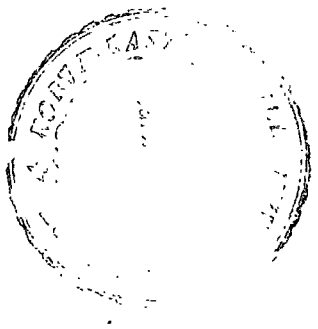
REGULATION OF GLUTAMATE DEHYDROGENASE DURING POSTNATAL DEVELOPMENT OF MICE

ABSTRACT



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Introduction

Glutamate dehydrogenase occupies a central role in nitrogen and carbon metabolism in practically all organisms. The reaction which it catalyses feeds the tricarboxylic acid cycle with carbon intermediate necessary for generating energy as well as precursors for other biosynthetic purposes such as in gluconeogenesis. GDH is also considered as an accessory enzyme to the urea cycle because of its involvement in NH_4^+ metabolism. It also plays an important role in the metabolism of the excitatory neurotransmitter glutamate in the central nervous system signifies the potential of this enzyme in influencing a wider aspect of metabolism in higher organisms, have gathered significant attention. However, its role in the development and/or aging of organism has not been adequately explored.

This study aims to determine the tissue distribution of GDH in mice at different postnatal ages and to gain insight into its regulation by various hormones at different postnatal ages. Further, this enzyme was purified from immature and matured ages to determine if any changes occur in its physico-chemical properties during the course of postnatal development.

Age and tissue distribution

GDH exhibited an age- and tissue-specific pattern of distribution in terms of activity with the highest activity observed in the liver, followed by the kidney, brain and heart. Except for the brain, which showed a gradual increase in activity with age, GDH from the liver, kidney and heart exhibited peak activity during the first 10 days after birth which declined to a more or less stable adult level after 30 days postnatally.

Hormonal responses

Administration of hormones to the immature and matured animals point towards an age- and tissue-specific pattern of response. Thus, it was observed that, T_3 administration resulted in 2.5 fold increase in GDH activity in the heart of the immature mice as compared to the mature mice which exhibited no response to this hormone. Whereas, dexamethasone treatment exerted response in the heart tissue of matured mice only. The brain tissue showed an overwhelming response to T_3 administration in terms of GDH activity with the immature mice exhibiting an excess of 2 fold over its matured counterpart and dexamethasone treatment evoked response only in the matured animal. T_3 administration also resulted in significant increase in GDH activity in the kidney and liver with the matured animals showing higher induction than the immature mice. Dexamethasone treatment however, induced similar responses in both the liver and kidney tissues with an approximately 2 fold increase in GDH activity for both the ages studied. Testosterone administration alone did not exert any significant changes in all of the tissues at both ages studied. Treatment with hormone combinations also resulted in differential response in the various tissues at the two ages. Thus, dexamethasone-triiodothyronine (dex-T_3) administration resulted in a significant 4 fold increase in GDH activity in the heart of the immature mice only with respect to the control, whereas testosterone-T_3 (test-T_3) treatment did not evoke any significant enhancement of GDH activity in this tissue at both the ages studied. In the brain, dex-T_3 administration resulted in a significant synergistic increase (5 fold) of GDH activity in the immature mice however; it was apparently antagonistic in the matured animal. Test-T_3 combination treatment exerted higher increase of GDH activity of the immature compared to the matured mice. In the kidney, dex-T_3 combination

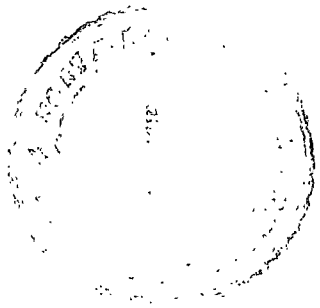
treatment resulted in significant increase (3 fold) of GDH activity in the matured mice compared to the 1.7 fold increase in the immature mice. A moderate (1.6 fold) increase in GDH activity was observed in this tissue of the matured mice in response to test-T_3 administration. The liver tissue also exhibited a synergistic response to dex-T_3 treatment with a 2.8 fold increase in enzyme activity of the immature mice and 4 fold increases in the mature mice. Test-T_3 treatment also showed similar synergistic response vis-à-vis enzyme activity in the adult mice, however no significant response was observed in the immature mice in this tissue.

Physico-chemical comparison of GDH

The physico-chemical properties of dialysed GDH from immature and matured mice indicated that there was no alteration in terms of buffer ionic optima, pH and temperature stability. The enzyme from both the ages also showed an overall similar pattern of response to the effect of various co-substrates and co-enzymes. Inhibition and inactivation studies on the enzyme did not reveal any significant differences between the two ages and further comparison of the total and subunit mass also showed no differences. However, the only difference observed was in the kinetic behaviour of the enzyme towards its substrate; α-ketoglutarate with the matured mice showing higher K_m, compared to its immature counterpart. The antigenic property of glutamate dehydrogenase from the liver of immature and matured mice from both the ages remained unchanged.

In conclusion, this study has given some insight into the endogenous activity of GDH which was marked by age- and tissue-specific pattern of distribution and have also indicated the larger role played by hormones like T_3 and dexamethasone in the regulation of this enzyme..

REGULATION OF GLUTAMATE DEHYDROGENASE DURING POSTNATAL DEVELOPMENT OF MICE



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I certify that the thesis entitled *" **Regulation of glutamate dehydrogenase during postnatal development of mice"*** submitted by Mr. **James Wallang** for the degree of Doctor of Philosophy of the North Eastern Hill University, Shillong, embodies the record of original investigation carried by him under my supervision. The thesis presented is worthy for the award of the Ph.D. degree. This work has not been submitted by him for any other degree of this or any other university.

Place: Shillong

Date: 17th April, 2007.

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LISTS OF ABBREVATIONS

ADP	Adenosine diphosphate
α-KG	alpha-ketoglutarate
APS	Ammonium persulphate
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
Bis	N, N' methylene bis acrylamide
BSA	Bovine serum albumin
CBB	Coomassie brialliant blue
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
GTP	Guanosine triphosohate
NADH	Nicotinamide adenine dinucleotide, reduced
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
PAGE	Polyacrylamide gel electrophoresis
R_m	Relative mobility
SDS	Sodium dodecyl sulphate
TEMED	N,N'N,N'-tetramethylethylene diamine

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CHAPTER - I

INTRODUCTION

The concept of life is an old one. Aristotle divided life into three stages- growth, stasis and decline which was modified later in Greek medicine and physiology into four stages- childhood, youth, maturity and old age. In the broadest sense, lifespan is divided into two main periods: *prenatal*, which include three stages- oval, embryonic and fetal, the latter two characterized by the most striking physiologic events and, *postnatal*; a period which begins with birth and is followed by intense development and growth; this constitute the developmental phase which is then followed by the adulthood or reproductive phase and finally senescence or old age.

