiotropic in nature, being the result of normal by-products of living processes. It may be as a result of evolutionary non-selected endogenous properties of an organism. Aging can be defined as the time-dependent drifting away of cells, cell organization and homeostatic control from their most optimum state of functions (Cutler, 1984). For the mammalian species, two classes of pleiotropic aging processes were postulated to exist. One is associated with energy metabolism and has been called the 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 of aging processes might play the most important role in causing aging. Medawar (1957), Williams (1957), and Hamilton (1960) have proposed that aging is caused by a special class of pleiotropic gene whose expression is linked to development. 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 cell, cell organization in tissues and the general homeostatic state of organisms.

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 lifespan (Timiras, 1994). Longevity is determined by processes governing the time-dependent stability and differentiation. These processes are encoded by longevity-related genes and their levels of expression are governed by other regulatory genes. Environment supplies the external factors that make development possible and allow inherited potentials to find expression.

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-cell interaction (Lewin, 1993).

One of the classical examples of sequential activation and repression of genes during development is that of hemoglobin during gestational period in human (Zucker Kandle, 1965) The hemoglobin, a tetramer protein, consists of $\alpha_2\epsilon_2$ chains in the fetus at the age of 1 or 2

months of gestation. The α chain remains the same while the ϵ chain is replaced by the γ chain in later phases of gestation. Just before birth, the γ chain is further replaced by the β chain which gives rise to the adult hemoglobin ($\alpha_2\beta_2$). The synthesis of individual chain is governed by specific gene. These genes are sequentially activated and repressed by certain factors during development of the human fetus.

Genetic control of development can best be 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) yielded valuable informations relating to switching on and 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 are a continuing processes and may operate throughout the lifespan of an organism.

On the other hand, during senescence, which is a characteristic of all multicellular organism, the functional abilities of most organs and the organism decrease. The decline becomes apparent towards the later part of the reproductive phase. Thus, the reproductive phase smoothly merges into the senescence phase, unlike the transition from the developmental to the reproductive phase in which specific genes are expressed and confer reproductive ability on the 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).

Various theories have been proposed to explain the phenomena of aging. All these theories can be broadly divided into molecular, cellular and system level theories (Sharma, 1988,94).

Molecular Theories — Molecular theories begin with the following assumptions : (i) All individuals within a species have an almost similar length of life. (ii) Individuals from different species have different lifespan. According to this theory, it is presumed that there is some genetic programme which determines the maximum lifespan for each species. An equally significant contribution to a genetic basis of aging is deduced from the duration of the three phases of lifespan. Molecular theories of aging include :

a) *Codon restriction theory*- This theory 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* — According to this theory mutations that occur randomly and spontaneously destroy genes and chromosome in post-mitotic cells during the lifespan of an organism. This process causes a gradual increase in the mutation load which in turn decrease the production of functional proteins (Szilard, 1959)

c) *Error Theory* — Orgel (1963) postulated the 'error' theory of aging according to which error occurring during information transfer (i.e. transcription and translation) may cause accumulation of defective proteins that may lead to the aging of an organism.

d) Gene *regulation theory*- Kanungo (1970,75,80,94) proposed 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 i.e. a sequential activation and repression of certain genes which are unique to these phases.

Cellular Theories- They relate to changes that occur in structural and functional elements of cell with the passage of time. These theories include : a) Wear and tear b) Age-pigments c) 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 compared since organisms have a well defined self controlled repair mechanisms and that when these mechanisms fail then they destabilize the system lending to aging and death.

b) Age *pigments*- Regular change that occurs in the cell composition during aging is the increasing accumulation of age pigments (lipofuscein) 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 neuron in old age (Brizee **et al.**, 1969,'75, Miquel **et al.**, 1978).

c) *Free-radical and cross-linking theory*- Harman (1956, 86, 91) proposed that the decrease in the adaptability of an organism during aging may be in part due to the free radical-mediated damages in the body. Both the 'Cross linking' theory of Bjorksten (1964) and the free radical theory 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- They include neuroendocrine and immunologic theories. According to Shock (1979), 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. With aging, the homeostatic adjustments decline with consequent failure of adaptive mechanisms and that the aging and death may be viewed as the result of this failure (Frolkis, 1982; Timiras, 1994). Walford (1969) proposed that efficiency of immune system decreases as a function of age. Hormonal and neural influences on the immune system have also long been known. Since efficiency of endocrine and neural system declines with age, immune system also exhibits age-related decline in several functions.

Enzyme levels during development and aging :

Enzymes are specific proteins that catalyze chemical reactions in biological system. Essentially, all biochemical reactions are enzyme catalyzed. One of the most striking features of enzymes is their specificity. Each enzyme catalyzes only one type of reaction showing a high selectivity for both reactants and products. Within a single species, there may exist 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 example, malate dehydrogenase is 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).

Growth and development of mammals 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 (Böhme, 1986). Many studies

have reported changes in enzyme activities with development and aging (Benzi **et al.**, 1980; Novdenberg, 1981; Sharma, 1988, 94). Developmental expression of enzymes varies markedly with the age, sex and strain of an organism. The level of glucose-6- phosphate dehydrogenase increases in the kidney of aging male rat, whereas it decreases in the females (Wilson and 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 tissue of old female (Chainy and Kanungo, 1978). Pyruvate Kinase (PK II) isoenzyme is present in fetal liver but absent in adult liver (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 and 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 and Ursprung, 1962). LDH isoenzyme composition is not only tissue-specific but also changes in the same tissue during development (Markert and 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 an 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 extend 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 function 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 & Kanungo, 1968). Studies on cytoplasmic alanine aminotransferase (c-ALAT) of the old rat liver reveal that the phenomena of sequential changes 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- and 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 enzymes 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 suckling and weaning rats (Yamada and Wakabayashi, 1991). Developmental changes of lipogenic enzyme activities in brown adipose tissues and liver of the rat have been studied and entail that the activities of fatty acid synthase, citrate cleavage enzyme, malic enzyme and glucose-6-phosphate dehydrogenase essentially followed a similar course of developmental expression as that of lipogenesis in these tissues. In contrast to those enzymes, NADP-linked isocitrate dehydrogenase remained unaltered over the period. Developmental expression of adenosine deaminase (ADA) has been studied in spleen, stomach and liver of mice at various postnatal ages (Singh and Sharma, 1995). The level of ADA is very low in the spleen and stomach of 5- and 10- day old mice and increases (3 fold) in 20 and 30- day old animals. In contrast, the activity of ADA is higher in the liver of 5-and 10-day old mice decreases (2-5 fold) in 20- and 30- day old animals and shows a sharp increase again at 60 day. These findings suggest an age-and tissue-specific expression of ADA. Maximal activities of key enzymes of glycolysis, krebs cycle and pentose phosphates pathway of several tissues at different ages have been studied and they show different patterns of expression in different tissues at various ages. (Sharma, 1988; Keast,'89).

Enzyme induction during development and aging:

Just as the cellular requirement for different proteins vary, the mechanism by which their respective genes are regulated also vary. The degree and type of regulation obviously reflect the function of the protein product of the 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 that catalyze reactions to control metabolic pathways, such as the citric acid cycle fall into this category. These genes are often referred to as housekeeping genes. Seemingly unregulated expression of such genes is called constitutive gene expression. The amount

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 inducible and the process of increasing the expression of such genes is called induction. The 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, higher level of tryptophan leads to repression of the genes for the enzymes catalyzing tryptophan biosynthesis in bacteria.

An eukaryotic transcription unit generally contains a single gene and 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 bp 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 tissue (Gillies **et al.**, 1983).

The promoter region consists of TATA box (centered at-30 bp), CAAT box (-75 bp) and GC box (-90 bp). 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 genertic 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 Vit D etc. 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 define 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 Matinez, 1991; Wiseman **et al.**, 1991).

Any enzyme catalyzed reaction may be influenced either: 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 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 a decrease or increase in the magnitude of induction with no change in the latent periods, iii) The response alters both the latent period and the magnitude of induction and iv) Age-related changes do not occur in the induction pattern (Adelman, 1975; 81). 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 isoenzyme induction by hormones have been studied in great detail. Kanungo and Gandhi (1972) reported that c-MDH but not m-MDH is inducible by cortisone in the tissues of old rats. The impairment of m-MDH induction in old animals can be repaired in regenerating liver after hepatectomy. Patnaik and Kanungo (1974) have shown that c-AlAT is inducible in the liver of immature, adult and old rats by hydrocortisone, whereas m-AlAT is inducible only in the older rats. Furthermore, c and m-tyrosine aminotransferase of the liver are inducible maximally in young rats by hydrocotisone (Ratha, 1977). A characteristic age-dependent induction of hepatic enzyme by dexamethasone has been established (Böhme **et al.**, 1986). The degree of induction was found to be highest in adrenalectomized rats.

Hormones and their receptors during development and aging :

Hormones are chemical signals synthesized from specialized group of cells that influence wide variety of cellular and metabolic processes within the body. They modulate the cellular and molecular processes by interacting with their cognate receptors located either on the cell surface (protein and peptide hormones) or within the cell/ cytosol/nucleus (steroid and thyroid hormones). Hormones have long been attributed to control the homeostasis, development and growth of animals. Hence, the level of hormones and their receptor and post-receptor events might influence

the process of development, growth and reproduction in animals.

Hormones that play crucial role in development are adrenal hormones (glucocorticoids), sex steroids (estrogen and testosterone) and thyroid hormones (T_3 / T_4). Glucocorticoids play an important role in the development, growth and homeostasis. Cohen (1973) and Martin **et al** (1977) reported that glucocorticoids are present even before birth in rats and exhibit significant changes after birth and neonatal period. In avian system, this hormone is known to increase during the early stages of development with a significant rise at about day 15 (Wise and Frye, 1973). It is also reported that exogenous hydrocortisone induces several enzymes (Cohen **et al.**, 1972). The rate of synthesis of corticosteroid hormones decreases with age (Ramanoff **et al.**, 1961; Grad and Khalid, 1968; Serio **et al.**, 1969).

The binding of glucocorticoids to their target tissues also changes with age. A decrease in glucocorticoid binding protein has been reported in human liver with increasing age (Singer **et al.**, 1973). Similar observations have also been reported in rat tissues by *in vitro* experiments (Roth,1979). Giannopoulos (1975) reported a significant change in the cortisol receptors in the nuclei of fetal rabbit liver during development. A lower binding of dexamethasone to the liver of old rabbit as compared to that of adult has been observed (Giannopoulos **et al.**, 1974). Petrovic and Markovic (1975) reported a decrease in the corticosteroid binding proteins in the liver and thymus of rats with advancing age. The role of several hormones on the longevity of species has been studied by Everitt (1971; 74). According to Everitt (1973, 75), the rate of aging and the duration of lifespan is determined by both the genetic and environmental factors.

Triiodothyronine(T_3) and tetraiodothyroxine (T_4) have long been recognized for their importance in regulating general metabolism, development and tissue differentiation. These hormones effect various processes by interacting with their intracellular receptors located primarily in the nucleus of target cells. Thyroid hormone receptors are a member of steroid receptors superfamily. The receptors upon interaction with hormone bind to specific DNA sequences termed a thyroid response elements (TREs), mostly located up stream of the regulated gene. This leads to a modulation of gene expression and consequently alterations in the metabolic activity of cells. Thyroid hormone-dependent enzymes, such as pyruvate carboxylase, phosphoenolpyruvate carboxykinase, pyruvate kinase and mitochondrial glycerolphosphate dehydrogenase were studied under different states of thyroid function and have shown variations in activities due to involvement

of these enzymes in controlling the overall rate of hepatic gluconeogenesis (Betger **et al.**, 1970). It has been reported that, in thyroidectomized rats, metabolites between cytosol and mitochondria show a marked decrease in citrate, 2- oxoglutarate and glutamate with a smaller change in aspartate and malate. These changes are interpreted as providing evidence for the importance of modification of the malate-aspartate shuttle in hypothyroidism. A decrease in thyroid function during old age has been observed by Frolkis (1973), but the level of plasma thyroxine showed no significant change. Thyroid hormones are present in low level in fetal rat liver and have been implicated in the appearance of glucokinase and malic enzyme during the late suckling period (Wakelam et at, 1985; Heide and Endo Visser, 1980).

Tissues responsiveness to hormones are controlled by binding of hormones to specific receptors that may lead to a change in the enzymatic and metabolic activity by activation of hormone receptor complex and conveying the messages to cellular control centres. Steroid receptors are represented by a single polypeptide chain and are located within the cell cytoplasm/ nucleus rather than on cell surface. However, receptor complexes of this type translocate to the nucleus and modulate the rate of transcription (Yamamoto, 1985). This, in turn, produces change in enzyme availability and subsequently alter the related metabolic activities of the cell. Receptors also exert a certain degree of control over the responses which 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 nuclei has been observed in rat prostate (Strain **et al.**, 1973).

Glucocorticoid receptors are a group of trans-acting factors whose activity is controlled by specific binding of the hormone (Beato,1989). Several studies have suggested that the glucocorticoid receptor level in the cell is a limiting factor in the process of gene induction by the hormone (Orket, 1991). However, the post-receptor events such as activation/ transformation, nuclear translocation and chromatin organization might also influence the magnitude of biological effects by the hormone (Sharma, 1991; Perlmann, 1992; Smith, 1993).

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 glucocorticoid have been reported to be influenced by the level of receptors and also by the post-receptor events (Sharma, 1994). High affinity glucocorticoid binding is detected in fetal rat

liver 6 days before birth and after the birth, there is a marked increase in the concentration of glucocorticoid receptors (Feldman, 1974; Giannopoulos, 1975). It is reported that rat hepatic glucocorticoid receptor concentration increases during development, concommitantly with the ability to induce tyrosine amino-transferase (Singer and Litwact, 1971). It is also reported that rat pancreatic glucocorticoid receptor and total plasma corticosterone level changes during postnatal development and the peak value of binding corresponds to an increase in the plasma corticosterone level during weaning. This indicates a close relationship between plasma corticosterone level and pancreatic glucocorticoid receptors and both may, therefore, play a vital role in pancreatic development in the rat (Lu **et al.**, 1987).

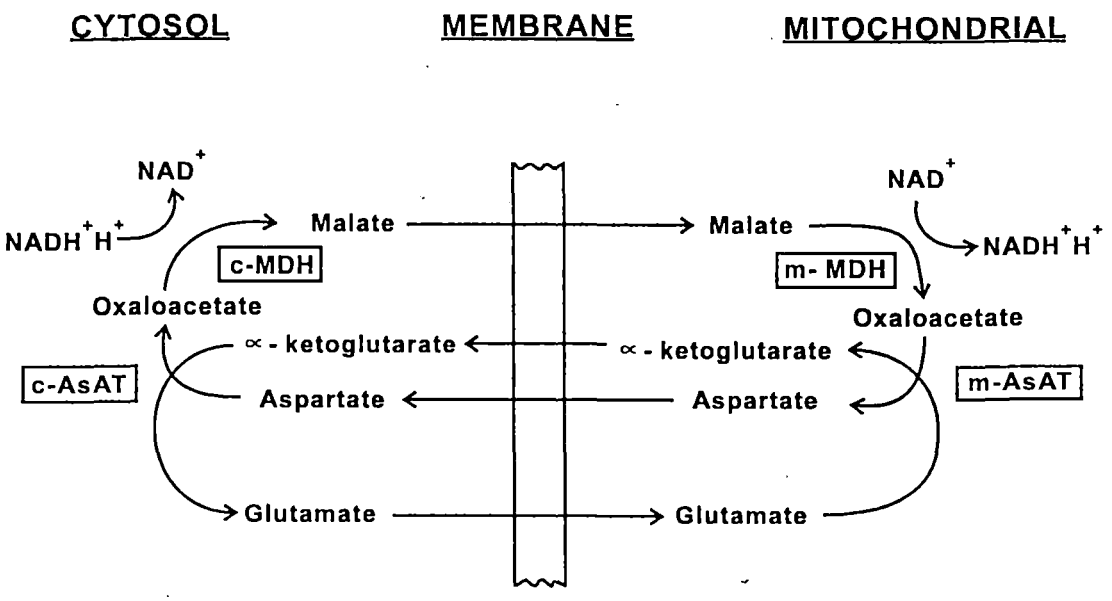
It is established that the steroid hormone action on target cells is controlled not only by the concentration of the individual hormones and their cognate receptors but may be modulated by the cascade of protein and peptide hormone action mechanisms (Sharma, 1991). Using biomodulators of glucocorticoid action, Katunuma **et al** (1988) reported that diacylglycerol (DAG), a potent activator of protein kinase C, enhances the induction of tyrosine aminotransferase (TAT) and ornithine decarboxylase (ODC) by dexamethasone, while itself has no effect on these enzymes induction in the absence of glucocorticoid. These findings established the involvement of protein kinase C in the expression of glucocorticoid action. The cross- talk between membrane hormone receptor is not confined to the steroid hormone action rather it also occurs with thyroid hormone action (Sharma, 1993).

Aggerbeck (1993) reported that the cytosolic aspartate aminotransferase (c-AsAT) displays liver-specific hormonal regulation. In the hepatoma cell line, *Fao*, both the activity and the mRNA level of c-AsAT are increased by glucocorticoid . The effect is potentiated by cAMP and inhibited by insulin and these effectors act at the transcriptional level. It was suggested that protein kinase A may also be involved in enhancing the binding of the glucocorticoid receptor to its target sites by a phosphorylation/dephosphorylation mechanism.

Malate-aspartate shuttle :

Lehninger (1951) showed that intact mitochondria are impermeable to externally added 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 TCA 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). This 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 reoxidize 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. Various mechanisms for the transfer of reducing equivalents from cytosol to mitochondria have been proposed, of which the glycerol phosphate and malate-aspartate shuttles are the most important (Meijer and VanDam, 1974; Dowson, 1974; Christen, 1985).



Schematic representation of malate-aspartate shuttle

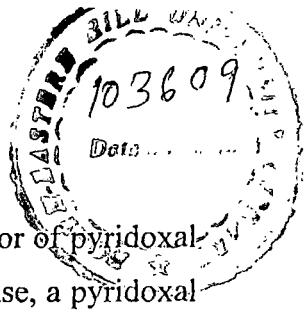
The most active NADH shuttle, which function in liver, kidney and heart mitochondria, is the malate-aspartate shuttle (Williamsom **et al.**, 1971; Cederbaum **et al.**, 1973; Ross **et al.**, 1977; Me Donald, 1983). The main enzymes of malate-aspartate shuttle are malate dehydrogenase(Grimm, S Doherty, 1961; Whitt, 1970) and aspartate aminotransferase (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 the enzymes have two homologous and genetically independent isoenzymes: one in the cytoplasm and the other in the mitochondrial fraction (Davidson, 1961; Bailey, 1969).

In this shuttle, the reducing equivalents of cytosolic NADH are first transferred to cytosolic oxaloacetate to yield malate by the action of cytosolic malate dehydrogenase (c-MDH). The malate formed, which carries the reducing equivalents donated by cytosolic NADH passes through the inner mitochondrial membrane into the matrix by a dicarboxylate 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 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 generation of cytosolic oxaloacetate to start another cycle of the shuttle and the details of schematic representation of malate- aspartate shuttle is shown previously.

The shuttle involves an influx of malate and glutamate and efflux of aspartate and α-ketoglutarate from mitochondria. The functional significance of malate-aspartate shuttle also unfolds the degree of control point 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). This carrier system, first described by Borst (1963), was designated as malate-aspartate shuttle. A directionality is imposed on the system by two carrier proteins.

The malate-aspartate shuttle can be reconstituted, using isolated mitochondria and the extra mitochondrial components of the shuttles (Cederbaum **et al.**, 1973; Tzu-chen yen **et al.**; 1989). 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 aminoxyacetate as a nonspecific inhibitor of pyridoxal-phosphate-dependent enzymes. Thus, it 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. 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 (Meloughlin **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, 1986).

Malate dehydrogenase :

Malate dehydrogenase (MDH ; EC 1.1.1.37) is widely distributed in mammalian tissues and plays an important role in carbohydrate metabolism. It catalyzes the oxidation of malate to oxaloacetate with a ΔG^0 of + 7 k cal/mole. MDH has been purified from wide variety of sources ranging from *E.*col to humans. It catalyzes 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 cytoplasm (c-MDH) and the other in the mitochondria (m-MDH). The two forms have different chemical, physical and kinetic properties and each has a characteristic electrophoretic pattern.

Larry and Johannes (1978) proposed that the mechanism of action of MDH is closely related to that of lactate dehydrogenase. m-MDH plays a crucial role in the reversible conversion of malate to oxaloacetate 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 oxaloacetate which is catalyzed by pyruvate carboxylase, a mitochondrial enzyme. Oxaloacetate 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 oxaloacetate. Further, oxaloacetate is converted to phosphoenolpyruvate 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. Davidson and Cartner (1967) have shown that c and m-MDH are under the control of two separate genes.

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 oxaloacetate and 48 μmol/ min/ g wet for malate oxidation at pH 7.4 (Crow **et al.**, 1982). The enzyme was also purified from the mitochondrial fraction of rat liver. The kinetic parameters for the enzyme were determined at pH 7.4. The V_{max} was found to be 38 μmol/ min/g wet wt for oxaloacetate reduction and 39 μmol/ min/ g wet wt for malate oxidation. Rates of the reaction catalyzed 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). Roderick and Banaszak (1986) determined the polypeptide chain conformation and coenzyme binding site of the crystalline porcine heart mitochondrial malate dehydrogenase by x-ray diffraction studies and reported that the overall polypeptide chain conformation, the location of the coenzyme binding site, and the location of catalytically important amino acid residues remain similar in mitochondrial malate dehydrogenase to that of cytoplasmic MDH and LDH isoenzymes.

Malate dehydrogenase shows an age-dependent increase of its activity in the liver, heart and brain tissues of rats (Ross and Ely, 1954; Schmukler, 1966; Singh and Kanungo, 1969; Sharma and Patnaik, 1982). The activity of c-MDH of young rat liver decreases after adrenalectomy and increases after administration of cortisone/ hydrocortisone to adrenalectomized animals (Kanungo and Gandhi, 1972; Sharma and Patnaik ,1982).

Berstein **et al** (1978) reported that the mechanism of substrate inhibition that occur with pig heart cytosolic and mitochondrial malate dehydrogenase is different from the lactate dehydrogenase from chicken heart. The inhibitor constant for oxaloacetate are 2.0 mM with the mitochondrial enzyme and 4.5 mM with the cytoplasmic enzyme. *In vivo* concentration of

oxaloacetate is 10 μM and so the substrate inhibition may not be significant under these conditions. Gelpi **et al** (1992) reported that m-MDH exhibits a complex regulatory pattern by citrate, which activates and inhibits mitochondrial malate dehydrogenase in the same direction ($NAD^+ \rightarrow$ NADH), and in the same reaction medium, depending on malate concentration. Zimmerle (1993) studied the chemical modification of porcine c-MDH and reported that it displays negative co-operativity in its substrate kinetics and co-factor binding activities.

Aspartate aminotransferase :

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 substrate. It catalyzes the transamination reaction representing key steps at the interconnection between the metabolic pathways of amino acid and dicarboxylic acid. AsAT effects the reversible transfer of an amino group from L-aspartate or L-glutamate to α-ketoglutarate and oxaloacelate (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 mitochondrial matrix (m-AsAT). The isoenzymes 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).

Aspartate aminotransferase isoenzymes 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 resolution and have reported that the enzyme molecule appears to consist of two dense and closely packed subunits with a dimension of 40 A^0 x 48 A^0x 60 A^0 each. Eichele et al (1979) have studied the crystal structure of m-AsAT from the chicken heart. In the electron density map, the enzyme is clearly seen as an isologous α_2 dimers (105°A 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 at., 1980).

The crystal structures of the stable, closed complexes of chicken mitochondrial aspartate aminotransferase with the natural substrate L-aspartate and L-glutamate have been resolved at 2.4 and 2.3A^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 Köhlen **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 been elucidated. The polypeptide chain of mitochondrial isoenzyme contains 40 3 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). The essential features of catalytic mechanism of both the isoenzymes are identical. A comparison of the amino acid sequence data of the cytosolic and mitochondrial aspartate aminotransferase from pig heart clearly indicates that these heterotopic isoenzymes are homologous protein. A comparison of the mitochondrial isoenzyme from two different species i.e. 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 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).

Hiroyasu et al (1978) have purified cytosolic and mitochondrial AsAT to homogeneity from human heart and shown that they 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 electrophoretic mobility, the human cytosolic isoenzyme differs from the corresponding isoenzyme from pig heart while no significant difference was observed between the mitochondrial isoenzymes from the two species (Boyd, 1961; Katunuma **et al.**, 1962; Kagamiyama & Wada, 1975).