The developmental phase includes an increase in the number and size of cells, their differentiation to perform specialized functions, and formation of organs. At the molecular level, specific genes that play specific roles have been identified, for instance the *myo* D gene for differentiation of skeletal muscles cells, homeotic genes for segmentation of the body and fibronectin gene for morphogenesis and organ formation. Several new proteins appear during this period, indicating the expression of hitherto inactive genes. The level of proteins change as cells differentiate and organs form, indicating changes in the expression of corresponding genes. Concomitantly the sizes of organs increase which result in the increase in the size of the organism and its functional ability. Adulthood is marked by the expression of certain genes such as those for hormones- follicle stimulating hormone (FSH), luteinizing hormone (LH) in vertebrates and for ovalbumin, vitellogenin and lysozyme for egg formation in egg laying vertebrates. The duration of this phase is more or less defined, particularly in females (Kanungo, 1994). Throughout infancy,

childhood and adolescence (for humans) come the completion of organs and a gradual remoulding of body shape (Arrey, 1966). These progressive changes continue and stabilize only on reaching adulthood and/or maturity. In higher animals, realization of the full genetic potential involves increase in size, changes in structure and function (Arrey, 1966; Kanungo, 1994).

A prerequisite for survival of any organism is an environment favourable to the optimal expression of its function and includes both external conditions (e.g., temperature, atmospheric pressure, food availability, etc) and conditions of the internal environment (metabolism, coordination and integration of multi-cellular and molecular function). In order to survive, an organism must vary all parameters of its internal environment and match them appropriately to environmental demands or in other words, stress (Timiras, 2003). Further, life has been compared to that of the general adaptation syndrome in which young age corresponds to the so-called "alarmed reaction" when many functions are acutely challenged, adulthood, with optimal efficiency of responses to stress; would response to the period of resistance and successful adaptation and, old age, with a declining ability to adapt to stress, would correspond to the period of exhaustion (Timiras, 2003).

The cessation of reproductive ability of the organism pronounces the beginning of senescence, a phase marked by a decline in the functional abilities of the organism. Senescence or aging has been defined as the collection of changes that render human beings progressively more likely to die (Medawar, 1952). As contended, one hallmark of aging in humans and many other species is an age-related increase in mortality rates. Aging has also been defined as a gradual deterioration of physiological function with age, including a decrease in fecundity (Partridge and

Mangel, 1999), or the intrinsic, inevitable, and irreversible age-related process of loss of viability and increase in vulnerability (Comfort, 1964). As reviewed, human aging has multiple causes which include genetic changes, damages by reactive oxygen species (ROS), inflammatory damage to tissues and hormonal imbalance. All of these can bring about major failure of organs (Holliday, 2004). The major theories of aging relates to particular causes of aging e.g. Free Radical Theory, Somatic Mutation Theory, Mitochondrial Theory, Immunologic Theory and several others (Harman, 2006; Comfort, 1964, 1979; Finch, 1990; Hayflick 1996; Holliday, 1995).

The developmental theory of aging, also called DevAge; defends that aging is a result of development, that aging and development are regulated by the same genetic mechanisms (Medvedev, 1990; Kanungo, 1994; Zwaan, 2003; de Magalhaes and Church, 2005). The theory proposes the idea that damage only begins accumulating after developmental processes are completes and it is this developmentally-triggered damage that causes most aspects of aging.

As argued, aging may be viewed as an unintended product of evolution, an unintended product of selection acting on development, even though in some species, aging may be a direct product of evolution. Evolution does not favour long life but rather, it optimizes developmental mechanisms for reproduction. Once an organism has passed its genes to the next generation maybe evolution gives up on it and the same genes responsible for the growth and maturation of that organism will inadvertently end up killing it (De Magalhaes and Church, 2005). Evolutionary, this can be seen as a form of antagonistic pleiotropy (Williams, 1957), one in which alleles beneficial early in life are harmful late in life.

Pathologic and Physiological Age-Related Changes

In humans, the body's functional decline tends to begin after the sexual peak, roughly at age 19 and some functions decline even sooner. Contrary to demographic measurements of aging, that show mortality rates increasing exponentially, the human functional decline is linear (Strehler, 1999). Succinctly, aging is characterized by changes in appearance, such as a gradual reduction in height and weight loss due to loss of muscle and bone mass, a lower metabolic rate, lower reaction times, declines in certain memory functions, declines in sexual activity and, in women, menopause, a functional decline in audition, olfaction, and vision, declines in kidney, pulmonary, and immune functions, declines in exercise performance, and multiple endocrine changes (Craik and Salthouse, 1992; Hayflick, 1994; Spence, 1995). However, apart from presbyopia, or farsightedness, which is caused by the continuous growth of the eyes' lenses and appears to be universal of human aging, and menopause, no single age-related change is inevitable. (Finch, 1990; Hayflick, 1994).

The incidence of a number of pathologies increases with age. These include diabetes, heart disease, cancer, arthritis, and kidney disease. Further, the incidence of some pathologies, like sinusitis, remains relatively constant with age, while that of others, like asthma, even decline. Therefore, aging is not merely a collection of diseases, since with age the organism become more susceptible to certain diseases, and more likely to die, becomes frail, and endure a number of physiological changes, not all of which lead to pathology.

Genetic factors

Aging has a strong genetic component. Even *damage-based theories* of aging recognize that certain genetic factors, such as defensive or protective genes, play a role in aging (Kirkwood and Austad, 2000). Likewise, *programmed theories* of aging recognize that some forms of damage contribute to aging and that environmental factors influence the outcome of aging to some degree. The difference between these two camps lies in the underlying mechanism: damage-based theories of aging argue that aging is predominantly a result of interactions with the environment (Holliday, 2004), while programmed theories argue aging is predetermined and occurs on a fixed schedule. Others have suggested similar segregations of theories of aging (Cutler, 1979). For instance, it has been proposed that aging could be: 1) a result of extrinsic or intrinsic factors that cause an accumulation of damage; or 2) that aging is a result of changes in gene expression that are either programmed or derived from DNA structural changes (Campisi, 2000).

It has been suggested that aging is multifactorial, a combination of age-related changes and diseases each timed by independent clocks (Olson, 1987) and that aging derives from the failure of multiple maintenance mechanisms (Holliday, 1995). However, it has been argued that aging is genetically programmed as evident in some species which age due to a precise, uniform genetic clock (Prinzinger, 2005). Semelparous species such as the salmon are such an example. In these organisms, aging and death follow a very specific, well-timed program analogous to development (Austad, 2004). Humans however show a gradual aging process, and therefore the mechanisms of aging in these organisms may not be similar.

The mortality rate doubling time (MRDT) is the approximation of the rate of aging; it varies widely among similar species. Even the pace and/or onset of age-related changes can be remarkably different between similar species. On the other hand, the MRDT is remarkably constant among human populations, even under different environmental conditions (Finch, 1990). This robustness of the aging process suggests a strong genetic component. Independently of the environmental conditions of both, a mouse will age 25-30 times faster than a human being. There are precise genetic factors contributing to the pace of aging among different species. There are compelling evidences that strongly imply that aging is programmed in genes i.e., there is a molecular clock regulating the aging process. This is further supported by the synchronization of life events among mammals (Finch, 1990). In fact, the aging phenomena amongst mammals often appears as the same process only timed at different rates, suggesting the existence of a molecular clock regulating cellular senescence (Holliday, 1995). Other examples such as the complex cascade of events in cellular senescence can be up-regulated by a few genes such as telomerase (Bodnar *et al.*, 1998; Wright and Shay, 2001).

The plasticity of lifespan in invertebrates shows how a few genes can regulate the entire aging process (Lin *et al.*, 1998; Vanfleteren and Braeckman, 1999; Benard and Hekimi, 2002). For instance, in mice, there are several examples of single genes that can extend longevity, in some cases more than 50%, increase in the MRDT, and delay the onset of multiple age-related changes and diseases (*Liang et al.*, 2003; de Magalhaes *et al.*, 2005a). Thus, Lin *et al.*, (1998) on their search for gene mutations that could extends life-span in *Drosophila melanogaster,* found that, a mutant line methuselah (*mth*) displayed approximately 35 percent increase in average life span and enhanced resistance to various forms of stress, including starvation, high

temperature, and dietary paraquat, a free radical generator. The gene *mth* predicted a protein with homology to several guanosine triphosphate-binding protein-coupled seven-transmembrane domain receptors. The involvement of signal transduction pathways to modulate stress response and life-span was advocated. Another such example is the *dauer* pathway in *C. elegans*, a nematode which is an alternative developmental pathway that results in a significant life-extension (Klass and Hirsh, 1976). In the *dauer* pathway, which can be activated by starvation and hence may be analogous to caloric restriction (CR), there is a developmental arrest, which has been argued that, at least in this model system, aging and development are coupled (Johnson *et al.*, 1984). Further genes influencing lifespan in *C. elegans* confirm a linkage between the timing of development and the timing of aging (Lakowski and Hekimi, 1996). In insects too arrested development due to environmental factors has been suggested to slow or even stop aging (Tatar and Yin, 2001). Other examples such as in the marine mollusk *Phestilla sibogae*, the length of larval life is determined by a chance encounter with a stimulus that causes metamorphosis (Brakefield *et al.*, 2005). Interestingly, the duration of post-larval life is unaffected by the length of the time it takes the larva to metamorphose. In other words, during the developmental hiatus from the onset of larval metamorphic competence to metamorphosis, aging is suspended (Miller and Hadfield, 1990). Similarly, semelparous species like the salmon clearly shows that developmental programs can cause aging, or a phenotype resembling aging, and death (de Magalhaes and Church, 2005). Further, there is a correlation in higher animals, including in mammals, between the time it takes to reach sexual maturity and how long, on average, they live afterwards (Charnov, 1993). This has been explained that it could be due to similar extrinsic mortality rates acting on animals, however, and may thus be a product of co-evolution rather than a

causal relation. These results clearly show that genetic mechanisms can, up to a certain degree, regulate aging in mammals. Moreover, it has been argued that the rapid evolution of longevity in the human lineage indicates that maybe a small number of genes are able to regulate the pace of aging (Cutler, 1975).

A large part of our understanding of aging has also come about due to studies on accelerated aging witnessed in humans and animals as a result of certain mutations. Progeroid syndromes, as they are called, are rare genetic diseases of which the three most impressive forms are Werner's (WS), Cockayne, and Hutchinson-Gilford's syndrome (Martin, 1978; Martin and Oshima, 2000). All these diseases originate a phenotype that is remarkably similar to an accelerated aging process, particularly in the case of WS. WS shows an early onset, compared to normal aging; of multiple age-related diseases like diabetes, cataracts, osteoporosis, baldness, and atherosclerosis (Goto, 1997) Though differences exist in terms of pathology, what most markedly distinguishes these syndromes is age of onset with Hutchinson-Gilford's and Cockayne syndrome almost exclusively affecting children while WS patients normally reach adulthood. There are also five reported cases of a neonatal form of progeria called Wiedemann-Rautenstrauch syndrome, in which babies appear to be born old (Rodriguez *et al.*, 1999).

In general, mammalian aging has been stated to be similar in different species, sometimes appearing as the same process only timed at different paces (Finch, 1990; Miller, 1999). The assumed link between the genetic mechanisms regulating development and aging has also been explained as to how aging has changed so rapidly in primates (Cutler, 1979; Allman *et al.*, 1993). One hypothesis is that, probably driven by an extended brain development (Cutler, 1979; Allman *et al.*, 1993;

Kaplan and Robson, 2002; Lee, 2003), hominid evolution led to an extension of development which in turn led to a delay of aging.

It was suggested that WS mimics about 50% of aging characteristics: early cataracts, old skin, grey hair, etc., but not brain aging (Martin, 1982; Gosden, 1996,). This is a high proportion since it is not clear that these diseases are indeed accelerated aging. Moreover, the WS phenotype tends to affect tissues where *WRN*, the gene in which mutations result in WS is expressed (Yu *et al.*, 1997; Motonaga *et al.*, 2002), so it makes sense that not all organs display signs of accelerated aging in WS. Such diseases demonstrate the hierarchical essence of aging in which a single gene can regulate a vast array of complex age-related changes.

DNA Damage Theory

The DNA, due to its central role in life, was bound to be implicated in aging. One hypothesis is that damage accumulation to the DNA causes aging, as first proposed by physicist Leo Szilard (Szilard, 1959, Gensler and Bernstein, 1981). The existence of rare genetic disorder like Progeroid syndromes which accelerates aging has been used to support this theory. Interestingly, the most impressive progeroid syndromes, *Werner's, Hutchinson-Gilford's*, and *Cockayne* syndrome originate in genes that are related to DNA repair/metabolism (Martin and Oshima, 2000). Werner's syndrome (WS) originates in a recessive mutation in a gene, *WRN*, encoding a RecQ helicase (Yu *et al.*, 1996; Gray *et al.*, 1997). Since WRN is unique among its family in also possessing an exonuclease activity (Huang *et al.*, 1998), it may be involved in DNA repair. Although the exact functions of WRN remain a mystery, it is undeniable that WRN plays a role in DNA biology, particularly in solving unusual DNA structures (Shen and Loeb, 2000; Bohr *et al.*, 2002; Fry, 2002). Studies

conducted have shown that cells taken from patients with WS have increased genomic instability (Fukuchi *et al.*, 1989) and these cells were found to be hypersensitive to topoisomerase inhibitors (Pichierri *et al.*, 2000). As such, WS is an indicator that alterations in the DNA over time play a role in aging. As with WRN, the protein whose mutation causes Hutchinson-Gilford's syndrome is also a nuclear protein: lamin A/C (Eriksson *et al.*, 2003). Recent results also suggest that some atypical cases of WS may be derived from mutations in lamin A/C (Chen *et al.*, 2003). The exact function of lamin A/C remains unknown, but is implied to be involved in the biology of the inner nuclear membrane. Further evidence suggests that the DNA machinery is impaired in Hutchinson-Gilford's syndrome (Wang *et al.*, 1991; Sugita *et al.*, 1995), again suggesting that changes in the DNA are important in these diseases and, maybe, in normal aging. The protein involved in *Cockayne Syndrome* Type I participates in transcription and DNA metabolism (Henning *et al.*, 1995).