Several workers (Fleisher **et al.**, 1960; Boyd,1961; Wada and Morino, 1964; Barra **et al.**, 1976) have purified both the isoenzymes of AsAT from various tissues of mammals and have shown that, at a fixed concentration of L-aspartate, the Km for α-ketoglutanate is much higher for mitochondrial than cytosolic enzyme. The reverse applies to the K_m for L-aspartate at a fixed concentration of α-ketoglutarate. The mitochondrial enzyme is more susceptible to the inhibition by DL glyceraldehyde-3-phosphate than that of the cytosolic enzyme (Kopelovich **et al.**, 1970). The inhibition of the mitochondrial enzyme is competitive with respect to α-ketoglutarate and noncompetitive with respect to L-aspartate. Aminoxyacetic acid and other aminoxyacetate are effective inhibitors of both c- and m-AsAT (Braunstein, 1973; Rej, 1977). The inhibitory effect of aminoxyacetic 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; Shrago and Lardy, 1966). Both the isoenzymes of AsAT are involved in the process of gluconeogenesis. Nakata **et al** (1964) reported that the specific activity of c-AsAT is higher in the adult as compared to that of fetal rat liver. Farre 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; Shrago and Lardy, 1966; Sharma and Patnaik, 1982, 84, 85).

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 3-4 weeks of postnatal age. The beginning of rapid AsAT accumulation in fetal liver coincides with the onset of thyroid and adrenocortical secretion. 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.

It has been reported that the removal of the hormone secreting organ from an animal causes a change in the level of many enzymes in different tissues. Adrenalectomy decreases the activity of c-AsAT in the liver of rats of all the ages (Sharma and Patnaik, 1982). Administration of hydrocortisone to adrenalectomized rats increases significantly the activity of this isoenzyme in the liver of rats. However, the degree of induction is highest in the liver of young rat and decreases with increasing age. The effect of testosterone on precursor m-AsAT mRNA was studied in rat ventral prostate and primary cell cultures of mini pig prostate. Testosterone induces a 2-3 fold increase in Pm-AsAT mRNA level in both ventral prostate and mini pig prostate culture (Franklin **et al.**, 1990). Aggerbeck et al (1993) reported that both the activity as well as mRNA level of c-AsAT are increased by glucocorticoids in hepatoma a cell line *Fao* and the effect is potentiated and inhibited by cAMP and insulin, respectively. They have also demonstrated these effects at the translational level. The presence of two regulatory regions in the c-AsAT promoter separates the positive cAMP effect from the negative insulin effect. Toussaint **et al** (1994) studied the expression and regulation of the rat testis c-AsAT gene and showed that the pattern of transcription initiation and the poly-adenylation site selection of a housekeeping gene can be tissue-specific. Abruzzese et al (1995) measured both mRNA level and activity level of AsAT isoenzymes in various rat tissues as a function of age. Patterns of mRNA expression for the two isoenzymes were similar in a particular tissue, whereas differed widely between the tissues. There seemed no correlation between mRNA levels and specific activities of the enzyme products. It was concluded that the translation of mRNA for the two isoenzyme is subject to tissue-specific regulation in an age related manner.

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 lifespan. And to study the activities of all the key enzymes of one particular metabolic pathway would provide a complete profile of their biological function during development and aging. Keeping in view the importance of studying all the enzymes of a particular metabolic cycle, the work embodies in this thesis is directed towards following:-

i) Assessment of endogenous activity levels of malate-aspartate shuttle enzymes in liver and kidney of mice at various postnatal ages (day 15, 30 and 60) in order to find out the age- and tissue-specific changes in the activity of this shuttle. Furthermore, data is to be confirmed by reconstituting the shuttle.

ii) Effects of adrenalectomy, glucocorticoid, combination of glucocorticoid and dibutyryl-cyclic AMP and thyroid hormone on the activity of shuttle enzymes in liver and kidney of mice at various postnatal ages (day 15, 30 and 60) to find out the role of these, in regulating the shuttle activity in a tissue- and age-specifc manner.

iii) Purification of one of the shuttle enzymes i.e., cytosolic aspartate aminotransferase from two different ages : one preweaned (15-day) and the other fully weaned and reproductively mature (180-day) to study its chemical and kinetic properties in order to find out, if there is any change in properties of shuttle enzyme as a function of age.

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EXPERIMENTAL

A. *MATERIALS*

Biochemicals and reagents :

Triton X-100, Oxaloacetate, NADH, ∝-Ketoglutarate, Malate dehydrogenase, Aspartate, Coomassie brilliant blue, Bovine serum albumin, Tris (Hydroxy amino methane), EDTA, Mercaptoethanol, ADP, Malate, Glutamate, Hydrocortisone, Triiodothyronine, Dibutyrylated-cyclic AMP were purchased from Sigma Chemical Company, USA.

Chromatography media and reagent for gel electrophoresis:

CM-Cellulose ion exchanger, Acrylamide, N'N' methylene bis acrylamide, NNN'N' Tetramethylene diamine, Ammonium persulfate, Bromophenol blue, Coomassie brilliant blue R-250, Glycine, O-Dianisidine tetrazotized salt were also purchased from Sigma Chemical Company, USA.

Other reagents and chemicals :

NaCl, Sucrose, K_2HPO_4, KH_2PO_4, Phosphoric acid, HCl, Mannitol, $MgCl_2$, KCl, Di-ethyl ether, Ethanol, Urea, $(NH_4)_2SO_4$, NaOH, Sodium acetate, Acetic acid, Glycerol, Methanol and all other chemicals were of analytical grade purchased from Sisco Res Lab, Hi-media and Qualigens, India.

Animals : Male Swiss Albino mice (Balb / c strain) were used for the experiments. They were maintained under normal laboratory conditions at 24 ± 2° C and fed with standard pellet diet (Amrut Laboratory, Pune) and tap water ad *libitum*. Male mice of three age groups (15-, 30- and 60- day) were used for developmental and hormonal studies. Male mice of age groups (15- and 180- day) were used for purification and kinetic studies. Each set of data was collected from 3-4 mice of the same age group. All the operations were performed at a fixed time of the day, in order to avoid fluctuations in enzyme levels due to circadian rhythm and the mice were sacrificed at 1800 hr.

INSTRUMENTATIONS :

pH measurement — A control dynamics digital pH meter model Apx 175 E/C was

routinely used for all pH measurements at room temperature and the calibration was done using standard buffer tablets of different pHs.

Absorbance measurements — A Hitachi U-2000 double beam spectrophotometer was used for all absorbance measurements in the visible and ultraviolet region using glass and quartz cuvettes of 1 cm path length, respectively.

Centrifugation — All centrifugations were carried out in a Hitachi model Himac CR 20B2 - high speed refrigerated centrifuge at 4° C.

Homogenization — Remi motor type RQ - 127A, HP8, rpm 8000 (1.1 Amps, 220/230 V) homogenizer fitted with a teflon pestle was routinely used. Glass homogenizing tubes (5-50 ml) were used for homogenizing tissues.

Electrophoresis — A Bio-Rod slab gel electrophoresis apparatus (Model 122/2.0) and mini protean II chamber was used for polyacrylamide gel electrophoresis.

Weighing Balance — Sartorius balance model 2434 (0.01 mg - 160 g), model 2405 (0.001 mg - 30 g) and an electronic tap pan balance (0.01 - 600 g) were used for weighing chemicals.

B. METHODS

Preparation of homogenates — The mice of various ages were killed by cervical dislocation. The liver and kidney were removed immediately and washed in ice-cold 0.9% NaCl. The adhering blood vessels were blotted dry on a filter paper and the tissues were stored at -70° C till further use. A 10% (w/v) homogenate of the tissues was prepared in ice-cold 0.25 M sucrose using a homogenizer fitted with a teflon pestle.

Fractionation of isoenzymes — The homogenates were centrifuged for 10 min at 800 xg at 2° C to sediment nuclei and cell debris. The resulting supernatant was further centrifuged for 40 min at 14,000 xg at 2° C to sediment mitochondria. The supernatant thus obtained was used for the assay of cytosolic aspartate aminotransferase (c-AsAT) and malatedehydrogenase (c-MDH). The mitochondrial pellet was washed once and suspended in a solubilizing medium (10 mM potassium phosphate buffer, pH 7.5 / 0.25 M sucrose / 0.5% Triton X-100) to make a

10% suspension. Various concentrations of phosphate buffer and Triton X-100 were used to solubilize the mitochondrial AsAT before using the most effective one mentioned above. For the assay of m-MDH, the mitochondrial pellets were suspended in 50 mM potassium phosphate buffer, pH 7.5 containing 0.25 M sucrose. Since it has been observed that the m-MDH is released maximally in this medium. Assays for m-AsAT and m-MDH were performed within 3 hr of mitochondrial suspension.

Assay of aspartate aminotransferase (AsAT) — The activity of both the isoenzymes of AsAT (c- and m- AsAT) was measured according to the method of Karmen (1955) with certain modification in the concentration of the substrates and cofactor (Hertzfeld, 1972; Sharma and Patnaik, 1982). According to the method, the rate of oxaloacetate formation is measured spectrophotometrically in a coupled reaction, catalyzed by malate dehydrogenase in the presence of NADH. The reaction occurs as follows:

$$\text{Asp} + \alpha\text{-KG} \overset{\text{AsAT}}{\rightleftharpoons} \text{Oxaloacetate} + \text{Glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \overset{\text{MDH}}{\rightleftharpoons} \text{Malate} + \text{NAD}^+$$

For each mole of oxaloacetate formed from aspartic acid, 1.0 mole of NADH is oxidized in the coupled reaction. NADH has a characteristic absorption maxima at 340 nm with extinction coefficient of 6.22×10^6 /mole. The rate of disappearance of NADH was measured at 340 nm using a cuvette of 1.0 cm light path. The final volume of the reaction mixture was 3.0 ml which contained :

Potassium phosphate buffer, pH 7.5	—	100 mM
α-Ketoglutarate	—	6.00 mM
Malate dehydrogenase	—	10 units
NADH	—	0.14 mM
Enzyme (suitably diluted)	—	20 μl
Aspartate	—	75 mM

The reaction was initiated by the addition of aspartic acid to the experimental cuvette and decrease in absorbance was recorded at 30 sec. intervals for 3 min. The linear decrease in absorbance per min was used for the calculation of the enzyme activity. The enzyme was so diluted that the decrease in absorbance did not exceed 0.1 per min.

One unit of enzyme activity was taken as that amount which catalyzed the conversion of one μ mole of the substrate per min at 25° C. The specific activity was expressed as units per mg protein

Assay of malate dehydrogenase (MDH) — Both the isoenzymes of MDH (c- and m-MDH) were assayed spectrophotometrically according to the method of Kitto (1969). The method is based on the measurement of the rate of oxidation of NADH (i.e. decrease in absorbance at 340 nm) in the presence of this enzyme and its substrate (oxaloacetate). The reaction occurs as follows :

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \overset{\text{MDH}}{\rightleftharpoons} \text{Malate} + \text{NAD}^+$$

The rate of disappearance of NADH was measured at 340nm in the spectrophotometer using a cuvette of 1.0 cm light path. The final volume of the reaction mixture was 3.0 ml which contained :

Potassium phosphate buffer, pH 7.5	—	100 mM
Oxaloacetate	—	0.5 mM
NADH	—	0.14 mM
Enzyme (suitably diluted)	—	20 μl

The reaction was initiated by the addition of oxaloacetate. The decrease in absorbance was recorded at 30 sec. intervals for 3 min and the enzyme activity was calculated from the initial rate of oxidation of NADH. The amount of enzyme used was adjusted such that the decrease in the absorbance was met 0.10 per min. One unit of MDH (both in isoenzyme) activity was defined as the amount which is required to oxidize 1 μ mole of NADH per min at 25 ° C. The specific activity was expressed as units per mg protein.

Protein estimation — Protein concentration was determined by the dye binding method of Bradford (1976) using bovine serum albumin as reference standard. The working reagents were as follows :

A. Coomassie brilliant blue G-250 (0.2% in 95% ethanol)

B. Phosphoric acid (85%)

Bradford stock solution was prepared by mixing reagents A and B and was stored in a brown bottle at 4° C for future use. Bradford working solution at the time of use was prepared diluting 15 ml of the Bradford stock solution to 100 ml with distilled water and filtered through Whatman No.1 filter paper. The final concentrations of the reagents were 0.01% (w/v) coomassie brilliant blue G-250, 4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid.

Appropriately diluted protein samples were made upto 1.0 ml with distilled water to which 5 ml of Bradford working solution was added and mixed thoroughly with the help of cyclomixer. Colour was allowed to develop (10-15 min) at room temperature and the intensity of the colour was measured at 595 nm against a reagent blank. Protein concentration was determined with the help of a computed standard curve prepared by taking BSA in the concentration range of 10 to 100 μg.

Reconstitution of malate-aspartate shuttle — For the reconstitution of the malate-aspartate shuttle, the tissues were homogenized in four volumes of homogenization buffer (50 mM Tris-HCl, pH 7.4/ 0.25 M mannitol / 1 mM EDTA/ 2 mM $MgCl_2$ / 30 mM 2-mercaptoethanol) and centrifuged at 800 xg for 10 min at 2° C to sediment nuclei. The supernatant was further centrifuged at 14,000 xG for 30 min to sediment mitochondria. The mitochondrial pellet was washed twice, suspended in homogenization buffer, and used for the reconstitution assay. The post-mitochondrial supernatant was dialyzed for 18 hr at 4° C against 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA, 2 mM $MgCl_2$, and 30 mM 2-mercaptoethanol. The dialyzed cytosol was centrifuged at 14,000 xg for 30 min to remove traces of particulate materials and the resultant clear supernatant was used for the reconstitution studies.

Reconstitution assay was done according to the method of Cederbaum (1973) and Dawson (1982) with some modifications of our own in the amount of protein taken and in the final assay

volume. The reaction mixture (final volume, 2.5ml) contained buffer incubation medium (300 mM mannitol/ 10 mM Potassiumphosphate buffer, pH 7.4/10 mM Tris HCl pH 7.4/ 10 mM KCl/ 5 mM $MgCl_2$/ 2 mM ADP/ 2 mM aspartate), 2 mg cytosolic protein and 1 mg mitochondrial protein. After setting the baseline to zero, 50 μl of 7 mM NADH was added to the sample cuvette giving the absorbance of 0.70. The slow steady fall in absorbance was monitored for 2 min and then 50 μl of a solution of 0.2 M each of L-malate and glutamate was added to both cuvettes. The decrease in absorbance was followed upto 10 min.

Hormone treatments — Effects of hydrocortisone, triiodothyronine and dibutyrylated cAMP on the activity of AsAT and MDH were studied in the liver and kidney of 15 -, 30- and 60- day old male mice. Different doses and time durations have been used to check the optimal dose and time response. Animals were grouped in two sets comprising of 3-4 mice per set. The first set of mice were administered intraperitoneally with hormone suspending medium and served as the normal. The second set of animals were administered with suspended test hormones. To avoid the fluctuation which may arise due to circadian variations, hormone administrations were done at a fixed time of day (0800 hr) in all cases.

Single hormone injection — When none of the different doses (0.1, 1.0, 2.5 and 5 mg/ 100 g body weight) of hydrocortisone administered as a single dose were effective on the shuttle enzymes of liver and kidney, some doses were checked as a repeated dose (for three days). Finally, hydrocortisone (1 mg/100 g body weight) was administered in a total volume of 0.3 ml normal saline containing 10% ethanol. Hormone was administered three consecutive days at the same time. Mice were sacrificed 6 hrs after the final injection and tissues (liver and kidney) were removed and washed in normal saline, blotted dry and stored at -70° C. These tissues were later processed and assayed for MDH and AsAT activities as described under method.

Dybutyrylated-cyclic AMP (Bt_2 - cAMP) — Different doses (0.1, 0.2, 0.5 and 1.0 mg/100 g body weight) of Bt_2 - cAMP prepared in normal saline (0.9% NaCl) were administered to the test animals as single dose. All the other conditions followed the similar patterns as described for hydrocortisone.

Triiodothyronine (T_3) — Different doses (50, 100 and 200 μg/100 g body weight) of triiodothyronine (T_3) suspended in saline (0.9% NaCl) and 10% ethanol mixture, were adminis-

tered as a single dose. Tissues were similarly removed and stored at -70° C and later used for assay of enzymes.

Effects of adrenalectomy and hydrocortisone treatments — Effects of adrenalectomy and the administration of hydrocortisone to adrenalectomised mice were studied on the activities of AsAT and MDH in liver and kidney of mice at three different postnatal ages. The mice of each age group were divided into three sets of 4-5 animals. Set I and II were used for the study of adrenalectomy and adrenalectomy plus hydrocortisone respectively and the III set was used as non-adrenalectomised control.

All the mice of set I & II were bilaterally adrenalectomised. These mice were given in addition to normal pellet diet, 0.9% NaCl instead of water for 5 days following adrenalectomy. During which, endogenous glucocorticoids become negligible in the blood (Raina and Rosen, 1968). On the 6th day, mice of set II were administered intraperitoneally with hydrocortisone (1 mg/100 g body weight) in 0.5 ml of 0.9% NaCl having 10% ethanol. Pilot experiments showed that i.p dose of 1 mg/100g body weight of hydrocortisone to the mice exhibited maximum effect on the activities of MDH and AsAT. The mice belonging to set I received the same amount of 0.5 ml suspension medium (10% ethanol and 0.9% NaCl) at a fixed time of the day for three days. All the mice were sacrificed after 6 hr of the final hormone injection and their tissues (liver and kidney) were taken out, washed in ice-cold saline, blotted dry and stored at -70° C till the assay of MDH and AsAT.

Effect of combination of hydrocortisone and Bt_2 -cAMP — Mice of three different postnatal ages were divided into two sets. Following similar pattern as of single hormone administration, combination of Bt_2 -cAMP (500 μg/100 g body weight) and hydrocortisone (1 mg/100 g body weight) was administered (i.p) in the test mice of set I in a total volume of 1 ml of 0.9% NaCl / 10% ethanol mixture. The second set received only the saline/ethanol mixture. Animals were sacrificed 6 hr after the injection. Tissues were similarly excised, washed in cold normal saline, blotted dry and stored at -70° C until assay of enzyme activities.

Purification of cytosolic aspartate aminotransferase (c-AsAT) — c-AsAT was purified from the liver of immature (15 day) and mature (180day) male mice according to the method of Marra **et al.** (1977) with some modifications as described earlier (Sharma and Patnaik,

1982). All the steps unless mentioned otherwise, were carried out at 4° C.

Step I — ***Crude extract***

The livers were collected from 10-12 male mice of immature and mature age groups. The adhering blood vessels from the tissue were removed. A 10% (w/v) homogenate of the liver was made in 0.25 M Sucrose using a glass homogenizer fitted with a teflon pestle. The crude homogenate was centrifuged at 22,000 xg for 30 min. The pellet was discarded and the resulting supernatant was used for further purification.

Step II — ***Ammonium sulfate fractionation***

The supernatant obtained from Step I was brought to 40% ammonium sulfate saturation through gradual addition of solid $(NH_4)_2SO_4$. The solution was slowly stirred for one hr and was then centrifuged at 10,000 xg for 10 min. The pellet was discarded.

The resulting supernatant of the first $(NH_4)_2SO_4$ precipitation was brought to 80% $(NH_4)_2SO_4$ saturation through a gradual addition of solid $(NH_4)_2SO_4$. After complete solubilization of the added salt into the crude enzyme solution, the pH was maintained at 7.4 by addition of 1 N NaOH. The solution was stirred slowly for 12 hr with the help of a magnetic stirrer. It was centrifuged at 20,000 xg for 30 min and the supernatant was discarded.

Step III — ***Dialysis***

The final precipitate obtained from Step II was dissolved in a minimum volume of 0.02 M sodium acetate buffer, pH 5.4 and was dialyzed for 36 hr against the same acetate buffer. Dialysate was then centrifuged at 10,000 xg for 30 min and pellet was discarded.

Step IV — ***CM-Cellulose chromatography***

The clear supernatant from Step III was applied on to a column (1.6 x 14 cm) of CM-Cellulose, which was pre-equilibrated with 0.02 M sodium acetate buffer, pH 5.4 at a flow rate of 30 ml/hr. After application of the sample, the column was extensively washed with 0.02 M acetate buffer pH 5.4. Subsequently, a linear gradient of sodium acetate buffer (0.06-0.16 M) was applied with the help of a gradient mixture to elute the bound fractions. The active peak fractions were collected, pooled and were concentrated by ammonium sulfate fractionation.

Step V — ***Ammonium Sulfate fractionation***

The enzyme-rich fractions obtained after CM-Cellulose chromatography were pooled together and were brought to 80% saturation by the addition of solid $(NH_4)_2SO_4$. The solution was stirred slowly for 12 hr and then centrifuged at 20,000 xg for 30 min. The supernatant was discarded and the pellet was dissolved in a minimum volume of 0.02 M sodium acetate buffer pH 5.4. This preparation was further dialyzed for 24 hr to remove the salt present with purified enzyme. The sample was then centrifuged at 10,000 xg for 30 min and the clear supernatant was used as the source of purified enzyme for the kinetic and other analysis.

Polyacrylamide gel electrophoresis of purified c-AsAT — Polyacrylamide gel electrophoresis (PAGE) of the purified c-AsAT from the liver of immature and mature mice was performed according to the method of Davis (1969) with slight modification. A 7.5% slab gel was prepared by taking : 2.5 ml of acrylamide / bis acrylamide / 3.73 ml of 1.0 M tris buffer (pH 8.8) / 3.70 ml of distilled water / 0.033 ml of APS (freshly prepared).

Prior to addition of APS, gel solution was degassed for 15 min under vaccum and was immediately casted into slab plates fitted with spacers, sealed at both sides and lower ends. The gel was allowed to polymerize and then pre-run for 15 min at 12 mA. Purified enzyme preparation (50 μg) from both the ages of mice were mixed with assay buffer, glycerol (10%) and bromophenol blue (0.1%). 20-40 μl of this preparation was carefully applied with the help of micro syringe onto gel lanes and electrophoresed for 30-40 min at 24 mA in the cold. Subsequently, one set of gel was removed, washed in distilled water and stained with 0.5% coomassie brilliant blue (prepared in 30% methanol and acetic acid) for 30 min. Background stain was removed by immersing the gel in destaining solution (20% methanol and 7% acetic acid).

The other set of gel, after electrophoresis, were processed for specific staining of c-AsAT according to the method of Doonan (1980) with certain modifications. The staining mixture contained L-aspartic acid (15 mM), ∝-ketoglutarate (6.8 mM), tris (100 mM; pH 7.5). Prior to use, O-Dianisidine-tetrazotized (Fast Blue B salt) was added to a final concentration of 10 mM and the staining mixture was stirred vigorously. The mixture was poured onto slab gel and the enzyme activity band appeared as violet colour within a short span of time. The staining mixture was then decanted and the gel were washed thoroughly to avoid any further development of background colour. These gels were stored overnight in a solution of distilled water, methanol

and acetic acid in the ratio of 5:3:1 (v/v) and subsequently photographed.

Kinetic studies

Effect of [L-aspartate] and [∝-ketoglutarate] on purified c-AsAT — The activity of purified c-AsAT from the liver of immature and mature mice was measured at various concentrations of L-aspartate (0-100 mM) and ∝-ketoglutarate (0.01-10 mM) in separate sets of experiments. The values of K_m for both substrates were determined by Michaelis-Menten and Lineweaver-Burk plots of data using Sigma enzfitter programme (Perella, 1988).

Effect of [Amino-oxyacetic acid] on c-AsAT — The effect of amino-oxyacetic acid (AoAA) on the activity of purified c-AsAT from the liver of immature and mature mice was studied by using various concentrations (0-0.2 mM) amino-oxyacetic acid at two different fixed concentrations of L-aspartate (10 and 40 mM) and ∝-ketoglutarate (1 and 2 mM). The K_i values were determined by Dixon's plot of the data using enzfitter programme.

Effect of urea on c-AsAT — Using varying concentrations (0.01-8 M) of urea as denaturant in potassium phosphate buffer, pH 7.5, the activity of purified c-AsAT from two different ages (immature and mature) were studied. The enzymes samples were incubated for 30 min at these concentrations of urea, while the other conditions of assay remained the same. The results similarly expressed as percentage activity retained in presence of specific urea concentration taking no urea as 100%.