Other progeroid syndromes exist, for example, *Nijmegen breakage syndrome*, which derives from a mutated DNA double-strand break repair protein (Carney *et al.*, 1998; Matsuura *et al.*, 1998; Varon *et al.*, 1998), has been considered as progeroid (Martin and Oshima, 2000). Accelerated aging is also seen in other life forms. For instance, mouse accelerated aging syndromes have also been implicated in DNA repair such as the mouse homologues of *xeroderma pigmentosum*, group D (de Boer *et al.*, 2002), *ataxia telangiectasia mutated* or ATM (Wong *et al.*, 2003), p53 (Donehower *et al.*, 1992; Donehower, 2002; Tyner *et al.*, 2002; Cao *et al.*, 2003), and Ercc1 (Weeda *et al.*, 1997). Thus many progeroid syndromes of mice involve the DNA machinery (Hasty *et al.*, 2003). It appears well-established that DNA mutations and chromosomal abnormalities increase with age in mice (Martin *et al.*, 1985; Dolle *et al.*, 1997; Vijg, 2000; Dolle and Vijg, 2002) and humans (e.g., Esposito *et al.*,

1989).These changes have not been ascertained s to whether they are causes or effects of aging. In addition, there is no consensus as to what type, if any, of DNA changes are crucial in aging. Correlations have been found between DNA repair mechanisms and rate of aging in some mammalian species (Hart and Setlow, 1974; Grube and Burkle, 1992; Cortopassi and Wang, 1996), though this may be an artifact of long-lived species being on average bigger (Promislow, 1994). On the other hand, mice overexpressing p48, which is important in repairing DNA damage deriving from UV radiation, had improved DNA repair mechanisms but still did not live longer (Tang *et al.*, 2000). Likewise, mice overexpressing a DNA repair gene called *MGMT* had a lower cancer incidence but did not age slower (Zhou *et al.*, 2001). Mice deficient in Pms2, another DNA repair protein, had elevated levels of mutations in multiple tissues and yet did not appear to age faster than controls (Narayanan et al., 1997). Embryos of mice and flies irradiated with X-rays do not age faster (Cosgrove *et al.*, 1993; Strehler, 1999), though one report argued that Chernobyl victims do (Polyukhov *et al.*, 2000). Certain mutations in DNA repair proteins, such as p53 in humans, despite affecting longevity and increasing cancer incidence, fail to accelerate aging (Varley *et al.*, 1997).

Reactive oxygen species (ROS) damage to DNA plays a role in aging, and some circumstantial evidence exists in favour of such hypothesis (Hamilton *et al.*, 2001). Damage from free radicals to nuclear DNA remains an unproven cause of aging but since ROS originate in the mitochondria, and since mitochondria possess their own genome, many advocates of the free radical theory of aging consider that oxidative damage to mitochondria and the mitochondrial DNA (mtDNA) is more important (Harman, 1972; Linnane *et al.*, 1989; de Grey, 1997; Barja, 2002). Some evidence exists that under caloric restriction (CR), oxidative damage to mtDNA is

more important than oxidative damage to nuclear DNA (Barja, 2002). Interestingly, disruption of the mitochondrial DNA polymerase resulted in an accelerated aging phenotype, for the first time directly implicating the mtDNA in aging (Trifunovic *et al.*, 2004). This appears to be unrelated to oxidative damage but instead result from increased apoptosis and accumulated mtDNA damage (Kujoth *et al.*, 2005; Singh, 2006). As such, mtDNA may play a role in age-related diseases and aging.

As contended, if progeroid syndromes represent a phenotype of accelerated aging then changes in DNA over time likely play a role in aging. Since many genetic perturbations affecting DNA repair do not influence aging, it is doubtful that overall DNA repair is related to aging or that DNA damage accumulation alone drives aging. In conclusion, changes in DNA over time might play some kind a role in aging, but the essence of those changes and the exact mechanisms involved remain to be determined.

The brain is also at the receiving end of various age-related ailments. Short-term memory loss, personality and cognitive changes with age, dementia, general decline of the nervous system and senses, and many other changes are likely to occur with aging (Craik and Salthouse, 1992; Hayflick, 1994, Zec, 1995). Until recently, it was thought that neuronal loss, due to the accumulation of damage, such as oxidative damage, was the main cause of brain aging. Nowadays, it appears that neurons can remain relatively healthy through life, with the exception of pathologies (Morrison and Hof, 1997). In fact, until recently, neurons were not thought to be able to divide. Now it appears that neurons can replicate in adult monkeys, in an area of the brain called hippocampus, which is used for long-term memory (Gould *et al.*, 1999). Similar results have been reported in humans (Eriksson *et al.*, 1998). Altman and Das,

(1965), and Fernando Nottebohm (1989) reported brain rejuvenation in birds. Instead of seeing brain aging as a mere consequence of the death of neurons, it appears that, even without neuronal death, biochemical and structural changes compromise neuron function (Teter and Finch, 2004). With age, what changes is the wiring, the complex network of connections between cells (Gopnik *et al.*, 2000). It has even been suggested that brain aging is an extension of brain development (de Magalhaes and Sandberg, 2005), in line with a linkage between development and aging.