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RESULTS

The results are presented in the tables which contain the mean values, standard deviation of the mean, level of significance and also per cent increase or decrease between two sets of data. The results are also presented in figures for clarity. Only the salient findings and not the detailed descriptions of the data are mentioned here. The activities of both the isoenzymes of malate dehydrogenase (c- and m- MDH) and aspartate aminotransferase (c- and m- AsAT) have been expressed as units / mg protein in the tables as well as in the figures. Activities of malate-aspartate shuttle enzymes and the shuttle activity were measured primarily during developmental period (preweaning, weaning and postweaning ages). Hormonal studies were also performed with the same ages. However, the kinetic characterization of cytosolic AsAT was done in the immature (15-) and mature (180-) mice in order to find the difference if any, in this isoenzyme as a function of age.

Malate-aspartate shuttle enzymes at various postnatal ages :

Malate dehydrogenase (MDH) — Specific activities (U/mg protein) of MDH isoenzymes (c- & m-) are significantly higher in the liver of 15-day old mice, declined thereafter at day 30, and remained unchanged until day 60 of postnatal age (Table-1; Fig- 1). In contrast, the activities of MDH isoenzymes (c- and m-) reach peak level at day 30 in the kidney of mice. It exhibits a decline at day 60 of postnatal age in the kidney of mice (Table- 1; Fig- 2). In both the tissues, the activities of mitochondrial MDH are significantly lower than that of cytosolic isoenzyme at all the postnatal ages studied.

Aspartate aminotransferase (AsAT) — Specific activity (U/mg protein) of isoenzymes of aspartate aminotransferase (c- & m-) shows a similar pattern as the isoenzymes of malate dehydrogenase in both the tissues i.e. liver and kidney. Levels of liver AsAT (c- & m-) are significantly higher at day 15, decline at day 30 and remained unchanged thereafter (Table-2; Fig- 3). In kidney, however, both the isoenzymes of AsAT are significantly higher at day 30. Like mitochondrial isoenzyme of MDH in both the tissues, the activity of mitochondrial AsAT is significantly lower as compared to cytosolic AsAT at all the postnatal ages. Further, it has been observed that the endogenous level of both the isoenzymes of AsAT are much lower as compared to the isoenzymes of MDH (Table-2; Fig-4).

TABLE - 1

Activities (U/mg protein) of malate dehydrogenase isoenzymes (cytosolic and mitochondrial) in the liver and kidney of normal male mice of various postnatal ages

Tissues	Age (Day)	*Cytosolic*			*Mitochondrial*		
		Mean	SD	P	Mean	SD	P
LIVER	15	12.02	± 0.43		3.47	± 0.19	
				<0.01			<0.001
	30	8.43	± 0.92		2.58	± 0.13	
				NS			NS
	60	8.28	± 0.70		2.53	± 0.16	
KIDNEY	15	18.56	± 2.01		1.52	± 0.25	
				<0.001			<0.01
	30	36.67	± 1.33		2.31	± 0.21	
				<0.01			<0.001
	60	28.27	± 2.46		1.05	± 0.23	

SD — Standard deviation; P — Level of significance; NS — not significance

TABLE - 2

Activities (U/mg protein) of aspartate aminotransferase isoenzymes (cytosolic and mitochondrial) in the liver and kidney of normal male mice of various postnatal ages

Tissues	Age (Day)	*Cytosolic*			*Mitochondrial*		
		Mean	SD	P	Mean	SD	P
LIVER	15	1.54	±0.15	<0.01	0.78	±0.06	<0.001
	30	0.75	± 0.07	NS	0.21	± 0.02	NS
	60	0.73	± 0.11		0.18	± 0.05	
KIDNEY	15	0.81	± 0.06	<0.001	0.13	± 0.02	<0.05
	30	1.20	± 0.12	<0.001	0.16	± 0.01	<0.001
	60	0.69	± 0.05		0.10	± 0.008	

SD — Standard deviation P — Level of significance; NS — not significance

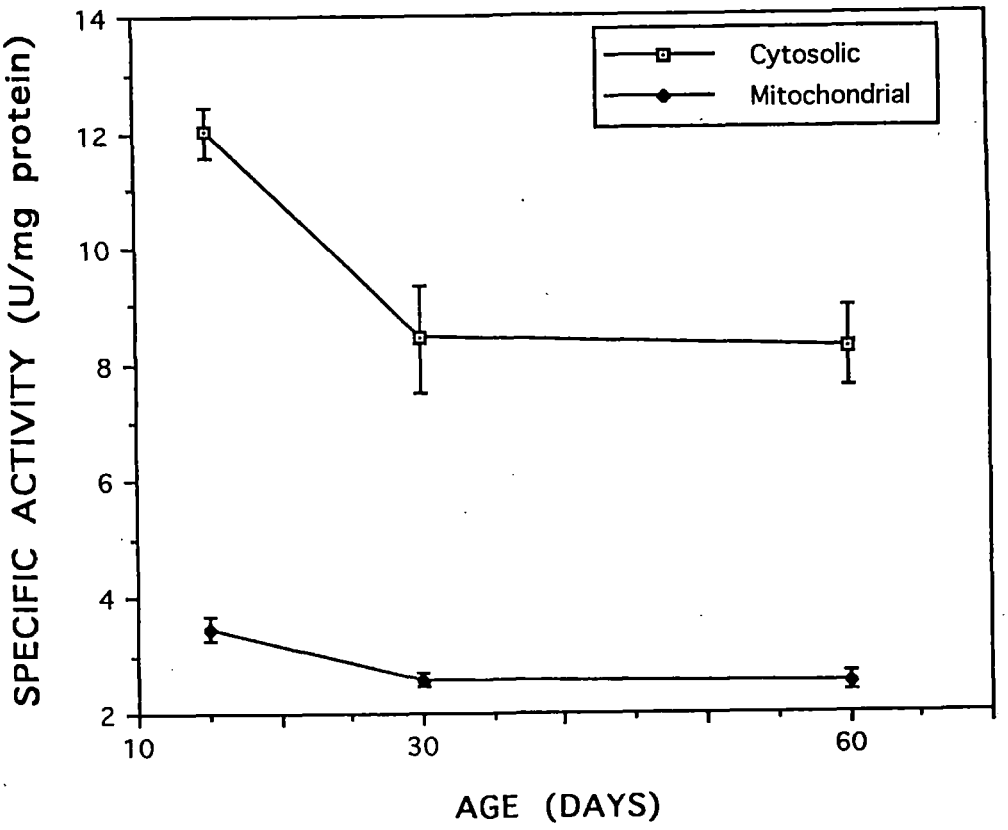


Fig. 1 Activity of malate dehydrogenase (MDH) isoenzymes (cytosolic and mitochondrial) in the liver of normal male mice of different postnatal ages. Values are means for 4-5 mice in each age group. Bars represent standard deviation.

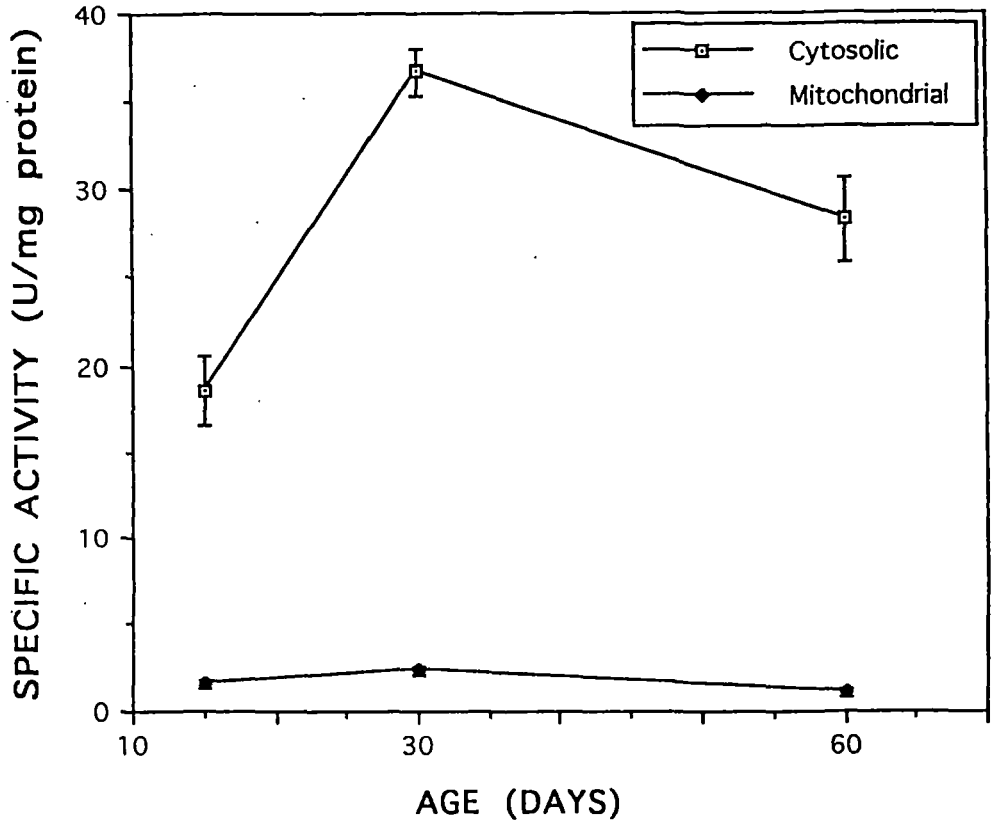


Fig. 2 Activity of malate dehydrogenase (MDH) isoenzymes (cytosolic and mitochondrial) in the kidney of normal male mice of different postnatal ages. Values are means for 4-5 mice in each age group. Bars represent standard deviation.

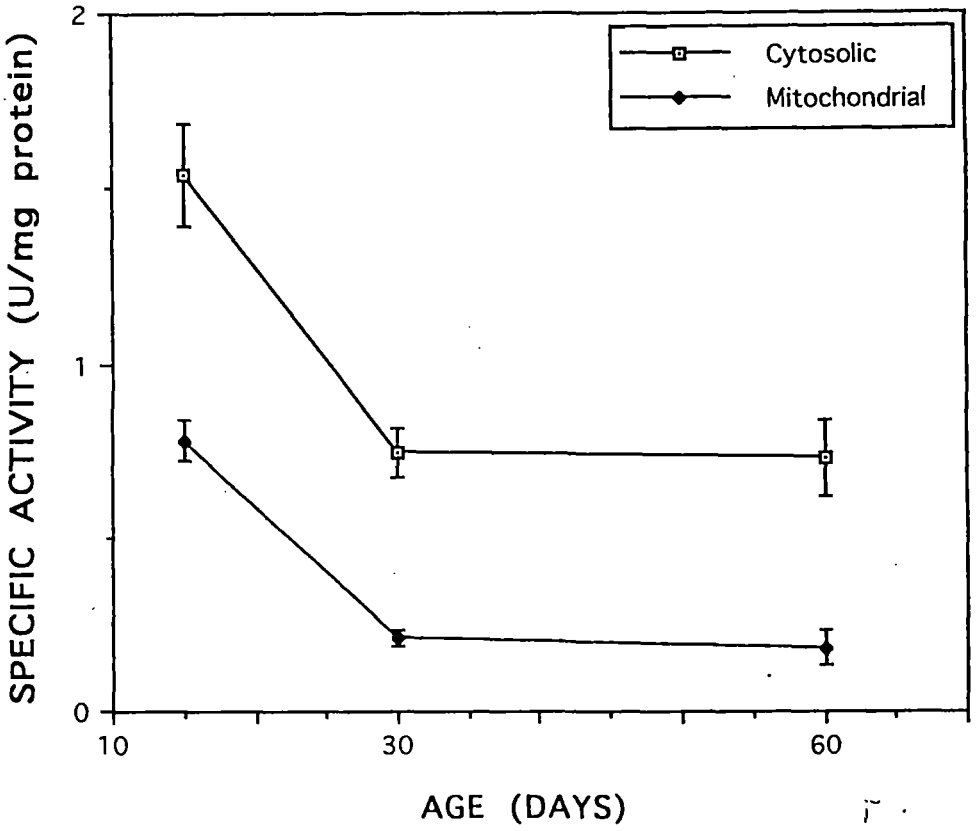


Fig. 3 Activity of aspartate aminotransferase (AsAT) isoenzymes (cytosolic and mitochondrial) in the liver of normal male mice of different postnatal ages. Values are means for 4-5 mice in each age group. Bars represent standard deviation.

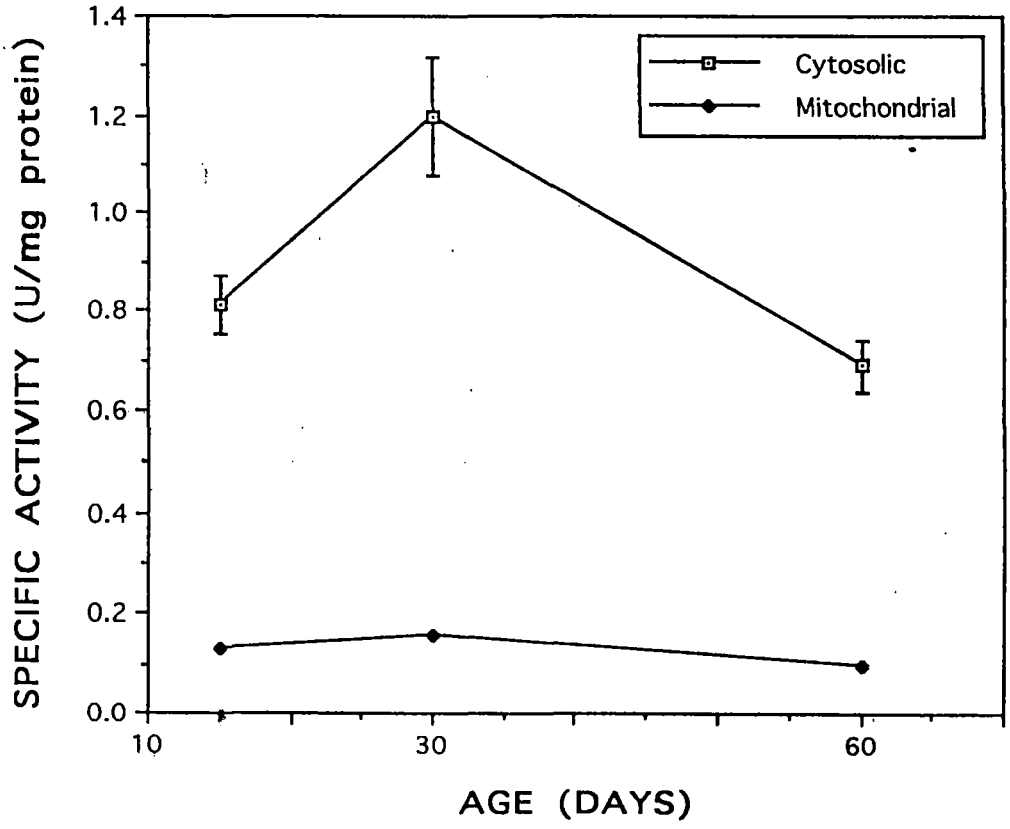


Fig. 4 Activity of aspartate aminotransferase (AsAT) isoenzymes (cytosolic and mitochondrial) in the kidney of normal male mice of different postnatal ages. Values are means for 4-5 mice in each age group. Bars represent standard deviation.

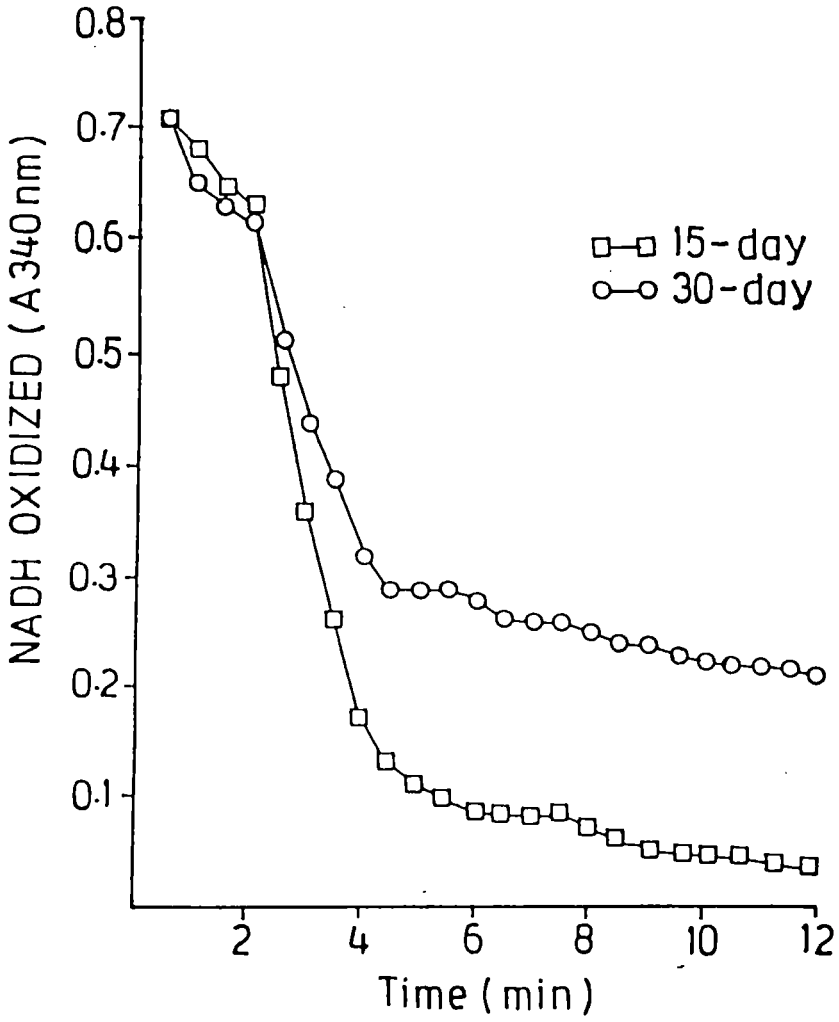


Fig. 5 Oxidation of NADH by reconstituted malate-aspartate shuttle in the liver of normal male mice. Equal amount of dialyzed clear cytosol (2 mg protein) and mitochondria (1 mg protein) from both the ages (15- and 30-day) were used for both the reconstitution assay. Details of experimental procedures are given in methods section. The traces depict the change in absorbance at 340 nm with the passage of time.

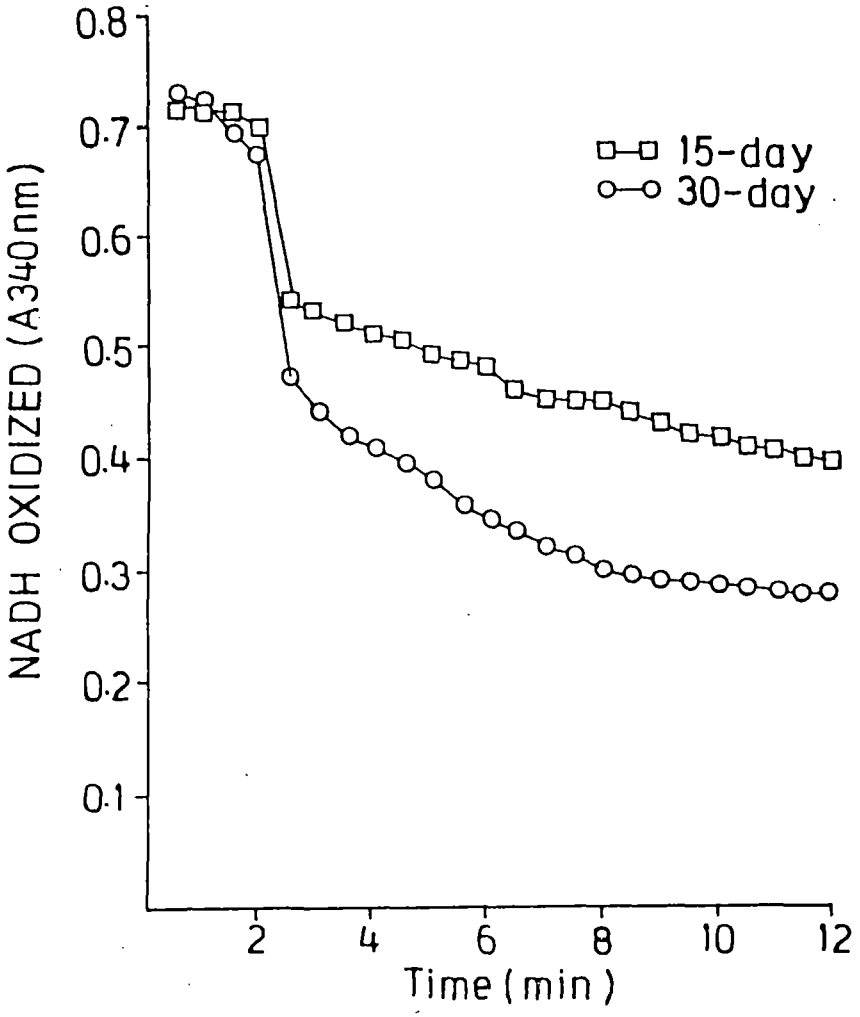


Fig. 6 Oxidation of NADH by reconstituted malate-aspartate shuttle in the kidney of normal male mice. Experimental procedures were same as those of Fig-5. The traces depict the change in absorbance at 340 nm with the passage of time.

Reconstitution of malate-aspartate shuttle :

Reconstitution of malate-aspartate shuttle *in vitro* confirmed the activity level of both the shuttle enzymes as reported above. Shuttle activity (oxidation of NADH as measured by decrease in absorbance at 340 nm) was significantly higher in the liver of 15-day old mice as compared to that of 30-day old mice (Fig-5). Whereas, the shuttle activity was significantly higher in the kidney of 30-day old mice than that of 15-day old mice (Fig-6). Similar to the expression of enzymatic activities, the shuttle activity showed an identical pattern in the liver and kidney of developing mice.

Hormonal regulation of malate-aspartate shuttle enzymes :

Effects of hydrocortisone triiodothyronine, Bt_2-cAMP were studied on the activity of shuttle enzymes in the liver and kidney of mice at three different postnatal ages (15-, 30- and 60- day) in order to understand the involvement of these hormones in regulating the levels of shuttle enzymes. Various doses (0.1, 1, 2.5 and 5 mg/100 g body weight) of hydrocortisone were administered as a single and also as repeated (3 days) doses at different postnatal ages of mice. There was no marked effect of single hydrocortisone administration on the activity of shuttle enzymes in either tissues at any of the ages studied. However, repeated injection of hydrocortisone (1 mg/100 g body weight for 3 days) showed around 15% induction of liver cytosolic AsAT in adult mice. It was thought that the endogenous level of adrenal steroid might have been enough to influence the shuttle enzymes, hence we adrenalectomized the mice to study the effect of exogenous administration of hydrocortisone.