The Endocrine System as the Pacemaker of Aging

The idea that hormonal changes drive aging is an old one (Gosden, 1996). Since the levels of certain hormones decline with age, like growth hormone (Ho *et al.*, 1987) and its downstream target insulin-like growth factor I or IGF-1 (Hammerman, 1987), many old and current anti-aging products are based on the idea of restoring these levels. Caloric Restrictions (CR) has been associated with hormonal alterations in rodents, such as decreasing plasma levels of insulin (Masoro *et al.*, 1992) and IGF-1 (Breese *et al.*, 1991), and an increase in growth hormone secretory dynamics (Sonntag *et al.*, 1999) and decrease in glucocorticoid functions (Sharma & Dutta, 2006). Interestingly, several genes have been identified in model organisms whose effects appear to mimic CR. The best example is probably the urokinase-type plasminogen activator (Miskin and Masos, 1997). Overexpression of this gene in the brain of mice causes a decrease in appetite resulting in a 20% decrease in food consumption and body mass, and a 20% increase in longevity Other genes which result in a phenotype similar to CR in generally affecting body size, growth hormone and IGF-1, and body temperature have been reported and reviewed (Bartke *et al.*, 2001a). For instance, mice homozygous for *Pit1* have lower growth hormone and IGF-1 levels; they are dwarf, live about 40% longer, their maximum lifespan is

increased, and their aging process appears to be delayed (Flurkey *et al.*, 2001). Mice mutant for *Prop1*, a transcription factor that regulates *Pit1*, live 50% longer (Brown-Borg *et al.*, 1996). In contrast, mice overexpressing bovine growth hormone have been reported to age faster (Bartke, 2003). Studies combining CR and mutations of one of these genes--the *Prop1* gene, are reported to increase in longevity, suggesting that distinct mechanisms may be at work (Bartke *et al.*, 2001b). Recent reports suggest that although the growth hormone/IGF-1 pathway is involved in CR, other mechanisms might also operate (Shimokawa *et al.*, 2003). Whatever the mechanisms, CR appears to operate through a neuroendocrine signalling cascade of which the GH/IGF-1 axis is a pivotal, though probably not the only, component (Masoro, 2005). As stated these results link some aspects of energy metabolism to aging via the GH/IGF-1 axis (Berner and Stern, 2004).

Experiments in different model organisms associated the insulin/insulin-like pathways with aging (Lin *et al.,* 2000; Clancy *et al.*, 2001). Smaller mice, horses, and dogs appear to live longer and this could be related to lower levels of IGF-1 (Miller, 1999; Miller *et al.*, 2002a). Some studies suggest that insulin-like growth factor I may affect human longevity (Bonafe *et al.,* 2003). Human patients with a mutated *Prop1* gene have been reported to live slightly longer (Bartke *et al.*, 2001a), though the matter is a bit more complicated because patients with deficiencies in GH and IGF-1 show signs of early aging but their lifespan may actually be increased (Laron, 2005) and untreated patients with growth hormone deficiency have a reduced longevity (Besson *et al.*, 2003). Nonetheless, it is clear that neuroendocrine systems can impact on aging and possibly on human aging as well (Bartke, 2005; de Magalhaes, 2005a).One emerging player in aging is the *klotho* gene, whose product acts as a circulating hormone. Mutations in *klotho* appear to accelerate the aging process

(Kuro-o *et al.*, 1997). In contrast, overexpression of *klotho* extends lifespan by about 30%. Its functions are largely unknown but it could be related to insulin/IGF-1 signaling (Kurosu *et al.*, 2005).

The exact mechanisms of action of these hormones still remain unknown. It has been proposed that the GH/IGF-1 axis regulates antioxidants (Brown-Borg *et al.*, 2005). Another hypothesis is that since growth hormone and IGF-1 are mitogens, lower levels of the GH/IGF-1 axis decreases cellular replication that impacts on some sort of cellular clock (Sonntag *et al.*, 1999). Similarly, it is thought that the GH/IGF-1 axis impacts on cellular processes like apoptosis or stress resistance and perhaps, hormonal changes regulate aging as indirect consequences of the developmental program. Overall, the GH/IGF-1 axis and associated neuroendocrine mechanisms, some of which still unknown; appear to influence mammalian aging.

The insulin-like pathway has also been implicated to play a role in animals choosing the *dauer* pathway (Wolkow *et al.*, 2000; Lin *et al.*, 2001). As mentioned above, endocrine regulation appears to have an effect on aging, while indirectly affect growth and maturation. The way neuroendocrine systems limit longevity suggests a link between reproduction and lifespan (Mobbs, 2004). It has been postulated that some hormones like growth hormone and genes involved in insulin-like signaling regulate growth and development early in life and later contribute to aging (de Magalhaes and Church, 2005). Early studies showed that CR stunted growth and sexual development, though the extent of which depends on the severity of the CR used (McCay *et al.*, 1935). In addition, high nutrition has been reported to accelerate maturation and decrease lifespan in ground squirrels (Harvey and Zammuto, 1985).

Tatar *et al.* (2001), have showed that the *Drosophila melanogaster* gene for insulin-like receptor (*InR*), homologous to mammalian insulin receptors as well as to *Caenorhabditis elegans* daf-2, a signal transducer regulating worm *dauer* formation, produced adult females with upto 85% extension in longevity and dwarf males with reduced late age-specific mortality. Further, studies have shown that these genotypes when treated with a juvenile hormone analog restore life expectancy toward that of wild-type controls. Thus, juvenile hormone deficiency which results from *InR* pathway mutation sufficient to extend life-span, and those in flies, insulin-like ligands, non-autonomously mediate aging through retardation of growth or activation of specific endocrine tissue.

Levels of enzyme during development and aging

The complexity of life requires that many thousands of proteins be present in each living cell at any given moment with each performing a specific task during the course of development and/or aging. To ensure that only reactions beneficial to the organism occur to any significant extent, cells need to be able to produce and degrade selected proteins upon demand and the protein molecules themselves, in addition, need to possess the ability to be regulated by external stimuli such as hormones, so that their activities can be turned on or off when needed. It is a combination of the relative quantities of different proteins in the cell and their individual levels of activity that determines the cellular status and it may be noted that the changes in the levels and biological activities of proteins have the potential to affect cellular conditions and to feature both in aging and disease (Gafni, 2001). A notable feature in age-related

changes is that, the rate of both synthesis and degradation of proteins is age related (Rattan, 1996).

Changes in the metabolic functions during development often accompany morphological changes. For instance, an increase in the DNA and mRNA content in the heart and liver by steroid hormone induction resulted in mitochondrial biogenesis and proliferation, steps which are necessary for the morphological differentiation of the murine mastocyma cells in culture (Laeng *et al*, 1998; Mutvei *et al*, 1998; Di Meo *et al*, 1992).

Previous studies have shown that development and/or aging of an organism is accompanied by alterations in mRNA synthesis and protein activity e.g. the mRNA level and protein content of kidney cytochrome-c oxidase increases as a function of age (Prieur *et al.,* 1998), the neuron specific protein norbin in mouse brain increase with age (Mani *et al.,* 2001), total HMG proteins decreases in the old rats (Prasad & Thakur, 1990), HMG nonhistone protein 14 and 17 increases whereas 1 & 2 decreases as a function of age in the rat liver (Thakur & Prasad 1991). Several reports and reviews have also shown that the change in gene expression in aging is associated with instability of the genome (Thakur & Kanungo 1981; Thakur, 1983, 1984; Thakur, *et al.,* 1999). Chaturvedi & Kanungo (1985) have shown that the rat brain chromatin undergoes increasing condensation as a function of age, resulting in decreased transcriptional activity in old age. Some of these alterations are mediated by hormones, such as testosterone and estradiol (Thakur *et al.*, 2000; Kumar & Thakur, 2004).

Enzymes are specific proteins which functions as biocatalyst, agents that serves to minimize the energy barrier during the formation of products from reactants.

Enzymes are responsible for all the structures and functions (phenotypes) of an organism, and represent specific genotypes of the organism. The initiation, duration and termination of various phases in the life of an organism such as differentiation, development and maturation possibly depend on various characteristics of enzymes such as their levels and isozyme pattern. Changes in their properties during lifespan may alter the activities of the organism. In vivo, the activity of these enzymes is strictly regulated and their rate of synthesis as well as their final concentration is under genetic control (Markert & Moeller, 1959). Some of the enzymes are synthesized as an inactive precursor form and are activated at physiological milieu (Mihara *et al.*, 1998). Other enzymes are controlled by the covalent insertion of small chemical groups whereas others through feedback inhibition by the end products. The presence and/or concentration of substrates, cofactors, activators and inhibitors play an important role in controlling the reaction rate of an enzyme.

Since enzyme catalyze all functions of the body, an understanding of the various aspects of enzymes such as their levels, isozyme patterns, inducibility and molecular properties during the lifespan may throw some light on the aging process and since they along with other proteins are coded by genes, understanding them may give an insight into the type of changes that occur at the genetic level. The activity level of some enzymes is also known to change with age. From within a class of enzymes, the activities of some enzymes of old rats and mice are lower than those of adult animals, and some others are higher and some do not change at all. For instance, isocitrate dehydrogenase (ICDH), exist in three isoforms in rats. The activities of all the three forms of ICDH are lower in the brain and liver of old rats compared to those of adult rats (Yadav & Singh, 1980; 1981). Another general observation is that the number of faulty enzymes increases with age. To cite a few examples, the levels of

purified superoxide dismutase (SOD), glyceraldehydes-3P-dehydrogenase and aldolases B and C were much lower in old animals; however the active-enzyme fraction present in the old animals has the same specific activity as that of the young animals (Reznick *et al.*, 1985).

Enzyme induction

There are various ways by which enzyme activity is enhanced (Walker, 1983). Karstrom (1936) introduced the term *adaptive* enzymes to describe those enzymes which are induced in a microorganism only in the presence of specific substrate, and another term *constitutive* enzymes which is present in the organism irrespective of composition of the medium it grows upon. The difference between these two enzymes lies in the conditions leading to their synthesis. To accommodate the variation observed in higher organisms including mammals, two new terms were introduced; enzyme *induction,* defined as a relative increase in the rate of synthesis of a specific apoenzyme resulting from exposure to a specific substance (Cohn *et al,* 1953) and enzyme *repression* to describe the reverse (Vogel, 1957).

Most induction studies involved the used of steroid hormones which binds to specific receptors in the cytosol, and the hormone-receptor complex binds to specific *cis*-acting elements in the promoter region of target genes to stimulate their transcription and binding of hormone-receptor complex to DNA is mediated by tissue-specific transacting protein factors. For example, acetylcholine esterase (AChE) is known to be induced by 17β-estradiol in the cerebral hemisphere of immature and adult ovariectomized rats, but not in old rats. The lack of response in the old rat is attributed to a decrease in the level of the estradiol-binding protein in the brain of old rats (Kanungo, *et al.*, 1975). Several other studies have also shown that the induction

in the old organism is lower than that of the adult, vis-à-vis glucose-6-phosphatase, fructose-1, 6-bisphosphatase, phosphofructokinase and hexose-P-isomerase (Singhal,1967a, 1967b; Singhal *et al.,* 1969) and malate dehydrogenase (Kanungo & Gandhi, 1972) Acetylcholine esterase (Moudgil & Kanungo, 1973) and choline acetyl transferase (James & Kanungo, 1978). However, there are few enzymes whose induction is the same in both the ages; these include tryptophan pyrrolase (Gregerman, 1959) aspartate aminotransferase (Patniak & Kanungo, 1976; Sharma & Patnaik, 1982) and transacetylase (Ratha & Kanungo, 1977). Another important observation on induction of enzymes is that it varies from organ to organ. For example the induction of pyruvate kinase is higher in the heart of old rats, whereas in the brains of the same rats, it is lower than that of the adult (Chainy & Kanungo, 1976, 1978a, 1978b). The age-related induction of phosphoenolpyruvate kinase by hydrocortisone in the liver and brain of rats has also been reported (Sharma & Patnaik, 1983).

Hormones

Cellular signaling constitutes an important part of the information flow in the biological systems and has been conserved from micro-organisms to humans. These signals include a wide variety of molecules starting from amino acids and their derivatives to proteins on one hand and the steroids and other lipid derivatives on the other (Sharma, 1999). In higher organisms, these signals or *hormones* as they are called are produced by specialized cells or tissues to coordinate the metabolism of diverse tissues so that they function for the benefit of the whole organism. Some hormones, as reviewed by Walker (1983), e.g. progesterone, have very specific effects on a limited number of target tissues; whereas others, e.g. thyroxine and the

glucocorticoids, interact with many tissues in the body to produce physiological responses.

Glucocorticoids

Glucocorticoids are produced by adrenal corticices under the control of the brain via the pituitary, hypothalamus and the limbic system and exert wide ranging effects including glucose homeostasis, reducing inflammation, maintain muscle strength and fluid promotion. Glucocorticoids generally have anabolic effects on protein and RNA metabolism in liver and catabolic effects on other tissues such as muscles, lymphatic and adipose tissues (Sharma, 1991). They are also known to have an overall enhancement of gluconeogenesis in which there is accelerated conversion of tissue proteins and amino acids to carbohydrate. This results in elevation in blood sugar and liver glycogen levels and the ultimate excretion of the liberated protein nitrogen as urea (Long *et al.*, 1940). In the course of promoting these processes corticosteroid exert characteristic and frequently opposite metabolic alterations in the various organs of the mammalian organism. Thus glucocorticoid cause marked metabolic depression in lymphoid and adipose tissues (Dougherty & White, 1944; Reynold *et al.,* 1960) yet accelerate biochemical processes in the liver (Feigelson *et al.*, 1964, 1965). In the search for causal sequences of events relating the various facets of glucocorticoid action, attention was drawn to hormonal enzyme induction as an early action which may precede and be responsible for many metabolic phenomena observed in liver and other target tissues. Glucocorticoids have been shown to act directly on the liver within a few hours markedly elevating the rate of synthesis and thereby the level of activity of a few enzymes e.g. tryptophan pyrollase and tyrosine-α-ketoglutarate transaminase (Knox *et al.*, 1956; Rosen & Nichol, 1963; Knox *et al*,

1965, Feigelson *et al.*, 1963, Feigelson & Greengard, 1962; Schmike *et al.*, 1965, Kenny, 1962; Segal & Kim, 1963). Other studies have shown that, in the rat glucocorticoids are present 4 days before birth. The increase and decline after birth and the neonatal period is characterized by relatively low level of glucocorticoid until 12-14 days after birth, thereafter a second surge of glucocorticoid is released by the adrenals between day 12 and 16 (Cohen, 1973; Martin *et al.*, 1977).