Effects of adrenalectomy and hydrocortisone on the activity of malate-aspartate shuttle enzymes :

Malate dehydrogenase (MDH) — Adrenalectomy causes a significant decrease (-20%, -30% & -25%, respectively) in the activity (U/mg protein) of liver cytosolic MDH of mice at all the postnatal ages studied (day 15, 30 and 60) (Table-3; Fig -7A). It also decreases (-26%, -23%, -26%, respectively) the level of liver mitochondrial MDH at those ages (Table3; Fig-7B). Administration of hydrocortisone (1 mg/100g body weight for 3 days) to adrenalectomized mice significantly increases (+49%, +46% and +27%, respectively) the activity of liver cytosolic malate dehydrogenase at all three postnatal ages studied. Administration of hydrocortisone also increases

TABLE - 3

Effects of ardenalectomy (A/d) and hydrocortisone (HC) on the activity (U/mg protein) of malate dehydrogenase isoenzymes in the liver of male mice of various postnatal ages

Isoenzyme	Treat-ment	15- Day				30- Day				60- Day			
		Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)
Cytosolic	Normal	14.19	± 0.94			7.92	± 0.20			7.88	± 0.34		
				<0.01	-20			<0.001	-30			<0.01	-25
	A/d	11.40	± 0.51			5.54	± 0.31			5.97	± 0.24		
				<0.001	+49			<0.001	+ 46			<0.01	+ 28
	A/d + HC	17.08	± 0.35			8.10	± 0.20			7.61	± 0.85		
Mitochondrial	Normal	1.20	± 0.09			0.90	± 0.10			0.89	± 0.08		
				<0.01	-26			<0.05	-23			<0.02	-34
	A/d	0.89	± 0.03			0.69	± 0.03			0.66	± 0.07		
				<0.001	+ 32			<0.001	+ 33			<0.05	+ 21
	A/d + HC	1.18	± 0.05			0.92	± 0.03			0.80	± 0.03		

SD — Standard deviation; P — Level of significance; % (+/-) — Percent increase or decrease

TABLE - 4

Effects of ardenalectomy (A/d) and hydrocortisone (HC) on the activity (U/mg protein) of malate dehydrogenase isoenzymes in the kidney of male mice of various postnatal ages

Isoenzyme	Treatment	15-Day				30-Day				60-Day			
		Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)
Cytosolic	Normal	8.78	± 0.42			18.01	± 1.69			9.07	± 0.70		
				NS	NE			<0.05	-24			<0.01	-22
	A/d	9.16	± 0.79			13.70	± 1.3			7.05	± 0.23		
				NS	NE			<0.02	+ 29			<0.01	+ 33
	A/d + HC	10.68	± 0.41			17.69	± 1.2			9.38	± 0.79		
Mitochondrial	Normal	0.81	± 0.05			2.00	± 0.22			0.55	± 0.02		
				NS	NE			<0.02	-29			<0.01	-22
	A/d	0.91	± 0.06			1.38	± 0.16			0.47	± 0.01		
				NS	NE			<0.01	+ 41			<0.02	+ 30
	A/d + HC	0.86	± 0.03			1.95	± 0.06			0.56	± 0.03		

SD — Standard deviation; P — Level of significance; % (+/-) — Percent increase or decrease,
NS — not significant; NE — no effect

TABLE - 5

Effects of ardenalectomy (A/d) and hydrocortisone (HC) on the activity (U/mg protein) of aspartate aminotransferase isoenzymes in the liver of male mice of various postnatal ages

Isoenzyme	Treat-ment	15- Day				30- Day				60- Day			
		Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)
Cytosolic	Normal	2.20	± 0.17			0.94	± 0.07			1.13	± 0.15		
				<0.02	-24			<0.02	-20			<0.05	-28
	A/d	1.68	± 0.08			0.75	± 0.05			0.81	± 0.11		
				<0.05	+ 39			<0.02	+ 36			<0.05	+ 28
	A/d + HC	2.34	± 0.28			1.02	± 0.10			1.04	± 0.15		
Mitochondrial	Normal	0.24	± 0.01			0.091	± 0.004			0.127	± 0.01		
				NS	NE			NS	NE			NS	NE
	A/d	0.22	± 0.01			0.088	± 0.005			0.136	± 0.007		
				NS	NE			NS	NE			NS	NE
	A/d + HC	0.25	± 0.03			0.092	± 0.005			0.120	± 0.02		

SD — Standard deviation; P — Level of significance; % (+/-) — Percent increase or decrease,
NS — not significant; NE — no effect

TABLE - 6

Effects of ardenalectomy (A/d) and hydrocortisone (HC) on the activity (U/ mg protein) of aspartate aminotransferase isoenzymes in the kidney of male mice of various postnatal ages

Isoenzyme	Treat-ment	15- Day				30- Day				60- Day			
		Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)
Cytosolic	Normal	0.94	± 0.02			1.11	± 0.03			0.63	± 0.07		
				NS	NE			<0.001	-20			<0.05	-22
	A/d	0.84	± 0.05			0.89	± 0.03			0.49	± 0.05		
				NS	NE			<0.01	-21			<0.05	-22
	A/d + HC	0.90	± 0.04			1.08	± 0.05			0.60	± 0.03		
Mitochondrial	Normal	0.11	± 0.01			0.23	± 0.01			0.08	± 0.002		
				NS	NE			NS	NE			NS	NE
	A/d	0.09	± 0.003			0.22	± 0.01			0.07	± 0.003		
				NS	NE			NS	NE			NS	NE
	A/d + HC	0.09	± 0.02			0.25	± 0.02			0.08	± 0.001		

SD — Standard deviation; P — Level of significance; % (+/-) — Percent increase or decrease,
NS — not significant; NE — no effect

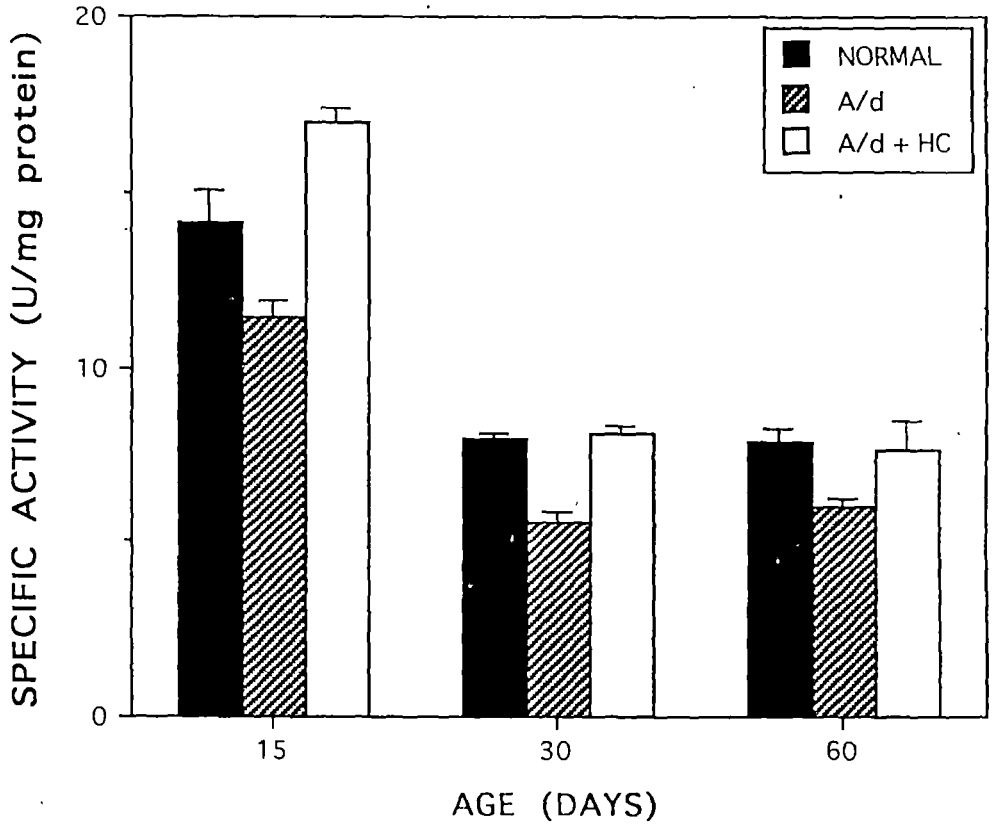


Fig. 7 (A) Effects of adrenalectomy (A/d) and hydrocortisone (HC) on the activity of cytosolic malate dehydrogenase isoenzyme in the liver of male mice at various postnatal ages. Hormonal treatments and other experimental conditions are described in method section. Values are means for 4-5 mice of each age group. Bars represent standard deviation.

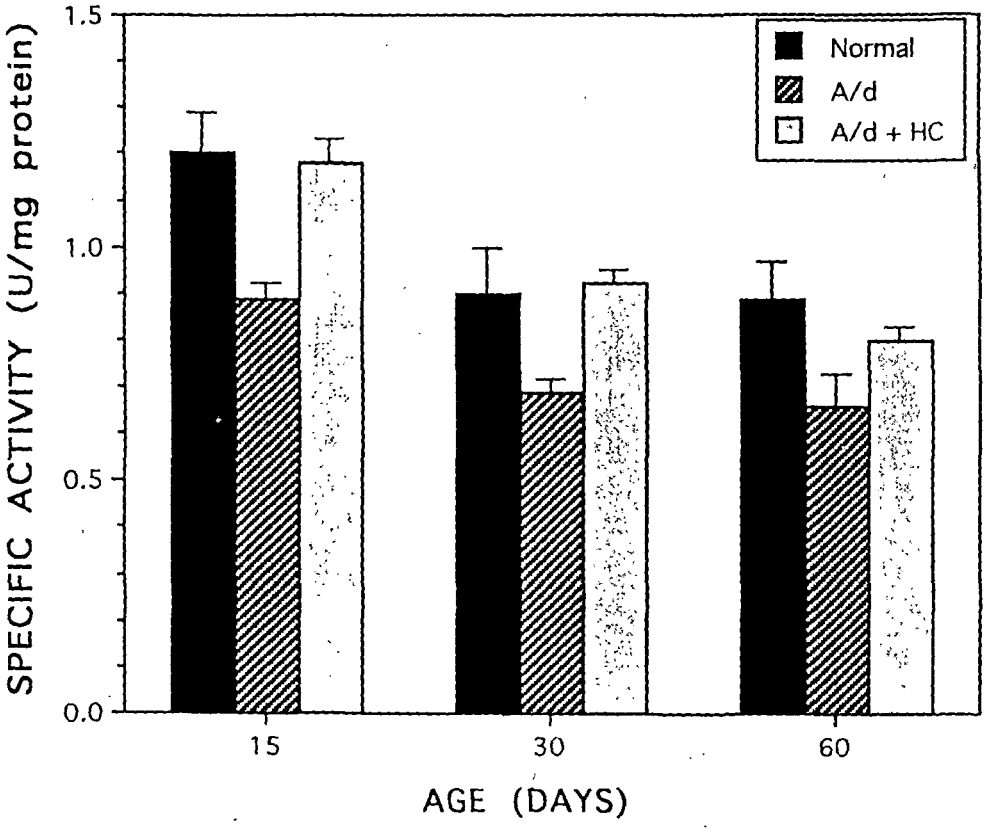


Fig. 7 (B) Effects of adrenalectomy (A/d) and hydrocortisone (HC) on the activity of mitochondrial malate dehydrogenase isoenzyme in the liver of male mice at various postnatal ages. Hormonal treatments and other experimental conditions are described in method section. Values are means for 4-5 mice of each age group. Bars represent standard deviation.

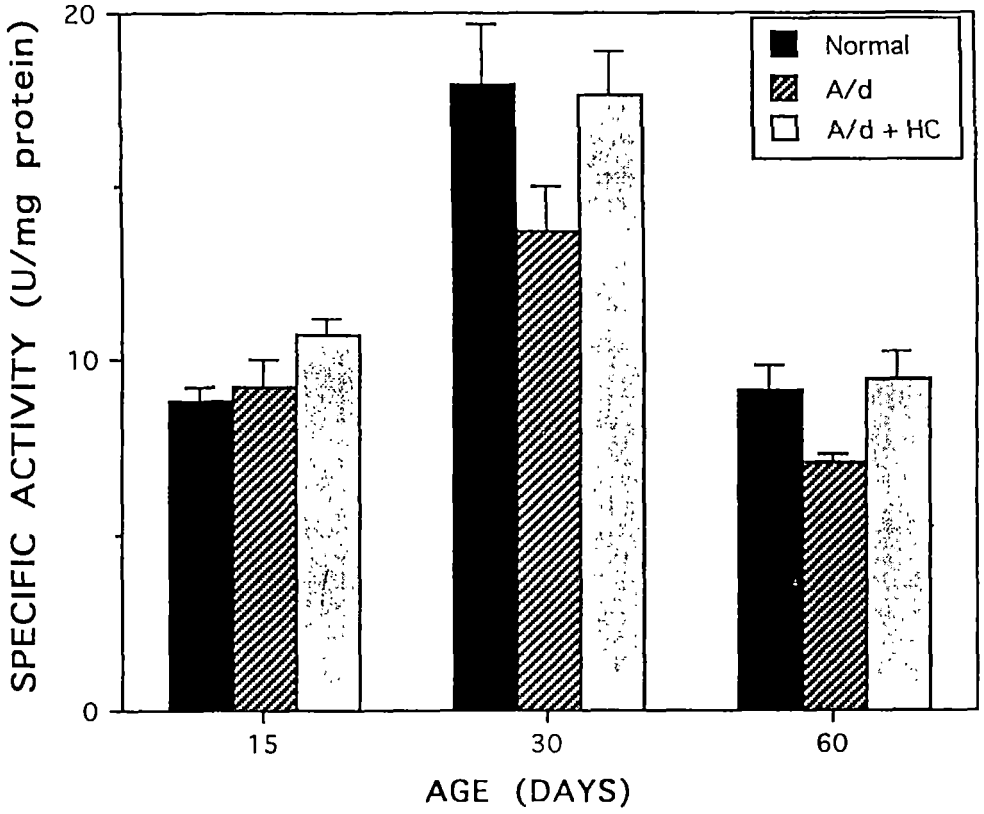


Fig. 8 (A) Effects of adrenalectomy (A/d) and hydrocortisone (HC) on the activity of cytosolic malate dehydrogenase isoenzyme in the kidney of male mice at various postnatal ages. Hormonal treatments and other experimental conditions are described in method section. Values are means for 4-5 mice of each age group. Bars represent standard deviation.

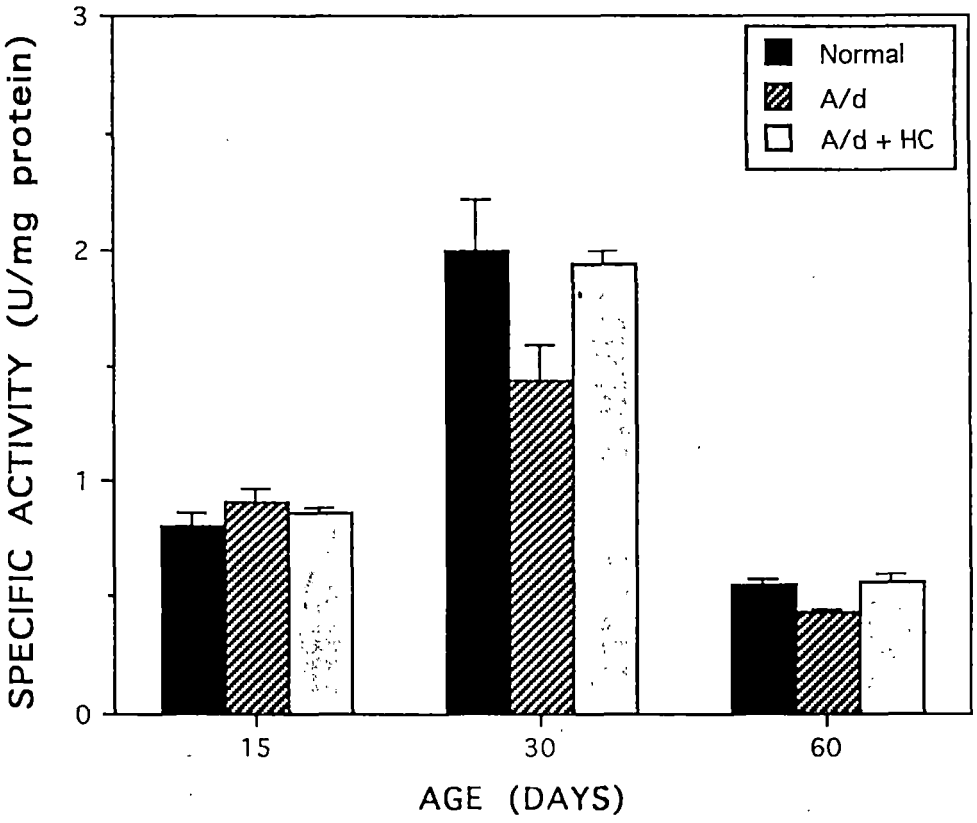


Fig. 8 (B) Effects of adrenalectomy (A/d) and hydrocortisone (HC) on the activity of mitochondrial malate dehydrogenase isoenzyme in the kidney of male mice at various postnatal ages. Hormonal treatments and other experimental conditions are described in method section. Values are means for 4-5 mice of each age group. Bars represent standard deviation.

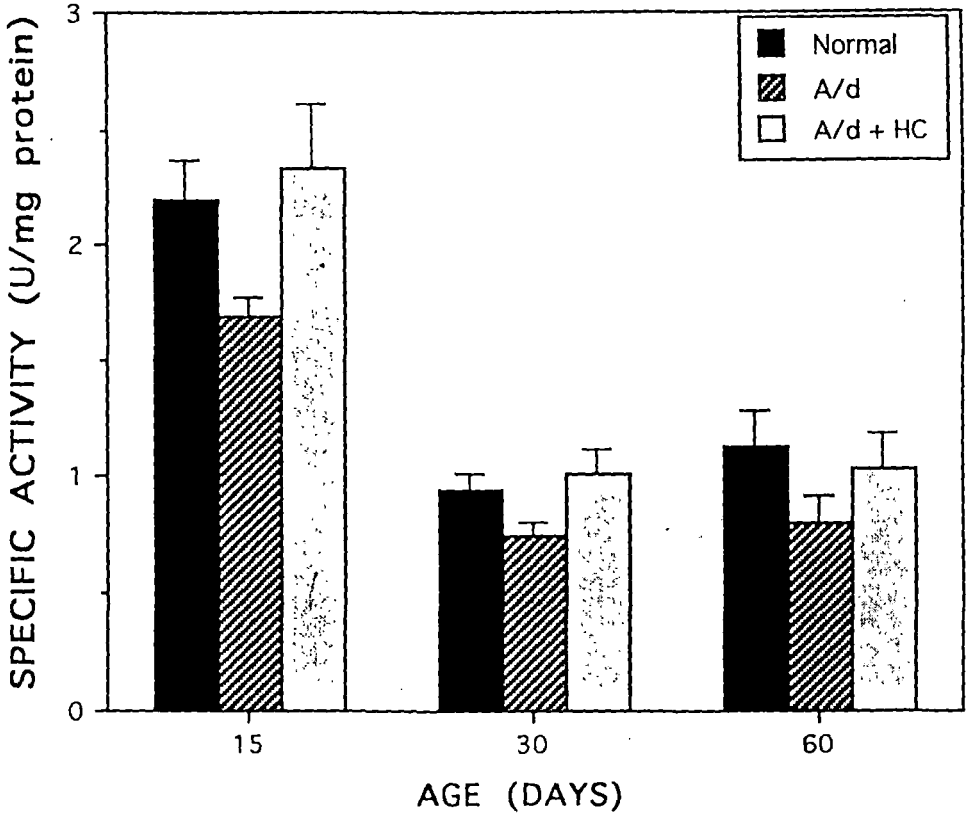


Fig. 9 (A) Effects of adrenalectomy (A/d) and hydrocortisone (HC) on the activity of cytosolic aspartate aminotransferase isoenzyme in the liver of male mice at various postnatal ages. Hormonal treatments and other experimental conditions are described in method section. Values are means for 4-5 mice of each age group. Bars represent standard deviation.

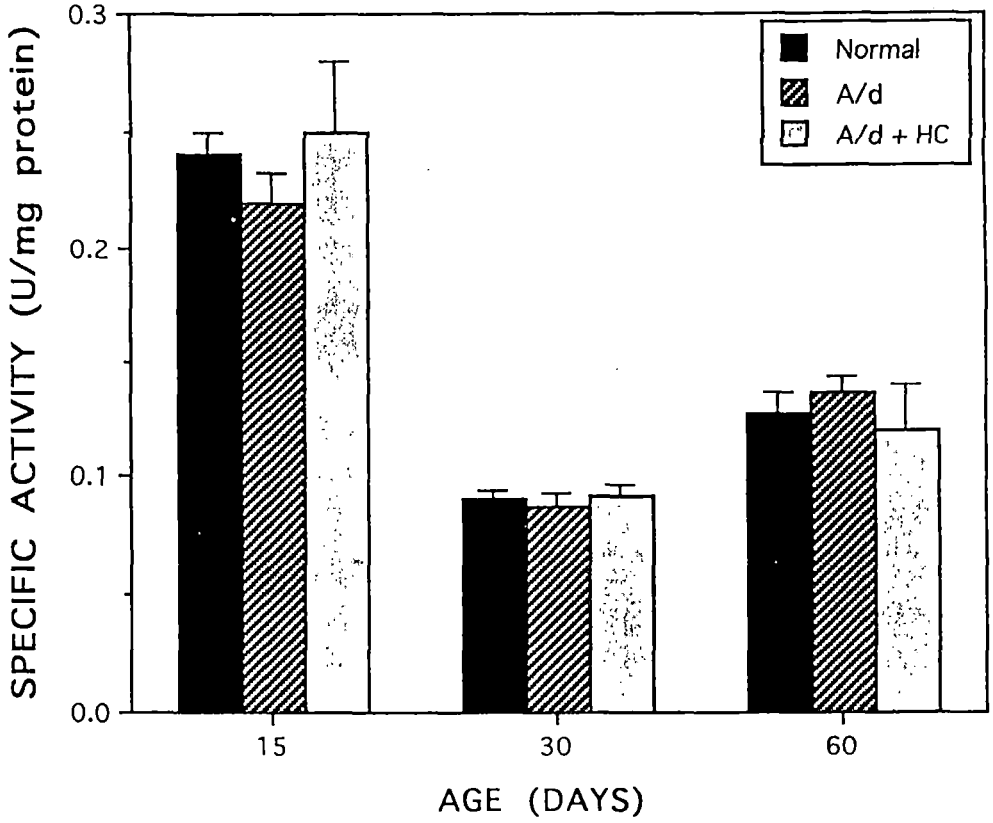


Fig. 9 (B) Effects of adrenalectomy (A/d) and hydrocortisone (HC) on the activity of mitochondrial aspartate aminotransferase isoenzyme in the liver of male mice at various postnatal ages. Hormonal treatments and other experimental conditions are described in method section. Values are means for 4-5 mice of each age group. Bars represent standard deviation.

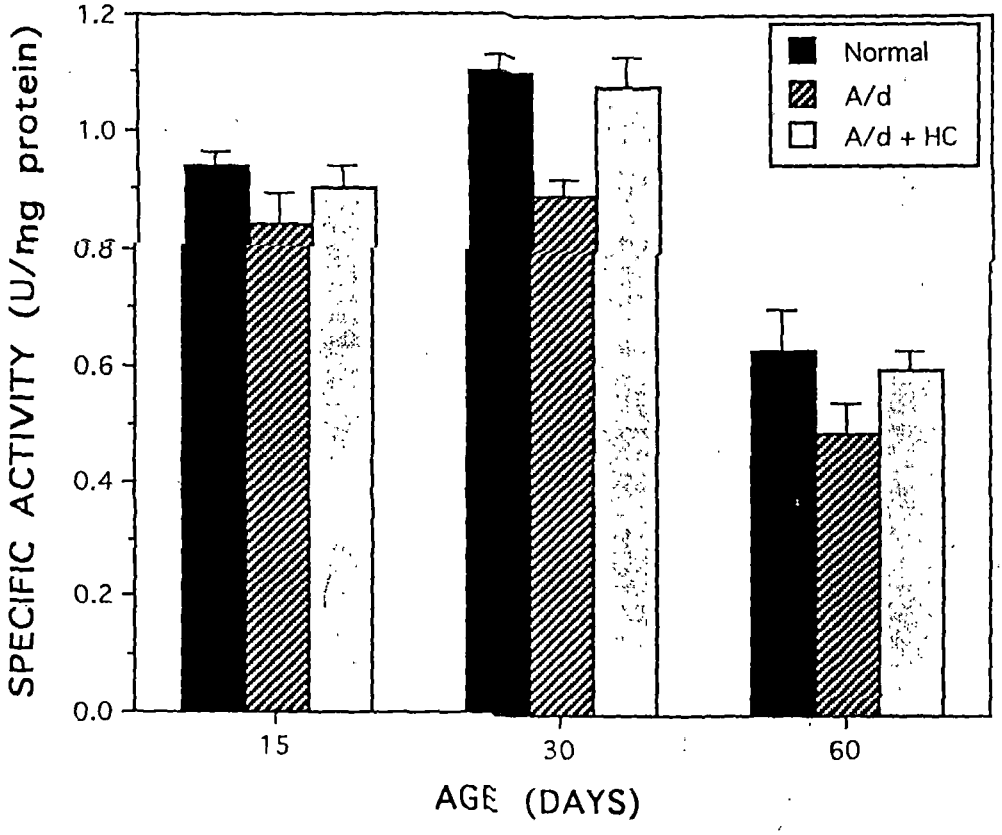


Fig. 10 (A) Effects of adrenalectomy (A/d) and hydrocortisone (HC) on the activity of cytosolic aspartate aminotransferase isoenzyme in the kidney of male mice at various postnatal ages. Hormonal treatments and other experimental conditions are described in method section. Values are means for 4-5 mice of each age group. Bars represent standard deviation.

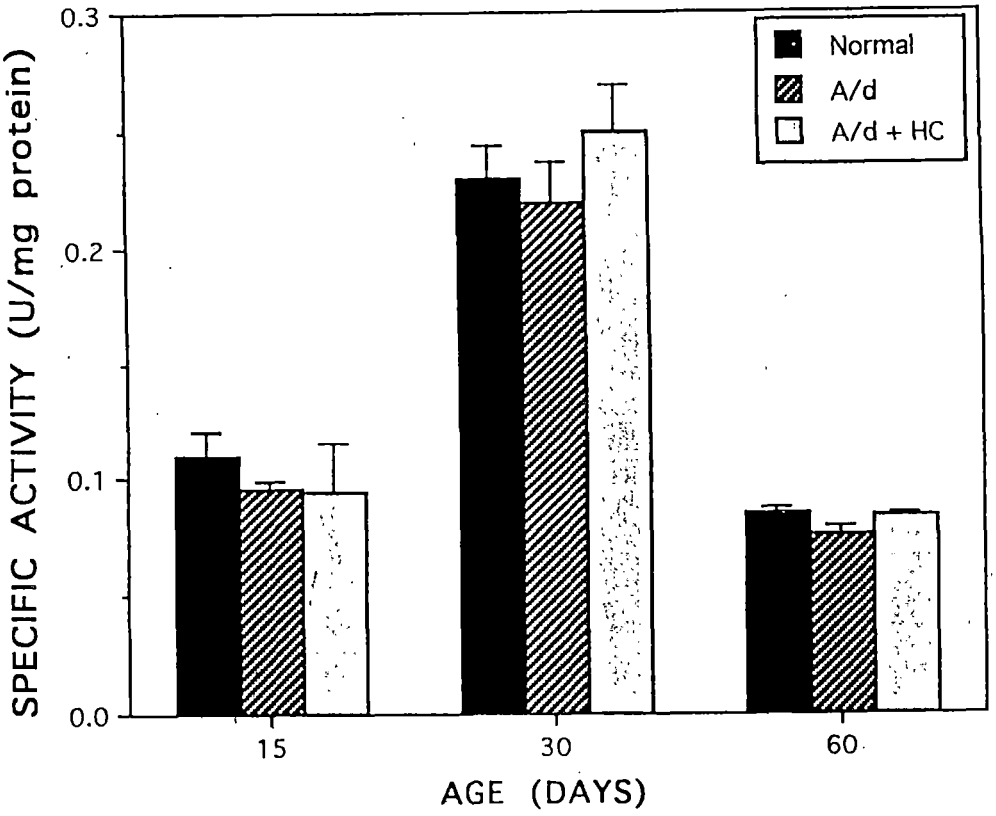


Fig. 10 (B) Effects of adrenalectomy (A/d) and hydrocortisone (HC) on the activity of mitochondrial aspartate aminotransferase isoenzyme in the kidney of male mice at various postnatal ages. Hormonal treatments and other experimental conditions are described in method section. Values are means for 4-5 mice of each age group. Bars represent standard deviation.

TABLE - 7

Effects of dibutyryladenosine 3' 5' cyclic monophosphate (Bt_2-cAMP) and hydrocortisone (HC) on the activity (U/mg protein) of malate dehydrogenase isoenzymes in the liver of male mice of various postnatal ages

Isoenzyme	Treat-ment	15- Day				30- Day				60- Day			
		Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)
Cytosolic	Normal	7.41	± 0.43			3.63	± 0.29			4.21	± 0.32		
				NS	NE			NS	NE			NS	NE
	Bt_2-cAMP + HC	7.23	± 0.56			3.11	± 0.04			3.97	± 0.38		
Mitochondrial	Normal	0.72	± 0.04			0.43	± 0.02			0.45	± 0.02		
				NS	NE			<0.001	28			<0.02	33
	Bt_2-cAMP +HC	0.73	± 0.05			0.55	± 0.01			0.60	± 0.07		

SD — Standard deviation; P — Level of significance; % (+/-) — Percent increase or decrease,
NS — not significant; NE — no effect

TABLE - 8

Effects of dibutyryladenosine 3' 5' cyclic monophosphate (Bt_2-cAMP) and hydrocortisone (HC) on the activity (U/mg protein) of malate dehydrogenase isoenzymes in the kidney of male mice of various postnatal ages

Isoenzyme	Treat-ment	15- Day				30- Day				60- Day			
		Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)
Cytosolic	Normal	9.98	± 0.10			20.21	± 1.60			14.78	± 0.39		
				NS	NE			NS	NE			NS	NE
	Bt_2-cAMP +HC	10.34	± 0.11			21.66	± 0.06			16.59	± 0.10		
Mitochondrial	Normal	0.90	± 0.07			1.49	± 0.05			0.64	± 0.01		
				NS	NE			NS	NE			NS	NE
	Bt_2-cAMP +HC	0.93	± 0.01			1.62	± 0.05			0.57	± 0.04		

SD — Standard deviation; P — Level of significance; % (+/-) — Percent increase or decrease,
NS — not significant; NE — no effect

TABLE - 9

Effects of dibutyryladenosine 3' 5' cyclic monophosphate (Bt_2-cAMP) and hydrocortisone (HC) on the activity (U/mg protein) of aspartate aminotransferase isoenzymes in the liver of male mice of various postnatal ages

Isoenzyme	Treatment	15-Day				30-Day				60-Day			
		Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)
Cytosolic	Normal	1.40	± 0.10			0.58	± 0.04			0.60	± 0.02		
				<0.01	30			P<0.01	28			P<0.001	31
	Bt_2-cAMP +HC	1.82	± 0.11			0.74	± 0.03			0.79	± 0.03		
Mitochondrial	Normal	0.37	± 0.07			0.13	± 0.01			0.19	± 0.01		
				NS	NE			NS	NE			NS	NE
	Bt_2-cAMP +HC	0.40	± 0.08			0.14	± 0.06			0.21	± 0.03		

SD — Standard deviation; P — Level of significance; % (+/-) — Percent increase or decrease,
NS — not significant; NE — no effect

TABLE - 10

Effects of dibutyryladenosine 3' 5' cyclic monophosphate (Bt_2-cAMP) and hydrocortisone (HC) on the activity (U/mg protein) of aspartate aminotransferase isoenzymes in the kidney of male mice of various postnatal ages

Isoenzyme	Treatment	15-Day				30-Day				60-Day			
		Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)
Cytosolic	Normal	0.94	± 0.02	NS	NE	1.64	± 0.05	NS	NE	0.88	± 0.06	NS	NE
	Bt_2-cAMP +HC	0.95	± 0.01			1.61	± 0.09			1.00	± 0.12		
Mitochondrial	Normal	0.11	± 0.009	NS	NE	0.19	± 0.05	NS	NE	0.085	± 0.005	NS	NE
	Bt_2-cAMP +HC	0.11	± 0.005			0.22	± 0.02			0.079	± 0.011		

SD — Standard deviation; P — Level of significance; % (+/-) — Percent increase or decrease,
NS — not significant; NE — no effect

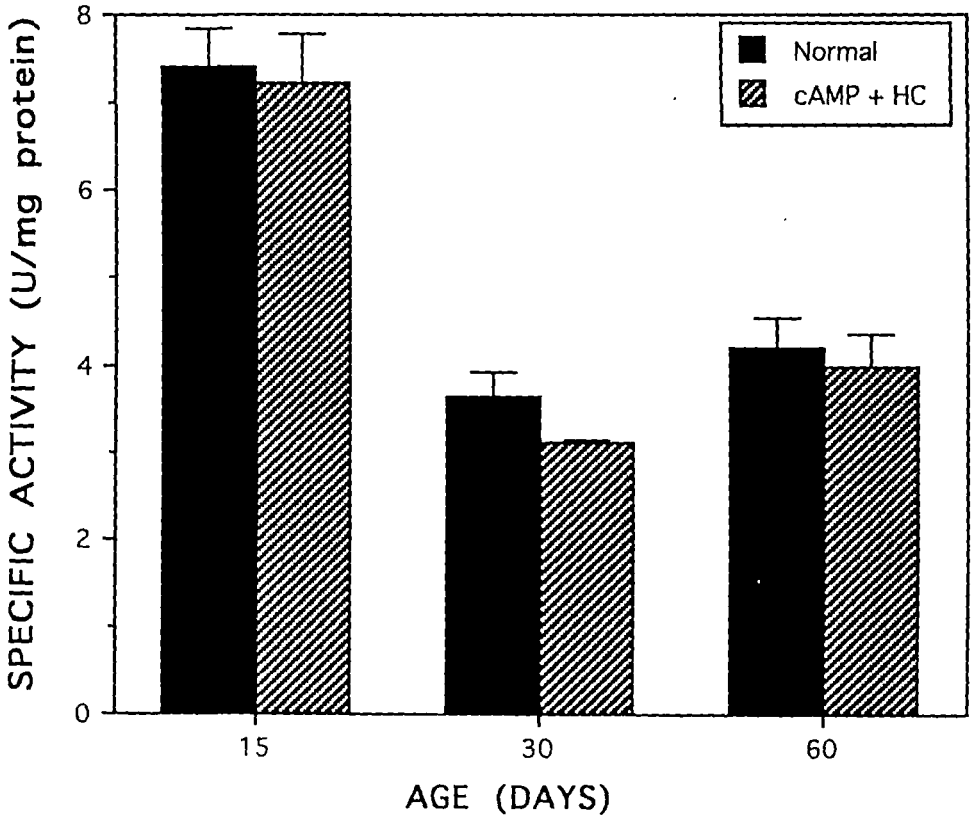


Fig. 11 (A) Effects of dibutyrylated - cAMP (Bt_2 - cAMP) and hydrocortisone (HC), on the activity of cytosolic malate dehydrogenase isoenzyme in the liver of male mice at various postnatal ages. Hormonal treatments and other experimental conditions are described in method section. Values are means for 4-5 mice of each age group. Bars represent standard deviation.

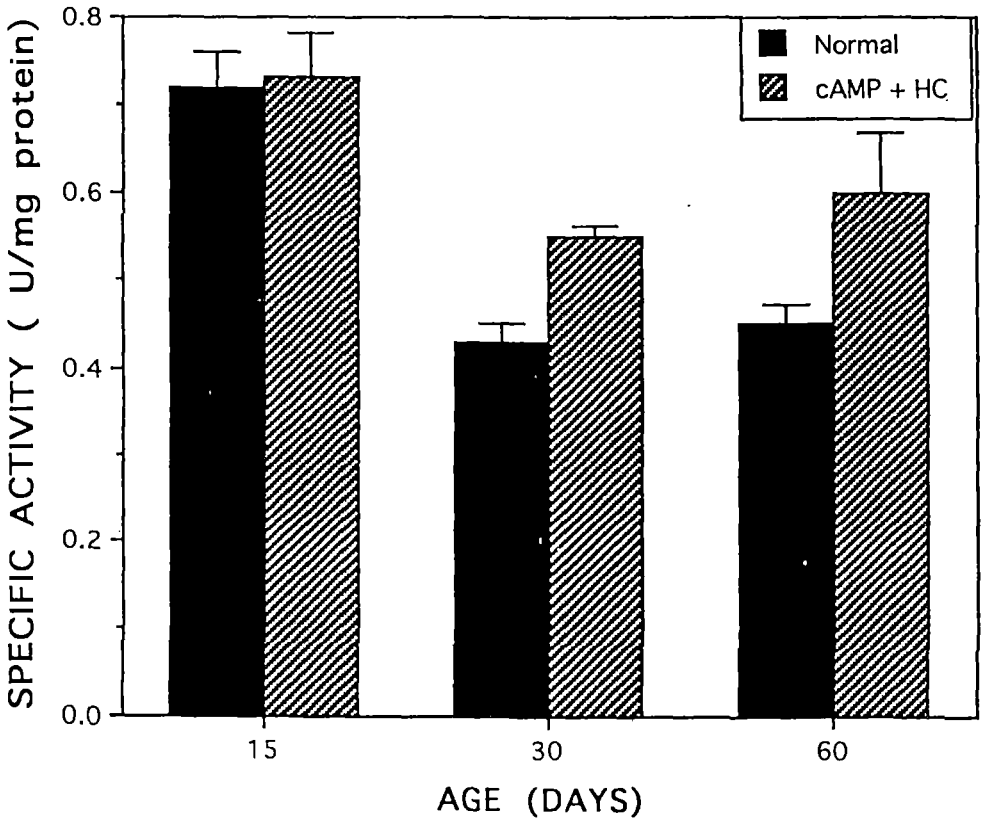


Fig. 11(B) Effects of dibutyrylated - cAMP (Bt_2 - cAMP) and hydrocortisone (HC), on the activity of mitochondrial malate dehydrogenase isoenzyme in the liver of male mice at various postnatal ages. Other details are same as Fig-11 (A).

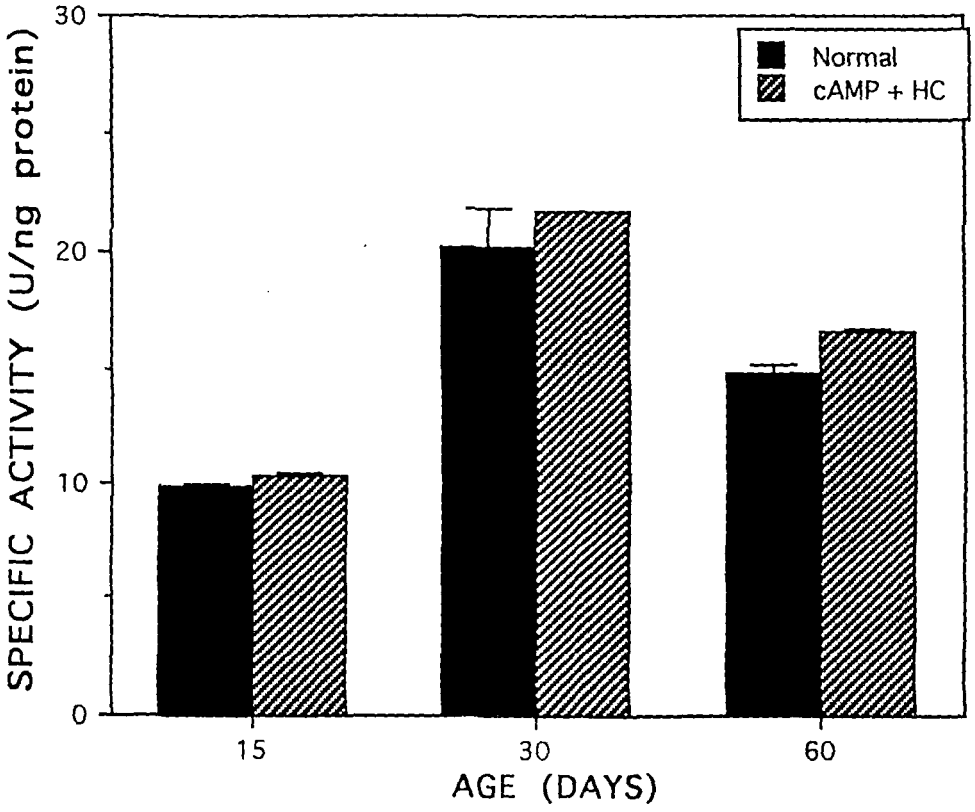


Fig. 12 (A) Effects of dibutyrylated - cAMP (Bt_2 - cAMP) and hydrocortisone (HC), on the activity of cytosolic malate dehydrogenase isoenzyme in the kidney of male mice at various postnatal ages. Other details are same as Fig-11 (A).

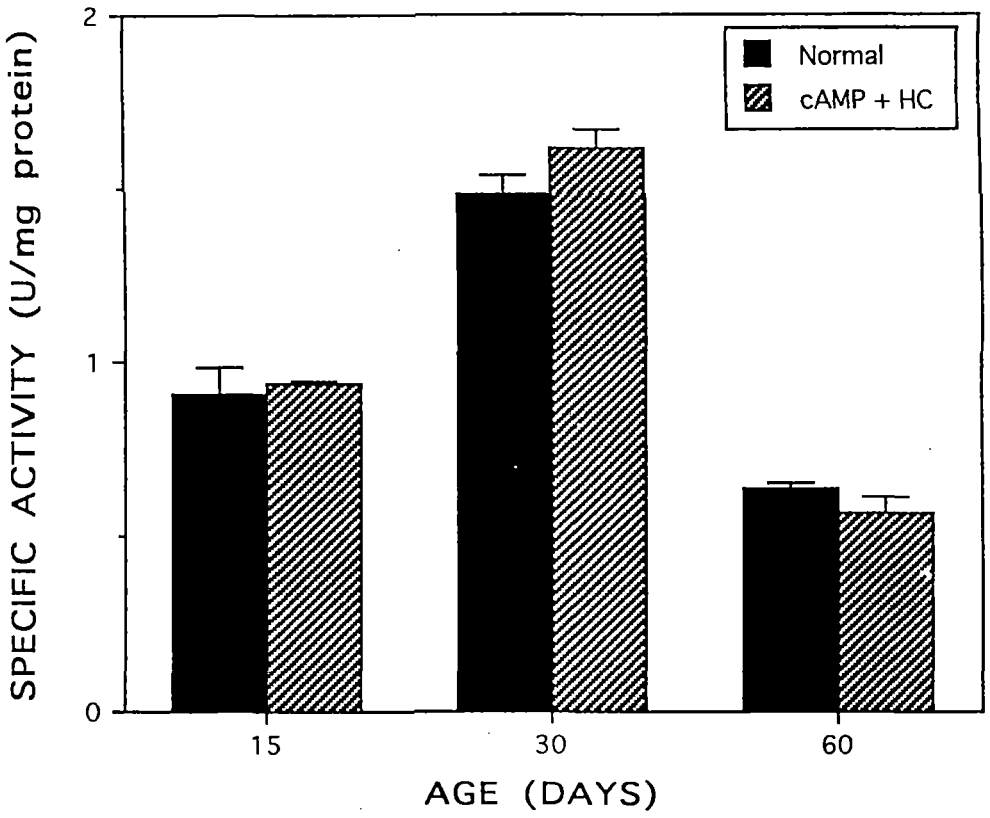


Fig. 12 (B) Effects of dibutyrylated - cAMP (Bt_2 - cAMP) and hydrocortisone (HC), on the activity of mitochondrial malate dehydrogenase isoenzyme in the kidney of male mice at various postnatal ages. Other details are same as Fig-11 (A).

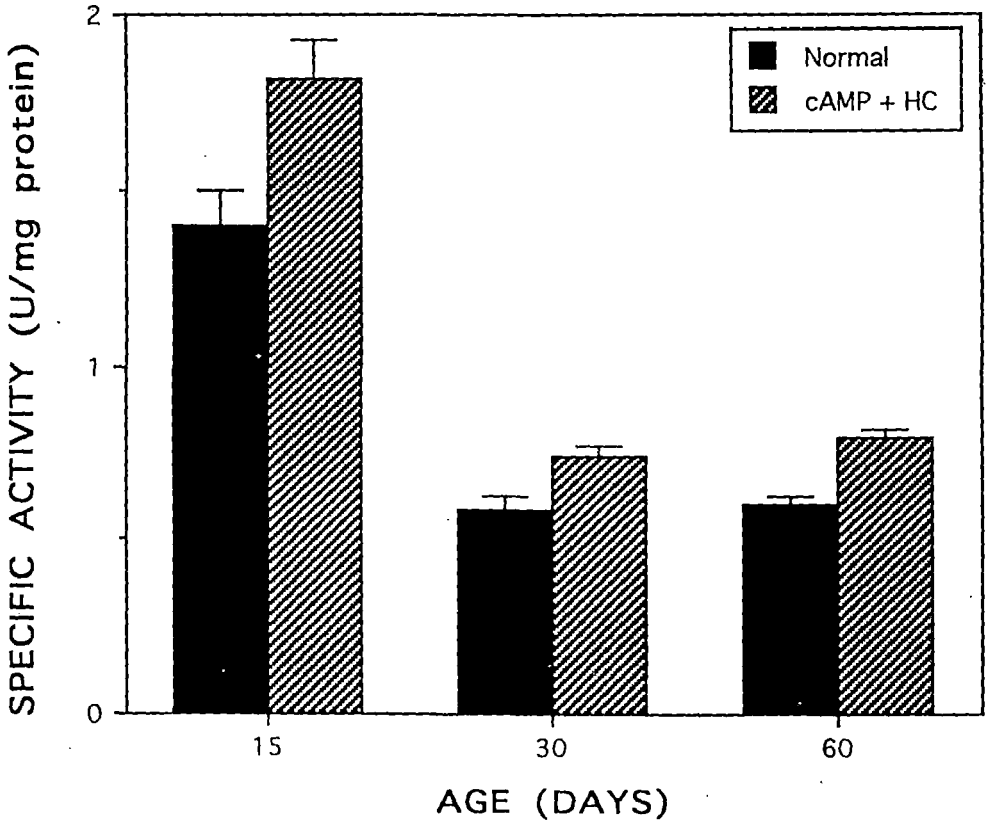


Fig. 13 (A) Effects of dibutyrylated - cAMP (Bt_2 - cAMP) and hydrocortisone (HC), on the activity of cytosolic aspartate aminotransferase isoenzyme in the liver of male mice at various postnatal ages. Hormonal treatments and other experimental conditions are described in method section. Values are means for 4-5 mice of each age group. Bars represent standard deviation.

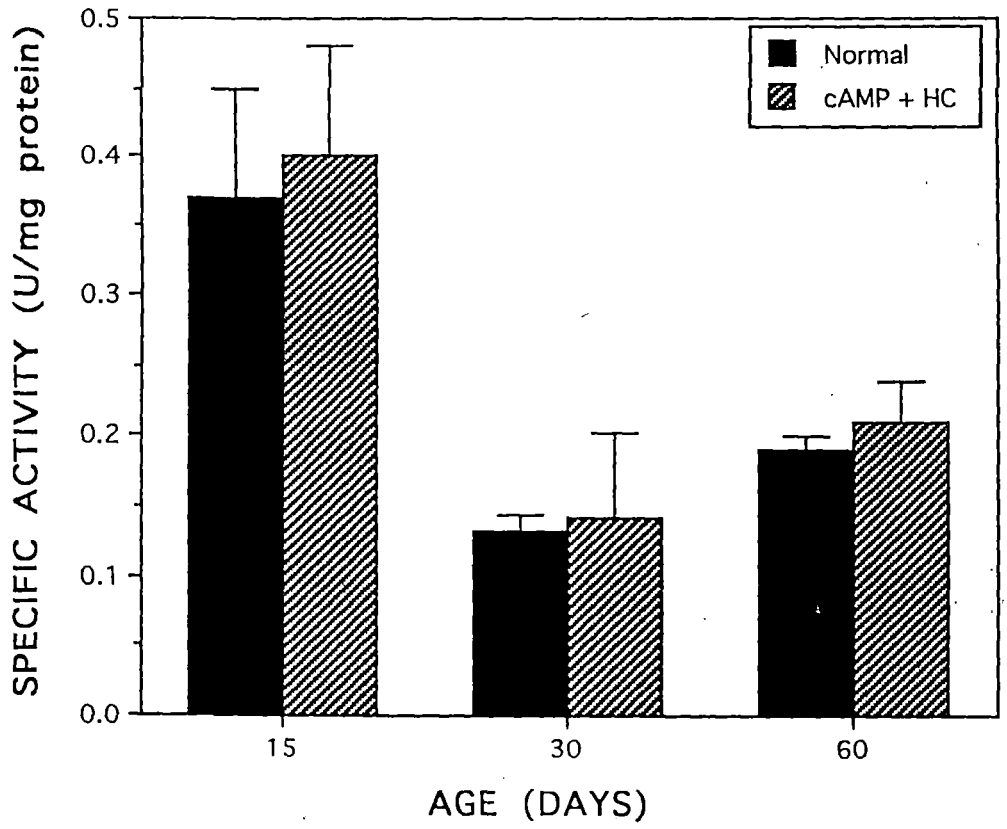


Fig. 13 (B) Effects of dibutyrylated - cAMP (Bt_2 - cAMP) and hydrocortisone (HC), on the activity of mitochondrial aspartate aminotransferase isoenzyme in the liver of male mice at various postnatal ages. Other details are same as Fig-13 (A).