Studies have shown that fetal dexamethasone infusion decreases fetal glutamate output (Timmerman *et al.*, 2000) and GDH activity is increased and altered to favour the forward reaction, i.e., the production of α-ketoglutarate and associated reductive units. This dexamethasone-induced change in GDH activity could, in the normal course of events, is ascribed to the increased need for TCA cycle intermediates for glycogen synthesis. In the fetal sheep, glucocorticoid infusions have been demonstrated to increase fetal liver glycogen concentrations (Eguchi *et al.*, 1992) presumably through glycogenic enzymes such as GDH and cytosolic phosphoenolpyruvate carboxykinase, changes that occur naturally as parturition approaches.

The role of glucocorticoid in the induction of GDH has been reported. Adrenalectomized rats showed decreased level of the enzyme GDH. This decrease in the level of the enzyme was restored to a normal level by a single injection of physiological doses of cortisol. Thus, it appears from these studies that the maintenance of normal level of GDH in adult animals is dependent on continued induction by glucocorticoid. Further, the inhibition of this response by actinomycin D and cycloheximide confirm the involvement of RNA and protein synthesis in this induction (Yaroni & Balinsky, 1984a, 1984b; McLean & Gurney, 1963).

Thyroid hormones

Thyroid hormones influence multiple physiological functions such as cell growth and differentiation, protein synthesis and basal metabolic rate. Their effects, especially on the mitochondrial metabolic activities are well documented (Tata *et al.*, 1963; Tata 1964, 1966; Satav *et al.,* 1973; Rajwade *et al.,* 1975; Katyare *et al.,* 1977; Nunez 1988). Hypothyroidism in general, results in decreased metabolic activities and the treatment of hypothyroid animals with physiologic doses of thyroid hormones restores these activities to an almost normal level (Tata *et al.,* 1963; Katyare *et al.*, 1970, 1977). Compared to other organs, the kidney is the most sensitive tissue to the thyroid status (Rajwade *et al.*, 1975; Satav and Katyare, 1982) and in addition thyroid hormone deficiency caused a non-synchronous turnover of kidney mitochondrial proteins (Rajwade *et al.*, 1975).

Thyroid hormone action is known to be tissue specific (Katyare *et al.,* 1977; Nikodem *et al.*, 1981; Satav & Katyare 1981, 1982). This tissue specific action of thyroid hormones has been explained partly on the basis of the number of nuclear binding sites present in the responsive tissues (Oppenheimer 1979, 1983). Although the mechanism of thyroid hormone action is not yet fully understood, there exist a good correlationship between the number of nuclear T3-receptors and thyromimetic action of hormone metabolites (Oppenheimer 1979, 1983; Muller *et al.,* 1984). It is believed that the early action of T_3 may be the regulation of synthesis of specific mRNA (Seeling *et al.*, 1982) whose translational product secondarily exerts effects on the DNA-dependent RNA polymerase, thus regulating the synthesis of specific mRNA species (Muller and Seitz 1984; Mutvei and Nelson 1989). The presence of thyroid hormone receptors or binding sites in mitochondria, plasma membranes and

cytosol has been reported (Oppenheimer 1983; Muller and Seitz, 1984; Nunez, 1988). Further, the presence of thyroid hormone receptor in mitochondria from various organs i.e. liver, kidney, lung, intestine heart, etc. has been well described (Oppenheimer, 1979; Hashizume & Ichikawa, 1982; Muller & Seitz, 1984; Nunez, 1988). Furthermore, in kidney mitochondria, it has been reported that there are four and two binding sites, for the outer and inner mitochondrial membranes, respectively (Hashizume & Ichikawa, 1982).

GDH was earlier shown to increase in activity during amphibian metamorphosis and following thyroxine treatment of tadpoles (De Groot & Cohen, 1962; Wiggert & Cohen, 1966). This increase can be correlated with the increased synthesis of urea during amphibian metamorphosis (Brown *et al.*, 1959). Although not strictly part of the urea cycle, GDH can be regarded as accessory to it (Cohen & Harvey, 1966) since it can fix free ammonia to form glutamate, the amino group of which may then be transferred to oxaloacetate by transamination to form aspartate. In turn aspartate as a substrate form arginosuccinate synthetase donates its amino group to give rise to one of the nitrogen group of urea.

In earlier studies, it was reported that the effect of thyroid hormones on GDH is rather a complex phenomena; with the normal adults showing no effect when administered with thyroid hormone (Lee & Lardy, 1965) however repeated treatment of neonates with thyroid hormones produced some increase in the level of GDH (Lamers & Mooren, 1981). It would appear therefore that the level of GDH in adult rats is not dependent on the induction by thyroid hormones. Studies in adult rats have shown that T_3 treatment increase the level of GDH, suggesting hormonal regulation of this gene (Yaroni & Balinsky, 1984b). It was also reported that the induction of GDH

activity by thyroid hormones is partly inhibited by blocking de *novo* protein synthesis, implicating T_3 is involved in altering gene transcription as well as specific enzyme characteristics (Yaroni & Balinsky, 1984a). Apart from stimulating enzyme activity, Wolff (1962) revealed that the thyroid hormones and other related compounds has the property of dissociating the GDH enzyme into subunits with sedimentation coefficient of ~14*S* from an original material of 26.4*S*. However, this effect can be reversed by ADP.

Gonadal hormones

The hormonal regulation of spermatogenesis has been extensively studied in mammals. The hypothalamic-pituitary-gonadal axis provides both positive and negative hormonal feedback necessary to ensure normal spermatogenesis. Gonadotropin Release hormone (GnRH) from the hypothalamus stimulates the release of Leutenizing (LH) and Follicle stimulating (FSH) hormone from the pituitary (Gharib *et al.*, 1990). LH in turn stimulates the synthesis of testosterone from cholesterol in the mitochondria of Leydig cells of the testis (Payne & O'Shaughnessy, 1996; Moyle & Armstrong, 1970).