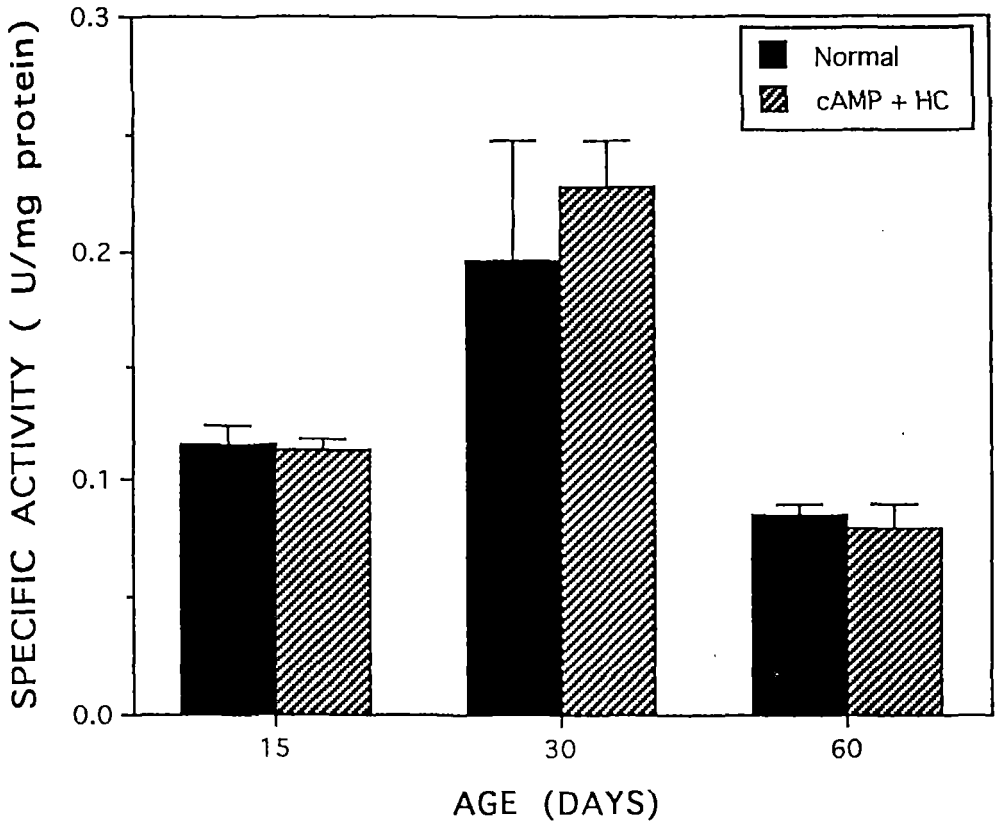


Fig. 14 (B) Effects of dibutyrylated - cAMP (Bt_2 - cAMP) and hydrocortisone (HC), on the activity of mitochondrial aspartate aminotransferase isoenzyme in the kidney of male mice at various postnatal ages. Other details are same as Fig-13 (A).

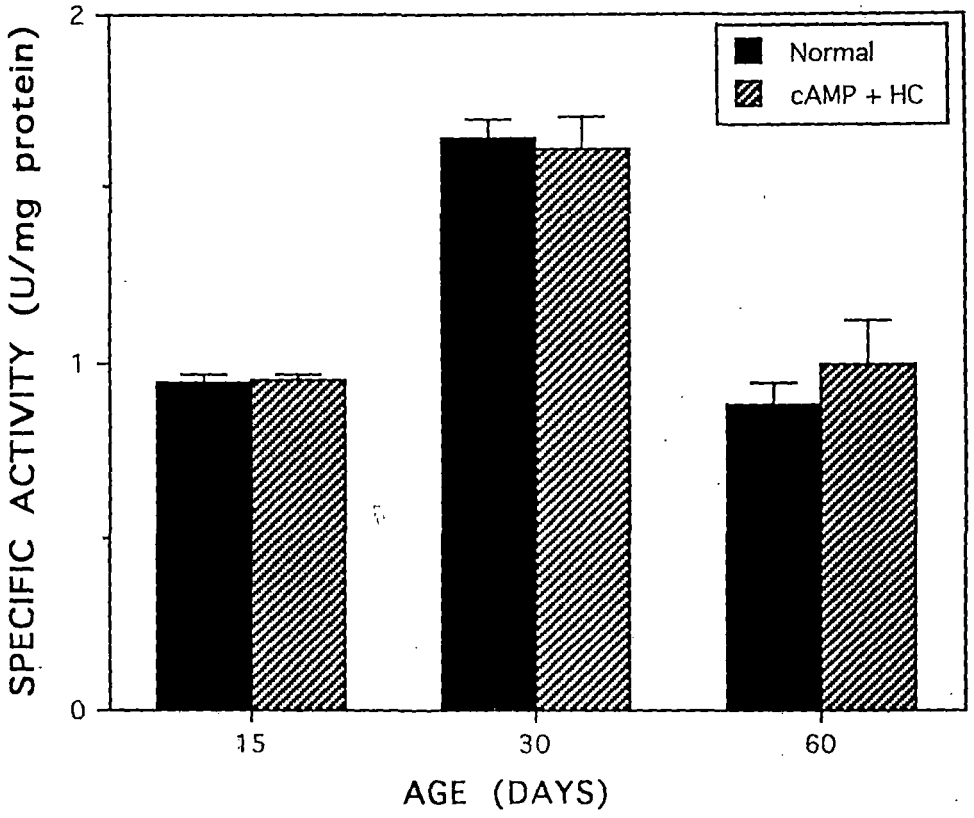


Fig. 14 (A) Effects of dibutyrylated - cAMP (Bt_2 - cAMP) and hydrocortisone (HC), on the activity of cytosolic aspartate aminotransferase isoenzyme in the kidney of male mice at various postnatal ages. Other details are same as Fig-13 (A).

(+32%, +33% and +21%, respectively) the activity level of mitochondrial malate dehydrogenase at all the three adrenalectomized postnatal ages of mice (Table-3; Fig-7A & B).

Adrenalectomy causes significant decrease (i.e. -24%, -22% for c-MDH and -29%, -22% for m-MDH) in the activity of kidney malate dehydrogenase at 30- and 60-day of postnatal ages (Table-4; Fig-8A & B). It does not show any effect on the activity of kidney malate dehydrogenase (cytosolic and mitochondrial) in preweaned mice (15-day old). Administration of hydrocortisone increases (+29%, +33% for c-MDH and +41%, +30% for m-MDH) the activities of both the isoenzymes of malate dehydrogenase at those postnatal ages (day 30, and 60) of mice (Table-4; Fig-8A & B).

Aspartate aminotransferase (AsAT) — Adrenalectomy (A/d) causes a significant decrease (-24%, -20%, -28%, respectively) in the activity (U/mg protein) of liver cytosolic AsAT of mice at the three postnatal ages studied (Table-5; Fig-9A). However, adrenalectomy (A/d) does not show any effect on the activity of mitochondrial AsAT in the liver of any of the three postnatal ages studies. Administration of hydrocortisone (1 mg/100g body weight for 3 days) shows a significant increase (+39%, +36% and +28%, respectively) in the activity of liver cytosolic AsAT at those postnatal ages of mice (Table-5; Fig-9A & B).

In kidney, adrenalectomy (A/d) causes a decrease (-24% and -22%) in the activity of cytosolic AsAT in postweaned mice only (i.e. day 30 and 60). Administration of hydrocortisone to adrenalectomized mice significantly increases (+21% and +22%) the activity of cytosolic AsAT in the kidney of those mice only (30- and 60-day). Adrenalectomy and hydrocortisone both have no effect on the activity of kidney mitochondrial AsAT in either of the postnatal ages of mice studied (Table-6; Fig-10A & B).

Effect of dibutyryl-cAMP (Bt_2 - cAMP) — Various doses (100, 200, 500 μg and 1 mg/100 g body weight) of dibutyryl -cAMP (Bt_2 - cAMP) were administered in different postnatal ages (15-, 30- and 60-day) of mice. It was observed that Bt_2 - cAMP has no effect on activity of both the isoenzymes of MDH and AsAT in the liver and kidney of mice.

Effects of Bt_2 - cAMP and hydrocortisone combinations on the activities of shuttle enzymes :

In order to find out the combinatorial effects of hormonal signals onto malate-aspartate

TABLE - 11

Effects of triiodothyronine (T_3) on the activity (U/mg protein) of malate dehydrogenase isoenzymes in the liver of male mice of various postnatal ages

Isoenzyme	Treat-ment	15- Day				30- Day				60- Day			
		Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)
Cytosolic	Normal	9.73	± 0.18	NS	NE	4.42	± 0.21	NS	NE	4.21	± 0.32	NS	NE
	T_3	11.28	± 0.58			4.93	± 0.26			4.34	± 0.39		
Mitochondrial	Normal	1.10	± 0.07	NS	NE	0.65	± 0.02	NS	NE	0.64	± 0.03	NS	NE
	T_3	1.20	± 0.05			0.75	± 0.04			0.64	± 0.11		

SD — Standard deviation; P — Level of significance; % (+/-) — Percent increase or decrease,
NS — not significant; NE — no effect

TABLE - 12

Effects of triiodothyromine (T_3) on the activity (U/mg protein) of malate dehydrogenase isoenzymes in the kidney of male mice of various postnatal ages

Isoenzyme	Treat-ment	15- Day				30- Day				60- Day			
		Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)
Cytosolic	Normal	9.74	± 0.043			21.39	± 1.90			14.79	± 0.29		
				NS	NE			NS	NE			NS	NE
	T_3	8.51	± 0.45			21.27	± 0.89			13.59	± 0.98		
Mitochondrial	Normal	0.94	± 0.025			1.80	± 0.32			0.64	± 0.013		
				NS	NE			NS	NE			NS	NE
	T_3	0.85	± 0.053			1.61	± 0.26			0.66	± 0.060		

SD — Standard deviation; P — Level of significance; % (+/-) — Percent increase or decrease,
NS — not significant; NE — no effect

TABLE - 13

Effects of triiodothyronine (T_3) on the activity (U/mg protein) of aspartate aminotransferase isoenzymes in the liver of male mice of various postnatal ages

Isoenzyme	Treat-ment	15- Day				30- Day				60- Day			
		Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)
Cytosolic	Normal	2.64	± 0.54	NS	NE	0.81	± 0.05	NS	NE	0.66	± 0.06	NS	NE
	T_3	3.15	± 0.22			0.83	± 0.05			0.65	± 0.08		
Mitochondrial	Normal	0.40	± 0.017	NS	NE	0.21	± 0.02	NS	NE	0.28	± 0.015	NS	NE
	T_3	0.35	± 0.007			0.18	± 0.01			0.23	± 0.003		

SD — Standard deviation; P — Level of significance; % (+/-) — Percent increase or decrease,
NS — not significant; NE — no effect

TABLE - 14

Effects of triiodothyronine (T_3) on the activity (U/mg protein) of aspartate aminotransferase isoenzymes in the kidney of male mice of various postnatal ages

Isoenzyme	Treat-ment	15- Day				30- Day				60- Day			
		Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)	Mean	SD	P	% (+/-)
Cytosolic	Normal	0.97	± 0.05	NS	NE	1.75	± 0.16	NS	NE	0.885	± 0.06	NS	NE
	T_3	0.98	± 0.05			1.65	± 0.13			0.944	± 0.01		
Mitochondrial	Normal	0.109	± 0.009	NS	NE	0.196	± 0.05	NS	NE	0.084	± 0.005	NS	NE
	T_3	0.083	± 0.072			0.203	± 0.02			0.076	± 0.004		

SD — Standard deviation; P — Level of significance; % (+/-) — Percent increase or decrease,
NS — not significant; NE — no effect

shuttle enzymes, a combination of Bt_2-cAMP (50 μg/100g body weight) and hydrocortisone (1 mg/100 g body weight) was administered in intact mice of three different postnatal ages (15-, 30- and 60-day). Administration of Bt_2-cAMP and hydrocortisone combinations has no effect on liver cytosolic malate dehydrogenase at all three postnatal ages. However, liver mitochondrial malate dehydrogenase shows an increase (+27% and +33%, respectively) upon administration of hormone combinations. The influence of combinatorial effect was observed in postweaned mice only (i.e. 30- and 60-day) (Table-7; Fig-11A & B). On the other hand in kidney both the isoenzymes of MDH remain unresponsive to the administration of c-AMP and hydrocortisone combinations (Table - 8; Fig-12A&B).

Administration of Bt_2-cAMP and hydrocortisone causes increase (+30%, +27% and +31%, respectively) in the activity of liver cytosolic AsAT of mice at all the three postnatal ages studied. However, mitochondrial AsAT shows no effect in the same tissue by administration of Bt_2-cAMP and hydrocortisone combination (Table - 9; Fig-13A&B). Like MDH isoenzymes, in kidney, both the isoenzymes of AsAT (cytosolic and mitochondrial) show no effect by the administration of Bt_2-cAMP and hydrocortisone combinations (Table - 10; Fig-14A&B).

Effect of triiodothyronine (T_3) :

Administration of T_3 shows no significant effect in the activity of cytosolic and mitochondrial malate dehydrogenase and aspartate aminotransferase in liver and kidney of mice at all the three (15-, 30- and 60-day) postnatal ages studied (Table -11 to 14).

Isolation and purification of liver c-AsAT :

Using similar experimental conditions, one of the shuttle enzymes i.e. cytosolic aspartate aminotransferase was isolated and purified from the liver of mice of two selected ages i.e. immature (15- day) and mature (180- day). Isolation and purification was done using the method described under experimental procedure and outlined in Table -15 for immature and mature ages, respectively. The degree of purification achieved was 30- and 33-fold for immature and mature ages, respectively. The enzyme was partially purified from the two ages of mice in order to compare the chemical and kinetic properties of this isoenzyme as a function of age. The elution profile of specific activity of this isoenzyme from the liver of immature and mature mice on CM-cellulose column is depicted in Fig-15. From the elution profile, it is clear that both the immature

TABLE 15

Purification protocol of cytosolic aspartate aminotransferase from the liver of immature (15-day) and mature (180-day) normal male mice.

AGE	Fractions	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification fold	Yeild (%)
15 Day	Supernatant (14,000 x g)	80	378.67	823.20	0.46	—	100
	Ist $(NH_4)_2SO_4$ (40%)	75	354.31	448.50	0.79	1.71	93
	IInd $(NH_4)_2SO_4$ (80%)	18	280.44	147.60	1.90	4.13	74
	Dialysis	30	259.26	87.00	2.98	6.75	68
	CM-cellulose	33	240.21	28.05	6.18	13.43	63
	IIIrd $(NH_4)_2SO_4$ (80%) & Dialysis	9	231.12	4.55	14.11	30.67	61
180 Day	Supernatant (14,000 x g)	79	364.79	935.36	0.39	—	100
	Ist $(NH_4)_2SO_4$ (40%)	75	314.64	456.60	0.69	1.76	86
	IInd $(NH_4)_2SO_4$ (80%)	20	255.36	182.40	1.40	3.50	70
	Dialysis	33	219.91	102.30	2.15	5.51	60
	CM-cellulose	30	174.04	29.40	5.92	15.17	47
	IIIrd $(NH_4)_2SO_4$ (80%) & Dialysis	8	161.84	5.20	13.15	33.17	44

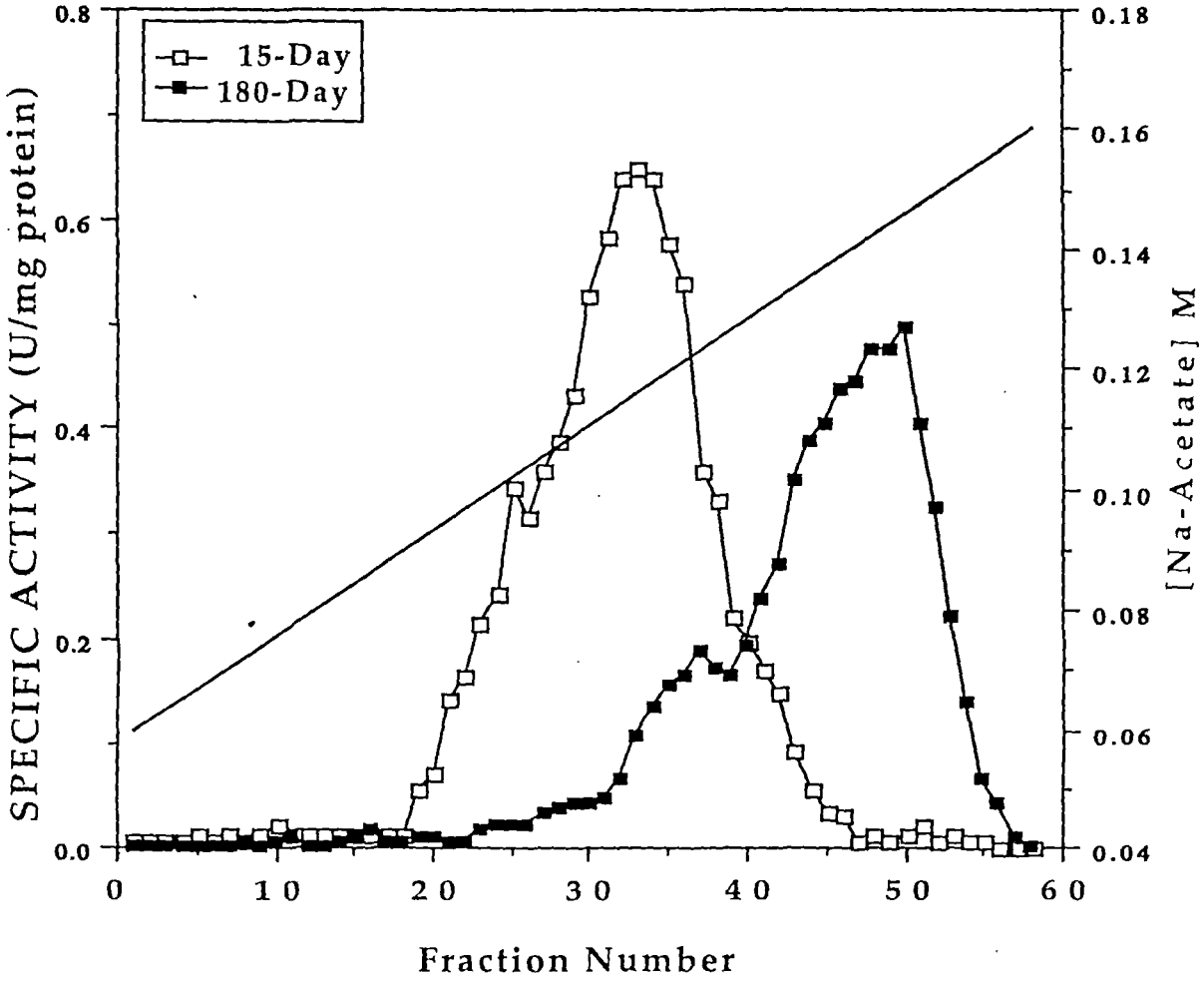
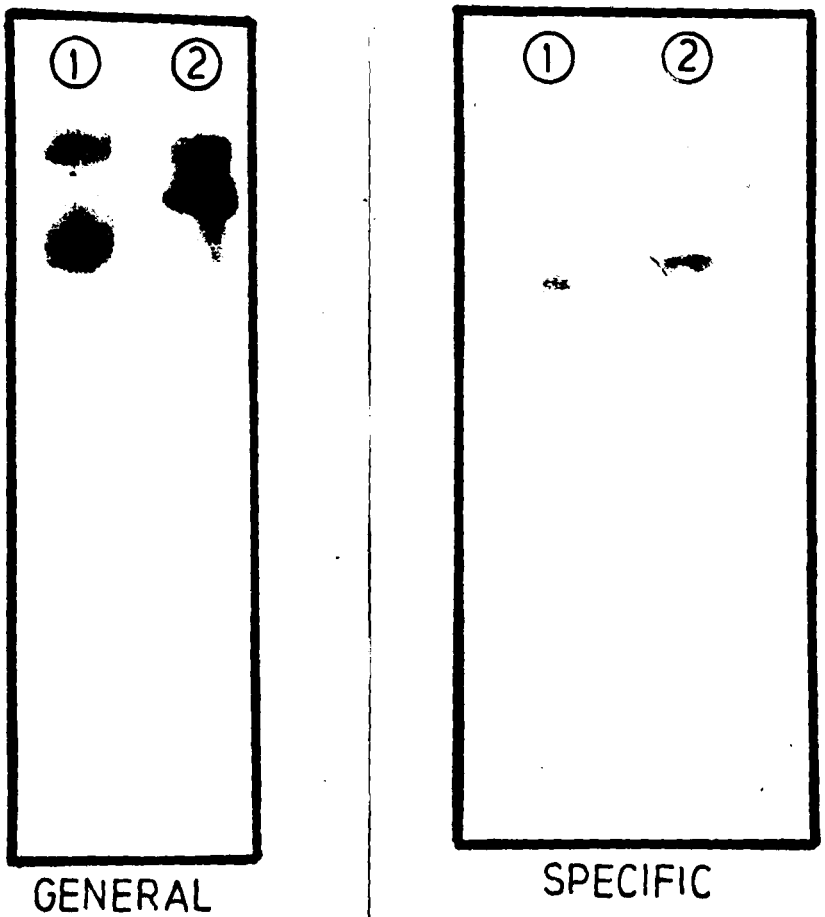


Fig. 15 Elution profile of cytosolic aspartate aminotransferase (c-AsAT) from the liver of 15- and 180-day old mice through CM-cellulose ion exchange. Details of experimental conditions are described in method section. c-AsAT was eluted applying linear gradient of sodium acetate buffer (0.06 - 0.16 M).

Fig. 16 Polyacrylamide gel electrophoresis of purified c-AsAT from the liver of 15-day (lane 1) and 180-day (lane 2) old male mice.

A. Stained for general protein using coomassie brilliant blue (R-250).
B. Stained specifically for c-AsAT using specific staining procedure as detailed in method section of the thesis.


①
②
GENERAL
①
②
SPECIFIC

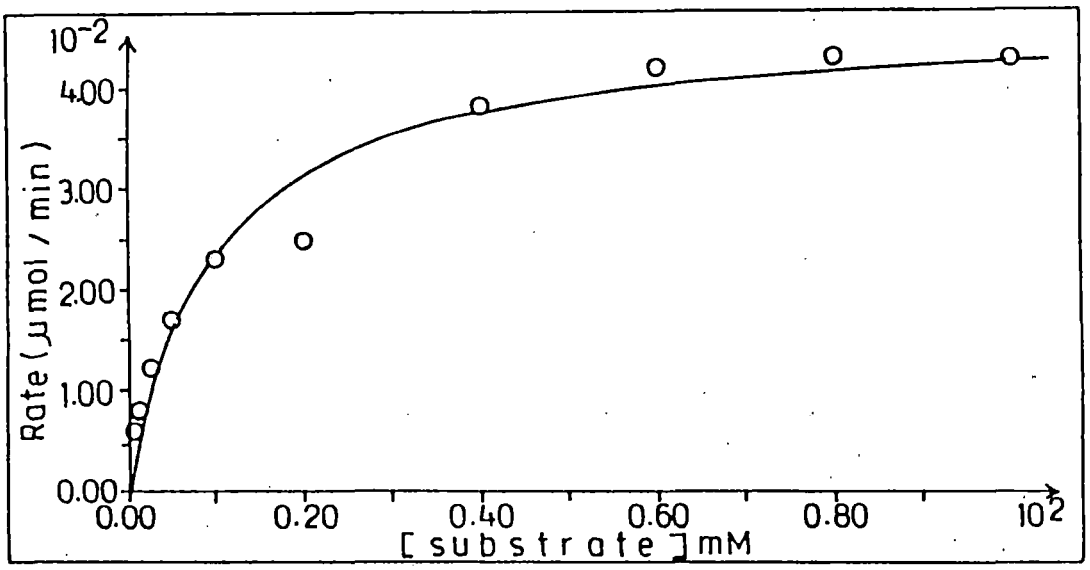


Fig. 17 (A) Michaelis-Menten plot for cytosolic aspartate aminotransferase (c-AsAT) from the liver of immature (15-day) mice with respect to aspartate as variable substrate. Data were computed and drawn using the enzfitter programme of sigma.

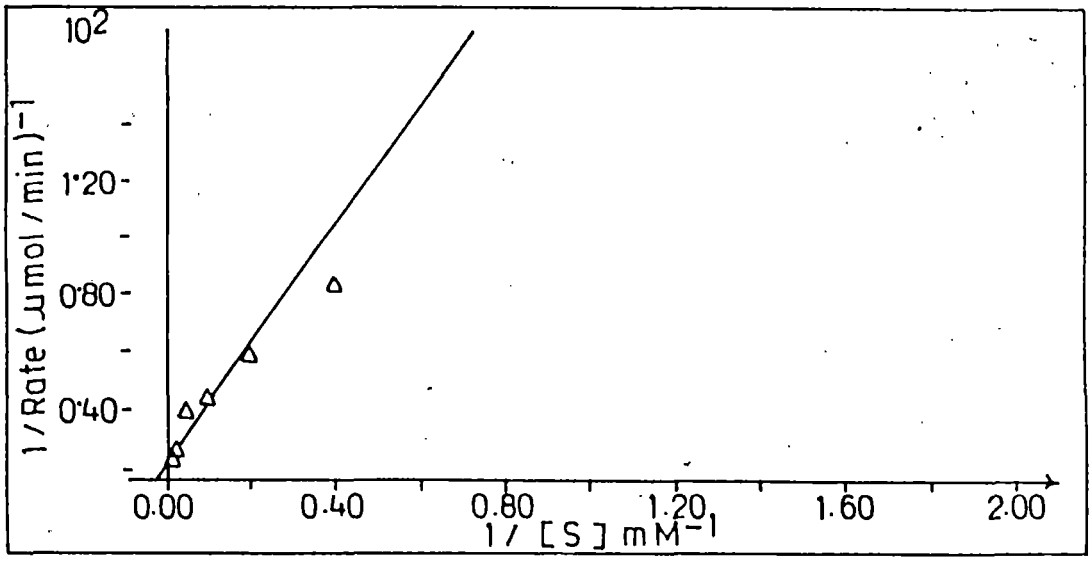


Fig. 17 (B) Lineweaver-Burk plot of the same.

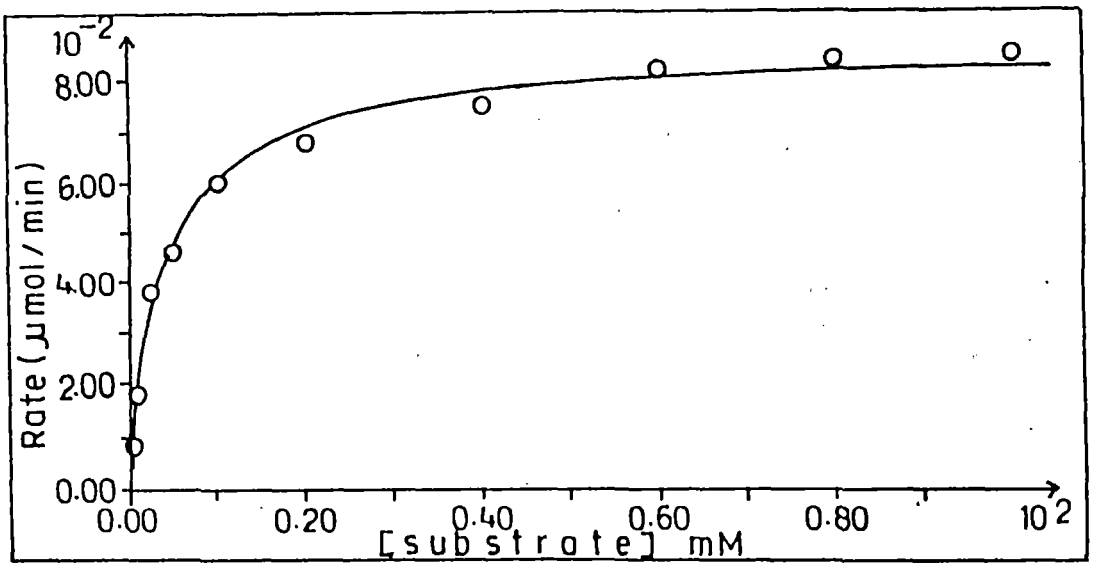


Fig. 18 (A) Michaelis-Menten plot for cytosolic aspartate aminotransferase (c-AsAT) from the liver of mature (180-day) mice with respect to aspartate as variable substrate. Data were computed and drawn using the enzfitter programme of sigma.

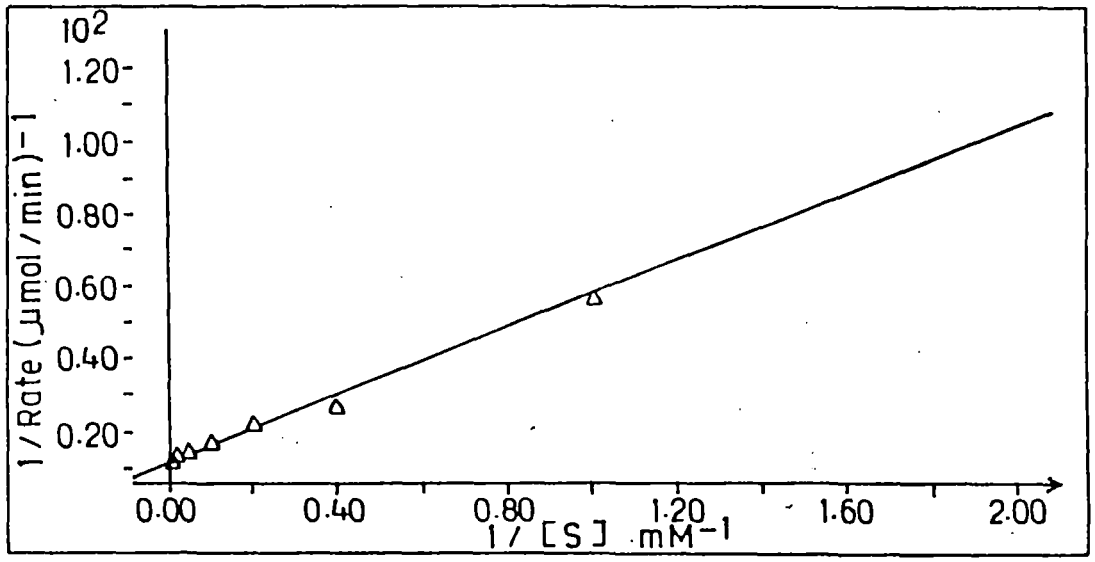


Fig. 18 (B) Lineweaver-Burk plot of the same.

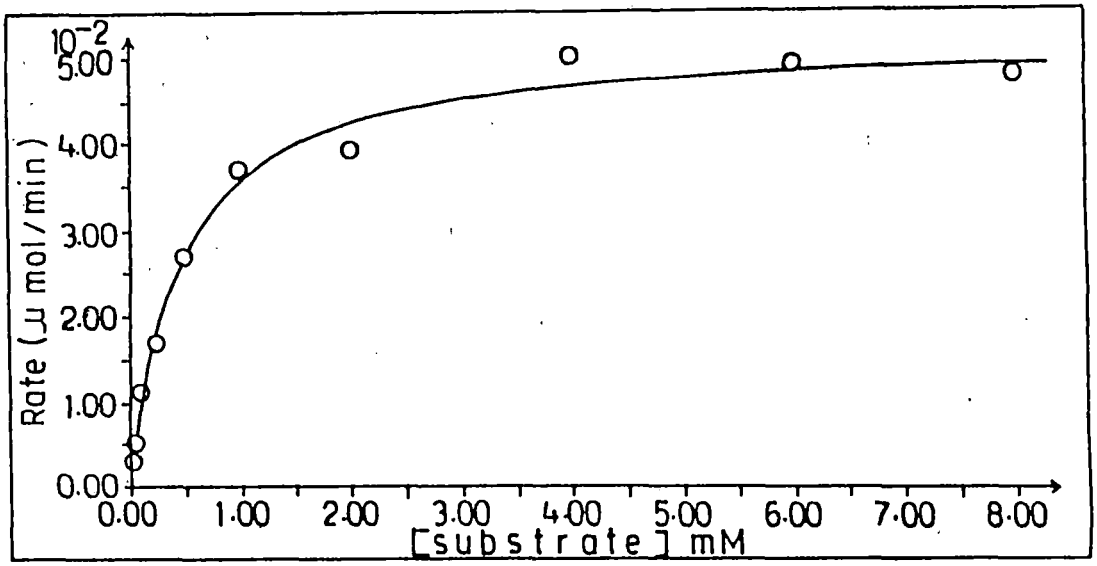


Fig. 19 (A) Michaelis-Menten plot for cytosolic aspartate aminotransferase (c-AsAT) from the liver of immature (15-day) mice with respect to α-ketoglutarate as variable substrate. Data were computed and drawn using the enzfitter programme of sigma.

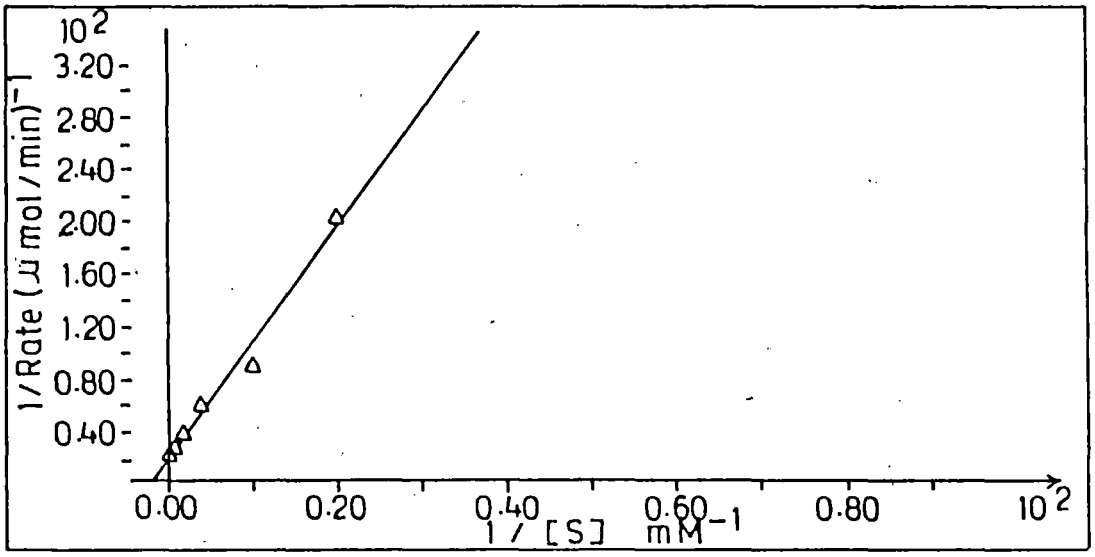


Fig. 19 (B) Lineweaver-Burk plot of the same.

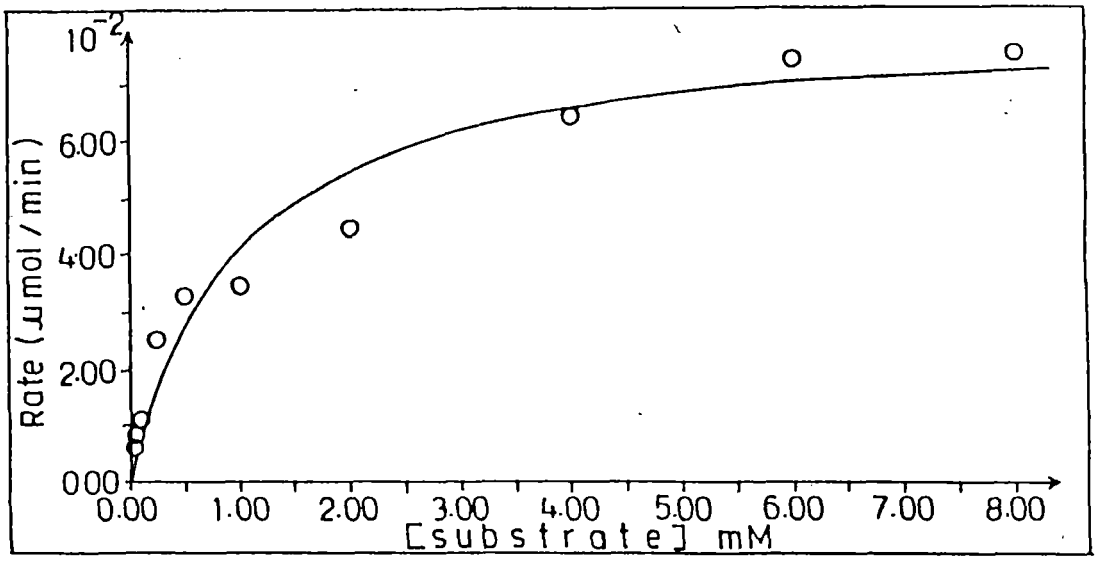


Fig. 20 (A) Michaelis-Menten plot for cytosolic aspartate aminotransferase (c-AsAT) from the liver of mature (180-day) mice with respect to α-ketoglutarate as variable substrate. Data were computed and drawn using the enzfitter programme of sigma.

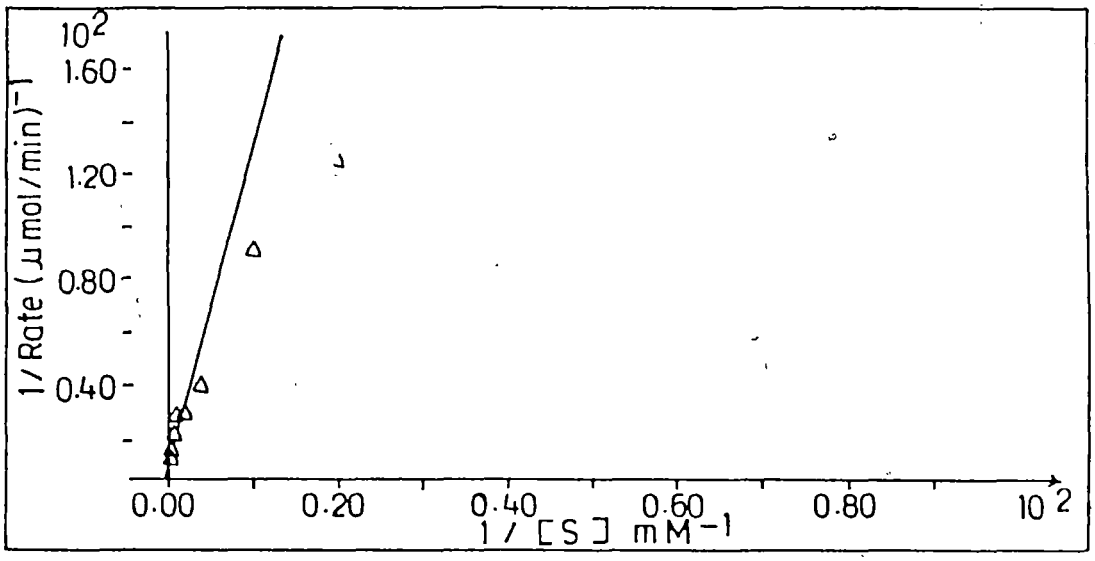


Fig. 20 (B) Lineweaver-Burk plot of the same.

TABLE - 16

Kinetic data of purified cytosolic aspartate aminotransferase from two different ages.

AGE (DAY)	PARAMETERS	ASPARTATE (Substrate)	α-KETOGLUTARATE (Substrate)
15	K_m (mM)	4.5	0.26
	V_{max} μmol/min	0.045	0.046
	K_{cat} (sec^{-1})	0.041	0.047
180	K_m (mM)	4.2	0.23
	V_{max} μmol/min	0.085	0.071
	K_{cat} (sec^{-1})	0.087	0.064

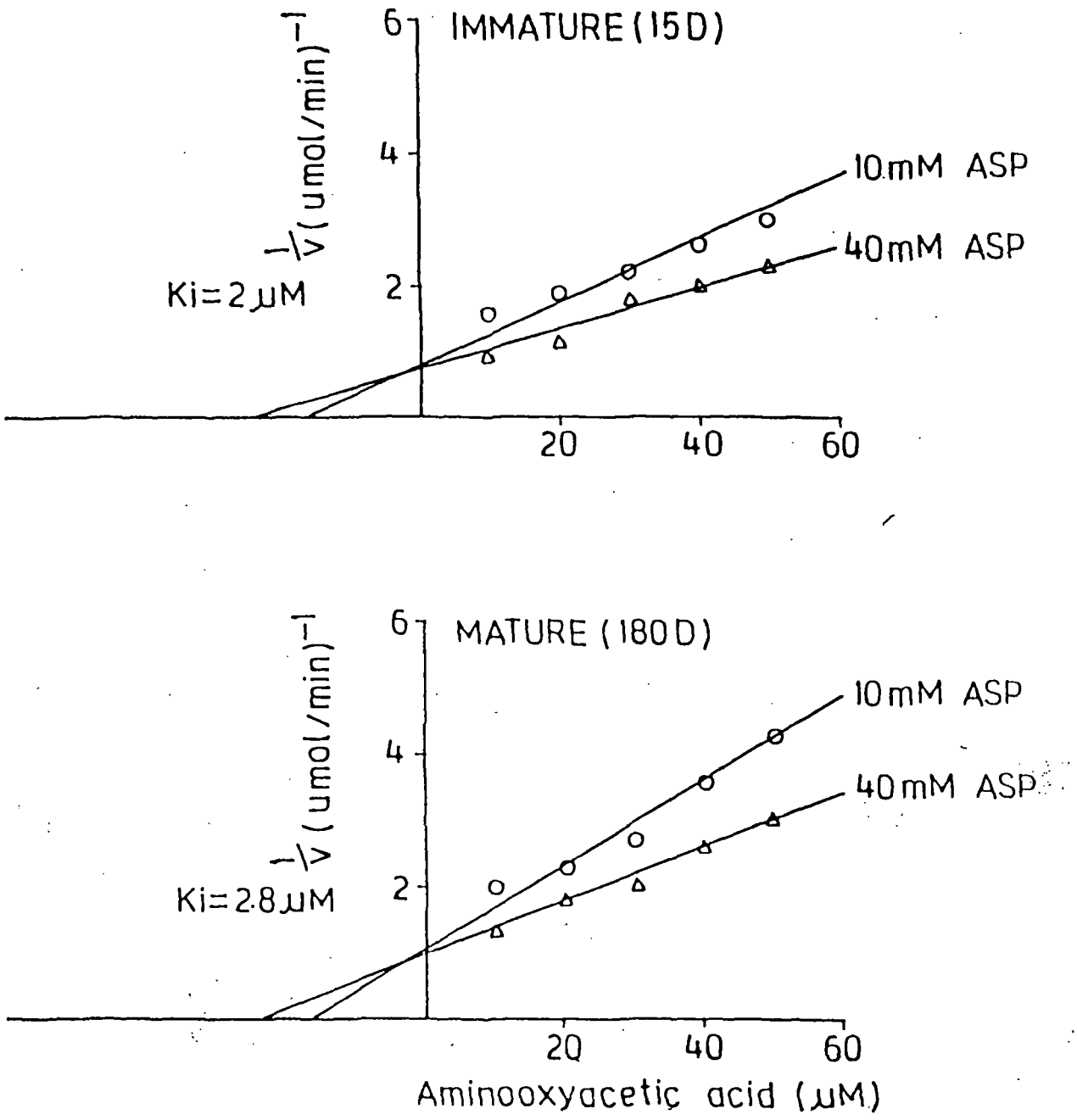


Fig. 21 Inhibition of cytosolic aspartate aminotransferase from the liver of immature (15-day) and mature (180-day) male mice by amino-oxyacetic acid with respect to aspartate (DIXON'S PLOT).

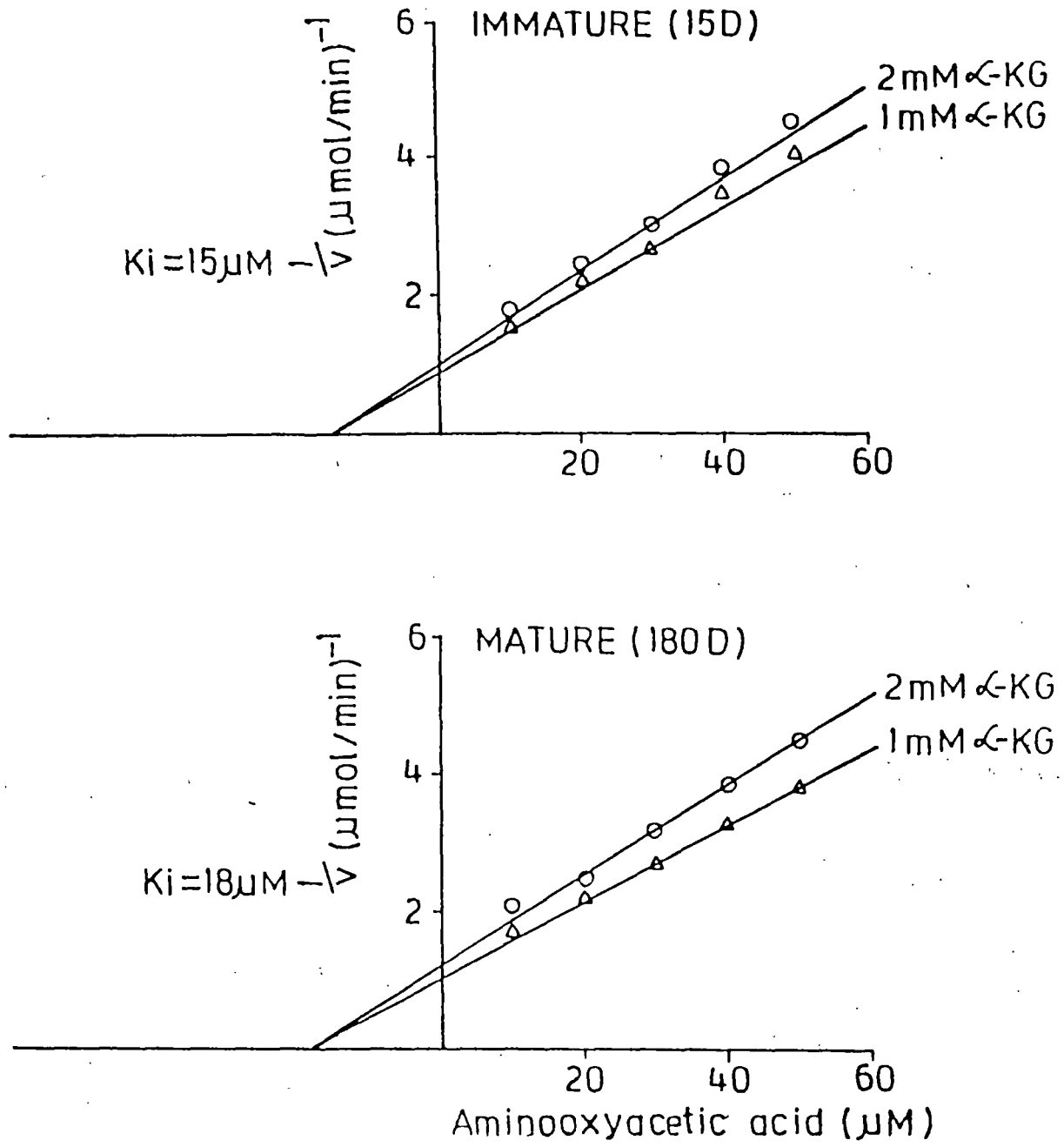


Fig. 22 Inhibition of cytosolic aspartate aminotransferase from the liver of immature (15-day) and mature (180-day) male mice by amino-oxyacetic acid with respect to α-ketoglutarate (DIXON'S PLOT).

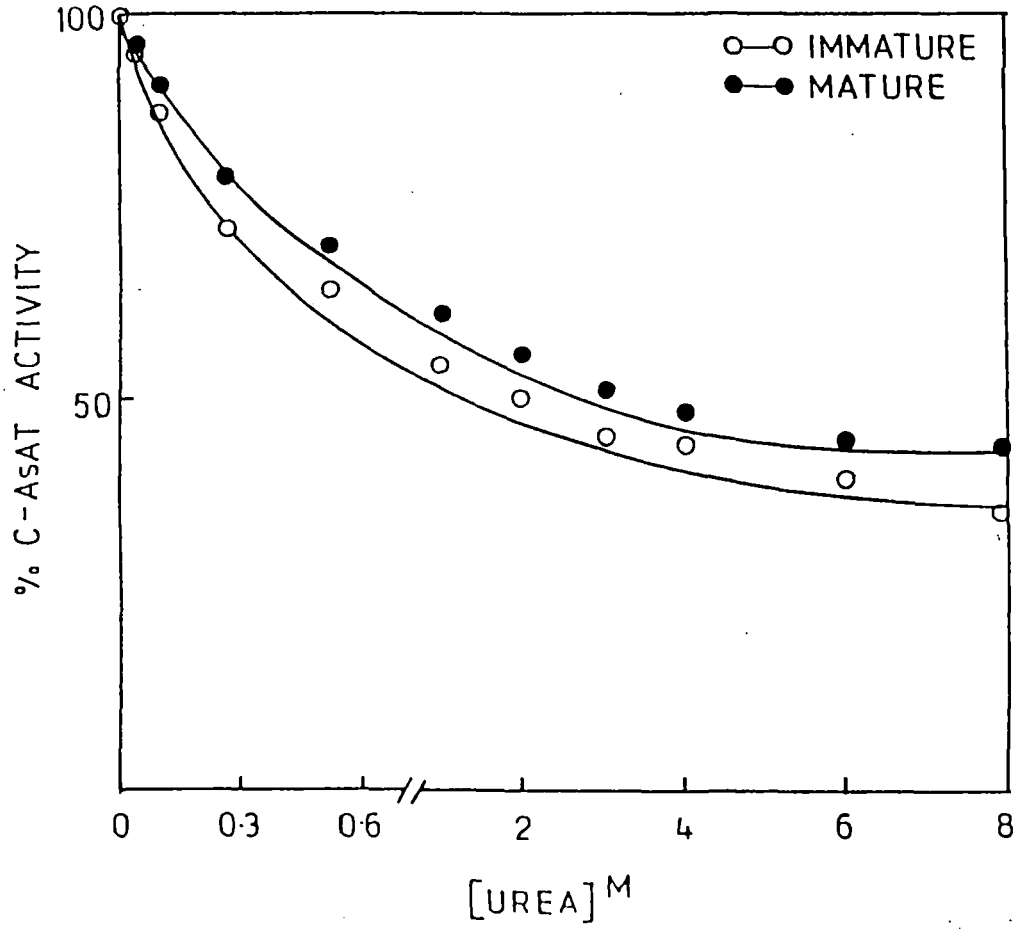


Fig. 23 Inactivation profile of liver cytosolic aspartate aminotransferase from immature (15-day) and mature (180-day) old mice, using varying concentrations of urea. Values are expressed in per cent residual activity.

and mature c-AsAT eluted at two different ionic strength of buffer. The immature c-AsAT elutes at the strength of 0.11 M sodium acetate buffer and the mature one elutes at 0.14 M sodium acetate buffer.

Polyacrylamide gel electrophoresis (PAGE) of purified c- AsAT :

Using non-denaturing polyacrylamide slab gel of 7.5% cross linking, the preparation representing the immature (15-day) and mature (180-day) c-AsAT was electrophoresed.

The one half of gel was stained for general proteins by coommassie blue and the other half was stained for the enzyme by specific staining of the gel as described in experimental procedure section. Both the gels show differential migration of c-AsAT isoenzyme from two age groups (Fig-16). This differential migration of c-AsAT isoenzyme from the liver of immature and mature ages confirms the result of the differential elution profile on CM-cellulose column. This may indicate the charge differences in the liver c-AsAT of immature and mature mice.

Kinetic properties of purified c-AsAT :

c-AsAT activities of the purified preparation from the liver of two age groups (immature and mature mice) were studied at varying concentrations of substrates i.e. L-aspartate and ∝-ketoglutarate using normal assay procedure. Data obtained were analyzed, computed for K_m, V_{max} and K_{cat} using the enzfitter programme (Table-16). The figures were drawn using the Michaelis-Menten equation and the insets to these figures were drawn using the Lineweaver-Burk transformation.

Effect of L-aspartate on purified c-AsAT — The K_m values of liver c-AsAT for L-aspartate were found to be 4.5 and 4.2 mM for the immature and mature mice, respectively. It indicates no significant difference between the K_m values for L-aspartate in immature and mature mice. The V_{max} and K_{cat} values of the enzyme are 0.045 μmol/min and 0.041 sec^{-1} for immature and 0.085 μmol/min and 0.087 sec^{-1} for mature, respectively. It indicates that c-AsAT from the mature mice showed higher turnover compared to the immature one (Fig-17 A & B and 18 A & B).

Effect of ∝-ketoglutarate on purified c-AsAT — The K_m values of c-AsAT for ∝-ketoglutarate were found to be 0.26 and 0.23 mM for immature (15-day) and mature (180-day) mice. Similar to L-aspartate, there is no significant difference between the K_m values of this

enzyme for ∝-ketoglutarate in immature and mature mice. The V_{max} and K_{cat} values of the enzyme of immature are 0.046 μmol/min and 0.047 sec^{-1} and of mature are 0.071 μmol/min and 0.064 sec^{-1}, respectively (Fig-19 A & B and 20 A & B).

Effect of [amino-oxyacetic acid] on purified c-AsAT — Fig -21 & 22 show the Dixon's plots of the data for the effect of inhibitor amino-oxyacetic acid (AoAA) on the activity of purified liver c-AsAT of immature and mature mice, respectively. Two different fixed concentrations of L-aspartate (10 and 40 mM) and ∝-ketoglutarate (1 and 2 mM) were used with varying concentrations of AoAA. Values obtained were plotted for Dixon's plot. The K_i values were 2 μM for immature (15-day) and 2.8 μM for mature (180-day) with respect to L-aspartate and 15 μM for immature (15-day) and 18 μM for mature (180-day) with respect to ∝-ketoglutarate (Fig-21 A & B and 22 A & B).

Studies on urea denaturation of purified c-AsAT :

Comparisons of the result of inactivation studies using different concentrations of urea on the purified liver c-AsAT of immature and mature ages of mice were performed. c-AsAT isoenzyme from immature gets 50% in inactivation (IC_{50}) at 1.2 M urea. Whereas, mature c-AsAT gets 50% inactivation (IC_{50}) at 2.3 M urea (Fig-23). This shows that immature isoenzyme is more susceptible compared to mature isoenzyme for urea denaturation.

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DISCUSSION

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

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

Enzymes are specific proteins that catalyze chemical reactions in biological system. Enzymes are the direct phenotypic expression of their genes. Considerable amount of literature has developed concerning changes in enzyme level as a function of age and has been well reviewed (Wilson, 1981; Sharma, 1988,94). At present, this collected information is hard to correlate owing to the variability and contradictory nature of the reported results. One of the problems in obtaining consistent results is that the enzymes have been studied individually without relationship to others in a particular metabolic pathway. But very few studies have reported the activities of all the key enzymes of a particular metabolic pathway which provide quantitative knowledge about the directionality of the pathway. Activities of several enzymes of glycolysis, Krebs cycle and pentose-phosphate pathway of several tissues in mature and old rats have been studied individually (Ardawi, 1982, '85; Dealmeida, 1989). Keeping in view the importance of studying all the enzymes of a particular metabolic cycle, the work embodies in this thesis was planned onto study the regulatory changes in enzymes of malate-aspartate shuttle to elucidate the mechanism of regulation of this shuttle during development and aging.

The malate-aspartate shuttle is primarily involved in the transfer of reducing equivalents from cytosolic NADH to the mitochondria in various tissues (McDonald, 1983). The reducing equivalents of cytosolic NADH are first transferred to cytosolic oxaloacetate to yield malate by the action of cytosolic malate dehydrogenase (c-MDH). The malate carrying reducing equivalents, passes through the inner mitochondrial membrane into the matrix, where the reducing equivalents are passed onto the matrix NAD^+ yielding oxaloacetate by the action of mitochondrial malate dehydrogenase (m-MDH). The shuttle involves an influx of malate and glutamate and an efflux of aspartate and ∝-ketoglutarate from mitochondria. Oxaloacetate is a physiologically important intermediate of several metabolic pathways. These pathways may either be catabolic (Krebs cycle) or anabolic (gluconeogenesis) in nature. Since oxaloacetate is impermeable to mitochondrial membrane, the malate-aspartate shuttle appears to be the primary mechanism for the transfer of reducing equivalents from cytosol to mitochondria or vice-versa.

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

i) the endogenous activity levels of shuttle enzymes at different postnatal ages and their tissue-specific patterns.

ii) regulation of shuttle enzymes by various hormones such as glucocorticoid and thyroid hormones during the same postnatal ages of mice.

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

Endogenous activities of malate-aspartate shuttle isoenzymes :

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

absorbance at 340 nm) in the liver of 15-day old mice compared to that of 30-day old animals. Whereas, the shuttle activity was significantly higher in the kidney of 30-day old mice than that of 15-day old ones. Similar to the expression of enzymatic activities, the shuttle activity showed an identical pattern in the liver and kidney of developing mice.

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

Hormonal regulation of shuttle enzymes :

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

Adrenalectomy decreases and administration of hydrocortisone to adrenalectomized mice increases the activity of kidney cytosolic and mitochondrial malate dehydrogenase only in 30- and 60-day old mice. It does not show any effect on the activity of kidney malate dehydrogenase

(cytosolic and mitochondrial) in preweaned mice (15-day old). These findings corroborate the observation (Herzfeld and Greengard, 1969) that : a) the same enzyme in different tissues of the developing animals need not be regulated by the same physiological stimuli. b) in the same tissue, the developmental formation of an enzyme may not be regulated by one particular signal but by the interaction of many others. c) the hormonal signals important for the developmental formation of the enzyme may or may not regulate the level of the same enzyme in adult tissues.

The genes responsible for the synthesis of cytosolic and mitochondrial malate dehydrogenase are reported to be different (Whitt, 1971,87; Basaghlia,1989). The inducibility of mitochondrial malate dehydrogenase by hydrocortisone is significantly lower than that of cytosolic MDH. This may be due to the differential responsiveness of both the genes of cytosolic and mitochondrial malate dehydrogenase isoenzymes towards hydrocortisone such as, their location on the chromosomes, availability for the inducer, nature of the trans-acting factors. Previous studies (Kanungo and Gandhi, 1972; Sharma and Patnaik, 1982) on inducibility of liver cytosolic and mitochondrial malate dehydrogenase isoenzymes by cortisone as well as hydrocortisone in the rats of various ages also indicate that the level of these isoenzymes are regulated by the adrenal corticoids.

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

Adrenalectomy decreases and the administration of hydrocortisone increases the activity of kidney cytosolic aspartate aminotransferase in post-weaned mice (30-and 60- day old). Since both the isoenzymes of AsAT are genetically independent (Braunstein, 1973), they differ from one another even in the pre-and post-mitotic tissues for their responses towards hydrocortisone. Although both the isoenzymes of AsAT are involved in gluconeogenesis, it is the cytosolic isoen-

zyme whose activity is regulated by glucocorticoids (Shield and Roth,1965; Shrago and Lardy, 1966; Sharma and Patnaik, 1982, 84,85).

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

Regulation of malate-aspartate shuttle enzymes by dibutyryl cyclic AMP (Bt_2-cAMP) and Combination of Bt_2 -cAMP and Hydrocortisone — Past couple of years, group of workers visualized the cross-talk between the steroid and protein/peptide hormone action. The discovery of cross-talk between membrane - associated receptors and intracellular steroid and thyroid hormone receptors has gained much attention in recent years because of its multiple functional implications and biomedical significance (Sharma, 1993).

Various doses of Bt_2-cAMP, a membrane permeable analog of cyclic AMP, were administered in different postnatal ages of normal male mice (i.e. 15-, 30- and 60-day old). None of these single doses of Bt_2-cAMP were effective on both isoenzymes of malate dehydrogenase and aspartate-aminotransferase in the liver and kidney of mice. These findings indicated that none of the shuttle enzymes of mice liver and kidney are regulated by cAMP at those postnatal ages studied. Hydrocortisone alone on intact mice fails to give any effect on the shuttle enzymes,

when used as a single dose. Hence, in order to find out the synergistic or antagonistic role of cAMP on hydrocortisone action, a combination of Bt_2-cAMP with hydrocortisone was injected in the mice of above age groups. It has been seen that only liver mitochondrial malate dehydrogenase and cytosolic aspartate aminotransferase show an increase in the activity. In case of mitochondrial malate dehydrogenase, the increase in activity has been seen only at 30- and 60-day of postnatal age. The activity of both the shuttle enzymes show no change with this combination in the kidney of mice at either of the age groups. This indicates that only liver and not the kidney is possibly equipped with the cross-talk mechanism in regulating the enzyme activity. Aggerbeck and coworkers (1993) reported that cytosolic aspartate aminotransferase (c-AsAT) is a ubiquitous enzyme that displays liver- specific hormonal regulation. Both the activity as well as mRNA level of c-AsAT are increased by glucocorticoids and the effect is potentiated and inhibited by cAMP and insulin, respectively in cultured hepatoma cell lines. The presence of two regulatory regions in the cytosolic AsAT promoter separate the positive cAMP effect from the negative insulin effect. Toussaint **et al** (1994) studied the expression and regulation of the rat testis cytosolic aspartate aminotransferase gene and showed that the pattern of transcription inhibition and polyadenylation site selection of a housekeeping gene can be tissue-specific. Aruzzese **et al** (1995) reported that the translation of mRNA for the two isoenzymes of AsAT is subject to tissue-specific regulation in an age-related manner.

Regulation of malate-aspartate shuttle enzymes by thyroid hormone — Thyroid hormones have been implicated in controlling development and differentiation of many animals. Böttger et al (1970) studied many gluconeogenic enzymes (pyruvate carboxylase, phosphoenol-pyruvate carboxykinase and pyruvate kinase) under different states of thyroid function and have shown variations in the involvement of these enzymes in controlling the overall rate of hepatic gluconeogenesis. This envisaged the role of thyroid hormones in gluconeogenesis. It has been reported that in thyroidectomized rats, there is a marked decrease in mitochondrial citrate, 2-oxoglutarate and glutamate with a smaller changes in aspartate and malate. These changes are interpreted as providing evidence for the importance of modification in the malate-aspartate shuttle in hypothyroidism, albeit to a moderate degree. In our study, administration of T_3 which is a potent thyroid hormone, in normal mice of three different postnatal ages (i.e. 15-, 30- and 60-day) showed no significant change in the activities of cytosolic and mitochondrial malate dehydrogenase as well as aspartate aminotransferase in the liver and kidney of mice. Most likely, this might be due to the tonic regulation of these enzymes by the endogenous circulating level of the

thyroid hormone.

Purification and properties of cytosolic aspartate aminotransferase :

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

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

purified c-AlAT of the liver of 5- 52-, and 100- week old female rats showed that the liver of the immature rat has only c-AlAT-A and the liver of old rats only c-AlAT-B. The adult rat liver has both the isoenzymes, but the level of c-AlAT-A was lower than c-AlAT-B. The A and B submits are under the control of two separate genes. Hence, the sequential appearance and disappearance of the two isoenzymes during the lifespan of the rat could be due to the sequential expression and repression of specific genes responsible for the synthesis of their subunits.

Kinetic studies on purified cytosolic aspartate aminotransferase :

Kinetic studies on the purified c-AsAT, of the liver of immature and mature mice were carried out to elucidate structural changes, if any, which occur in the active site of enzyme molecule as a function of age. For both the immature and mature mice liver c-AsAT, hyperbolic curve was obtained when the velocity of the enzyme catalyzed reaction was plotted against varying concentrations of both the substrates (i.e. L-aspartate and ∝-ketoglutarate) by using enzfitter programme (Perella, 1988). The figures were drawn using the Michaelis - Menten equation and the insets of these figures were drawn using the Lineweaver - Burk transformation. The plots indicate that none of the two substrates exhibit allosteric effect on the enzyme activity. Analysis of data indicates no significance difference between the K_m values of this enzyme for both the substrates in immature and mature mice. However, the enzyme from the mature mice showed higher V_{max} and K_{cat}, indicating higher turnover compare to the immature one. This indicates that the c-AsAT from mature mice catalyses the reaction at a faster rate than that of c-AsAT from immature mice, although the binding affinities for substrates remained the same. It may be plausible that the substrate binding sites of the enzyme are not affected for by the charge difference between the enzymes from the two ages. However, the charge difference in the c-AsAT at two ages might contribute to the catalytic turnover of the enzyme at respective ages. The higher catalytic rate of mature enzyme might extend an adaptation to control the homeostatic function of the metabolic demands of the mature mice since malate aspartate shuttle is one of the major control points for glycolysis, Krebs cycle and gluconeogenesis. The c-AsAT from the liver of mice of both the ages are competitively inhibited by amino-oxyacetic acid (AoAA) with respect to L-aspartate and non-competitively with respect to ∝-ketoglutarate. The K_i values of this enzyme for AoAA at immature and mature ages are similar. It is known that AoAA inhibits c-AsAT competitively with respect to its amino acid substrate and noncompetitively with respect to its keto acid substrate (Braunstein, 1973; Rej, 1976; Sharma and Patnaik, 1982)

The kinetic parameters of a number of enzymes have been measured as a function of age. Studies on pyruvate kinase (Chainy and Kanungo, 1978) of the brain, myosin ATPase (Koldor and Min, 1975; Srivastava and Kanungo, 1979) and aldolase (Gershon and Gershon, 1973) of skeletal muscle, cytosolic alanine- aminotransferase of liver (Patnaik and Kanungo, 1976) cytosolic aspartate aminotransferase of rat liver (Sharma and Patnaik, 1982) showed that, in general, there is no significant difference between K_m, K_i and molecular weight from young and old rats. Reiss and gershon (1976) and Gupta and Rothstein (1976) have proposed that the differences in the kinetic parameters of enzymes seen in old animals may be due to post translational modifications. Recently, a similar kinetic difference in the V_{max} and K_{cat} of inorganic pyrophosphatase in immature and mature chicken liver has been reported (Syiem, 1996). The kinetic differences in the catalytic efficiency of enzyme without affecting the affinities for substrate have been attributed to adaptational significance depending on the age-specific metabolic demand in animal's tissues.

Unfolding and inactivation of c-AsAT :

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

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

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SUMMARY

Enzymes are specific proteins that catalyze chemical reactions in biological systems. Living cells have evolved a complex regulatory mechanism to control the concentrations of their enzymes, particularly those catalyzing critical metabolic reactions. In an organism, several physiological and biochemical changes occur during development, growth, adulthood and senescence. The developmental phase includes an increase in the number and size of cells, and their differentiation to perform specialized functions. The metabolic events that occur during development might influence the later part of lifespan. Aging is the characteristic of all multicellular organisms. The functional abilities of most organs and the organisms decrease during senescence. The decline becomes perceptible towards the later part of the reproductive phase. Thus, the reproductive phase smoothly merges into the senescence phase, unlike the transition from the developmental to the reproductive phase in which specific genes are expressed and specific structures and functions appear that confer reproductive ability to the organism. During senescence, adaptability to external and internal stresses decreases and the homeostatic mechanisms deteriorate and that increase the susceptibility in old age.

During development and aging, different metabolic adjustments take place as an adaptation to the changing demand made upon them. Study of all the enzymes of a particular metabolic pathway provides a complete profile of their biological functions. 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 regulatory changes in enzymes of malate-aspartate shuttle to elucidate the mechanism of regulation of this shuttle during development and aging.

The malate-aspartate shuttle appears to be the primary mechanism for the transfer of reducing equivalent from the cytosolic NADH to the mitochondria in many animal tissues. It has been seen that inner mitochondrial membrane is impermeable to NADH. The NADH formed during glycolysis in the cytoplasm by the oxidation of glyceraldehyde-3-phosphate must be regenerated to NAD^+ for glycolysis to operate. The shuttle involves an influx of malate and glutamate and efflux of aspartate and ketoglutarate from the mitochondria. The main enzymes of the shuttle are malate dehydrogenase and aspartate aminotransferase. Both these enzyme have two homologous and genetically independent isoenzymes. One in the cytosolic and the other in the

mitochondrial fraction.The present study aims :

(a) to assess the endogenous activity level of shuttle enzymes in a postnatal age - and tissue - specific manner

b) regulation of enzymes of shuttle by various hormones such as glucocorticoid and thyroid hormone during development of mice

c) lastly, to purify one of the shuttle enzymes that is cytosolic aspartate aminotransferase and to study its chemical and kinetic properties in order to find out changes, if any, in properties as a function of age.

Endogenous level of shuttle enzymes :

The endogenous activities of isoenzymes of malate dehydrogenase and aspartate aminotransferase show a significant change during postnatal development of mice. The activities of both the isoenzymes (cytosolic and mitochondrial) of malate dehydrogenase (MDH) and aspartate aminotransferase (AsAT) were significantly higher in the liver of mice at day 15, declined at day 30 and remained unchanged thereafter until day 60. In contrast, the activities of these isoenzymes showed a lower value at day 15, increased to a peak value at day 30 in the kidney of mice. It indicates an early developmental expression of shuttle enzymes in the liver than in the kidney of mice which may in turn exhibit an early involvement of malate-aspartate shuttle in the transfer of reducing equivalents to compensate the metabolic demands of this tissue in growing mice. Reconstitution studies confirmed the observation of malate- aspartate shuttle enzymes in liver and kidney during postnatal development of mice.

Hormonal regulation of shuttle enzymes :

It was observed that adrenalectomy decreases and administration of hydrocortisone to adrenalectomized mice increases the activity of cytosolic and mitochondrial malate dehydrogenase and cytosolic aspartate aminotransferase in the liver of 15-, 30-, and 60 -day old mice. Per cent decrease following adrenalectomy is almost similar in all the postnatal ages studied. This indicates that adrenal steroid do play a role in the regulation of the shuttle enzymes.

Adrenalectomy also decreases and administration of hydrocortisone to adrenalectomized

mice increases the activity of kidney cytosolic and mitochondrial malate dehydrogenase and cytosolic aspartate aminotransferase only in 30- and 60- day old mice. It has not shown any effect on the activity of these enzymes in preweaned mice (15-day old). It may be due to the differential level of glucocorticoid receptors and other trans-acting factors in the liver and kidney of mice during this phase of postnatal development. These findings corroborate the observations that the same enzyme in different tissues of the developing animals might be regulated differentially by the same physiological stimuli. The hormonal signals important in the developmental formation of the enzymes may or may not regulate the level of the same enzyme in adult tissues. Differential hormonal regulation of AsAT isoenzymes indicate that they are subject to different physiological controls in different tissues.

Various doses of Bt_2-cAMP, a membrane permeable analog of cyclic AMP, were administered in different postnatal ages of normal male mice. None of these single doses of Bt_2-cAMP were effective on both isoenzymes of MDH and AsAT in the liver and kidney of mice. The finding indicated that none of the shuttle enzyme of mice liver and kidney are regulated by cAMP at those postnatal ages studied. Last couple of years, group of workers visualized the cross-talk between steroid and protein/ peptide hormone action. Hence, in order to find out the synergistic or antagonistic role of cyclic AMP on hydrocortisone action, a combination of Bt_2-cAMP with hydrocortisone was injected in the 15-, 30- and 60- day old mice. It has been observed that only liver mitochondrial malate dehydrogenase and cytosolic aspartate aminotransferase show an increase in the activity. In case of mitochondrial malate dehydrogenase, the increase in activity has been seen only at 30- and 60- day of postnatal age. The activity of both the shuttle enzymes show no effect of this combination in the kidney of mice at either of the age groups. This indicates that only liver and not the kidney is possibly equipped with the cross-talk mechanism in regulating the enzyme activities.

It is seen that administration of T_3 which is a potent thyroid hormone on normal mice of three different postnatal ages (i.e. 15-, 30- and 60- day) showed no significant change in the activities of cytosolic and mitochondrial malate dehydrogenase as well as aspartate aminotransferase in liver and kidney. Most likely this might be due to the tonic regulation of the enzymes by the endogenously circulating level of the thyroid hormones.

Chemical and kinetic properties of c-AsAT :

To find out the change, if any as a function of age, in the chemical and kinetic properties, one of the shuttle enzyme i.e. c-AsAT was isolated and purified from the mice liver of two selected ages (i.e. 15- and 180- day) using similar experimental conditions. The enzyme preparations from both the ages were passed through the CM-cellulose column and the elution profile of the specific activities of this isoenzyme from the liver of two ages of mice exhibited the requirement of two different ionic strength. This indicates that there might be an overall charge difference on the isoenzyme from two different age groups. It was further confirmed by running enzyme preparations onto polyacrylamide gel electrophoresis and staining the gels with general and specific stains. The isoenzyme from immature and mature ages migrated at two different levels, confirming the change difference onto C-AsAT from two ages. Changes in the isoenzyme patterns and their electrophoretic mobilities have earlier been reported and reviewed. They can arise due to genetic variability or sometimes due to epigenic events (such as acetylation, phosphorylation and proteolysis) depending on the metabolic demand to commensurate the requirement at specific stage of development.

Kinetic analysis of data indicates no significant difference between the K_m values of this enzyme for both the substrates in immature and mature mice. However, the enzyme from the mature mice showed higher V_{max} and K_{cat}, indicating higher turnover compared to the immature one. This indicates that the c-AsAT from mature mice catalyses the reaction at a faster rate than that of c-AsAT from immature mice, albeit the binding affinities for substrates remained the same. It may reflect that the substrate binding site of the enzyme is not affected for by the charge difference between the enzymes from the two ages. However, the charge difference in the c-AsAT of two ages might contribute to the catalytic turnover of the enzyme at respective ages. The higher catalytic rate of mature enzyme might extend an adaptation to control the metabolic demands of the mature mice since malate-aspartate shuttle is one of the major control point for glycolysis, Krebs cycle and gluconeogenesis. Inactivation studies of the enzymes from both the ages depict differential folded structure as envisaged by the different requirement of urea for their 50% inactivation. It further corroborates our earlier assumption that there is a difference in the overall charge of the enzyme at two ages.

It may be concluded that the enzymes of malate-aspartate shuttle as well as the shuttle activity expressed differentially in different tissues of mice as a function of postnatal development.

And the shuttle enzymes are also regulated differentially by glucocorticoid where as they do not exhibit any change in intact mice with the exogenously added cAMP as well as thyroid hormones. However, a combination of cAMP and glucocorticoid regulates the shuttle enzymes differentially ia tissue- and age- specific manner. Purification and kinetic analyses show a definite charge difference in C-AsAT isoenzyme at two different ages i.e. immature and mature. The K_m remains the same while catalytic efficiency is higher in mature as compared to immature owing to greater adaptation in mature animals.

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