Testosterone is an androgen that generates direct genomic actions as a result of binding to the androgen receptor (AR). The AR belongs to the family of nuclear receptor that acts as ligand-responsive transcription factors (Jenster *et al.*, 2000). In the testis, AR has been localized to Leydig cells, pertibular cells and Sertoli cells (Bremner *et al.*, 1994; Shan *et al.*, 1995). Testosterone freely diffuses through the plasma membrane and binds AR, forming a complex that subsequently interacts with the androgen response element (ARE) in the promoter region of targeted genes. The transcript of targeted genes can be either induced or repressed depending on the

factors that associate with the ligand receptor complex bound to the ARE (Luisi *et al.*, 1991; Beato, 1989). Many evidences have shown that the concentration of testosterone in the testes of the adult rat ranges from 25-100 ng/ml (Sharpe, 1994) with a further increase at puberty (Jean-Faucher, 1978), suggesting that higher intratesticular concentration of this hormone have been correlated to initiation of spermatogenesis in mammals. In adult mammals, testosterone is probably the most important factor required in the maintenance of normal qualitative spermatogenesis since its withdrawal leads to incomplete spermatogenesis and infertility. To demonstrate the importance of testosterone in spermatogenesis, various experimental protocols including ethane dimethane sulfonate treatments, administration of antiandrogens immunoneutralization of LH and hypophysectomy have been used to generate testosterone depletion in rat testes (Kelce & Zirkin, 1993; Russel *et al.*, 1981; Chandolia *et al.*, 1991; Dym & Raj, 1997). Most of the molecular targets for the action of testosterone is still unknown, however studies have shown the presence of a testosterone-regulated gene which is regulated by androgen in both testis and in the epididymis in the placenta and embryo (Maiti *et al.*, 1996; Barbulescu *et al.*, 2001).

On the other hand, evidence exists to suggest the role played by testosterone in mammalian testis. It was observed that within 24 h of treatment with testosterone propionate, there were more mRNAs with reduced than increased abundance in testis of hypogonadal mice suggesting that the primary role of testosterone is to repress gene expression (Patricia *et al.*, 2004).

Androgens have been shown to increase GDH activity in neonatal female rats in the hypothalamic region especially in the paraventricular, lateral ventromedial, arcuate, medial mamillary and posterior nuclei of the hypothalamus (Packman *et al.*,

1977). There is also a report that showed that the female estrogenic inhibits GDH activity by causing the dissociation of the enzyme into smaller molecular weight forms (Yielding & Tomkins, 1960). Estrogens have been reported to play an important role in brain aging. The effect of this hormone is affected through their intracellular receptors, estrogen receptor ERα and Erβ, whose expression is also regulated by growth hormone and thyroid hormone. The levels of these receptors decrease during aging which in turn influence estrogen signaling leading to alterations in mouse brain functions (Thakur & Sharma, 2006). Testosterone has also been shown to have similar impact on mice, but in an age- and tissue-specific manner (Thakur *et al.*, 2000) besides it has also been reported to down regulate its own receptor mRNA in the brain of adult mouse (Kumar & Thakur, 2004). The effect of age on physico-chemical properties of the uterine nuclear estrogen receptors of albino rats have also been reported (Kaur & Thakur, 1991).

Hormone receptors

Glucocorticoid and Thyroid hormones receptor

Glucocorticoid receptors are a group of *trans*-acting factors present predominantly in the cytosolic fraction of many cell types. The untransformed native glucocorticoid receptor is found in the cytosol as a 9-10s complex of about 320kDa (Vedekis, 1983; Holbrook *et al.,* 1983). This complex is a hetero-oligomer that contains a single molecule of the steroid binding protein and two molecule of a non-steroid binding protein; the 90 kDa heat shock protein (hsp 90). Activation/transformation of glucocorticoid–receptor complex is one of the initial events that occur after binding of the hormone to the specific cytosolic receptor. Upon interaction with hormone, they translocate into the nucleus and interact with specific

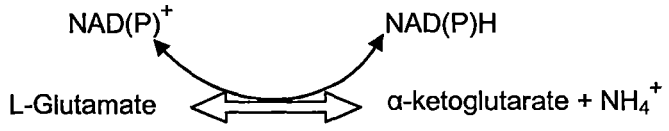
DNA sequences; the glucocorticoid response elements located generally 100-200bp upstream of the regulated gene(s). Binding of cognate hormone receptor complexes to GREs modulates the expression of gene which in turn influences the metabolic response (Beato, 1989). High affinity glucocorticoid binding is detected in fetal rat liver six days before birth. With the onset of birth there is a marked increase in the concentration of glucocorticoid receptors (Feldman, 1974). Hepatocytes isolated from fetus 3-4 days before birth are reported to be stimulated to synthesize tyrosine amino transferase (TAT) when incubated with glucocorticoid (Cake *et al.*, 1981). TAT is normally synthesized just before birth. These findings along with the interpretations have been quite extensively reviewed (Walker, 1983). The changes in the level of glucocorticoid receptors as well as in the physicochemical properties have been well documented (Sharma, 1988). Glucocorticoid effect on various processes depends on the level as well as physicochemical properties of its receptor. Age-dependent changes in the inducibility of enzymes by glucocorticoid have been reported to be influence by the level of receptors and also by the post-events (Sharma and Timiras, 1986; Sharma, 1993).

Thyroid hormones receptors, like steroids receptors are intracellularly located. Activated ligand-receptor complex binds to specific elements in the DNA, the thyroid hormones response elements (TREs) and modulate gene expression which results in biological response. The presence of nuclear receptors for thyroid hormones has been well documented (Oppenheimer, 1979). Further thyroid hormone receptors or binding sites have been reported to be present in the plasma membranes, cytosol and mitochondria of various organs namely the liver, kidney, intestine, heart, lung etc. (Oppenheimer, 1979; Hashizume & Ichikawa, 1982; Nunez, 1988).

Interest has also been drawn towards the hormone action mechanism and cross-talk in signal transduction. It has been reported and reviewed that the hormone action on target cells of protein/peptide and steroid hormones may be interconnected and influenced by each other, whereby hormones or metabolite(s) in the hormone-action cascade exert either synergistic or antagonistic effects (Sharma, 1993). Thus diacylglycerol (DAG) an activator of protein kinase C, have been shown to enhance the induction of tyrosine aminotransferase (TAT) and ornithine decarboxylase (ODC) by dexamethasone, but showed no such effect on its own (Kido, *et al.*,1986), suggesting that an interrelationship between the steroid hormone receptor-mediated signal transduction system and the membrane-hormone receptor cascade (Sharma, 1991).

Glutamate dehydrogenase

L-Glutamate dehydrogenase (GDH; EC 1.4.1.3) is a pyridine nucleotide-dependent enzyme which catalyses the reversible oxidative deamination of L-glutamate to α-ketoglutarate and ammonia.



GDH is a ubiquitous enzyme present in practically all organisms. In plants GDH catalyzes the reversible reductive amination of α-ketoglutarate to form glutamate in the presence of the co-factor NAD(P)H. The isoforms have different intracellular locations and co-factor affinities, but share similar pH optima, relative molecular masses, and specificities for α-ketoglutarate and glutamate. Mckenzie and Lees (1981) demonstrated that GDH activity from root extract was associated with the

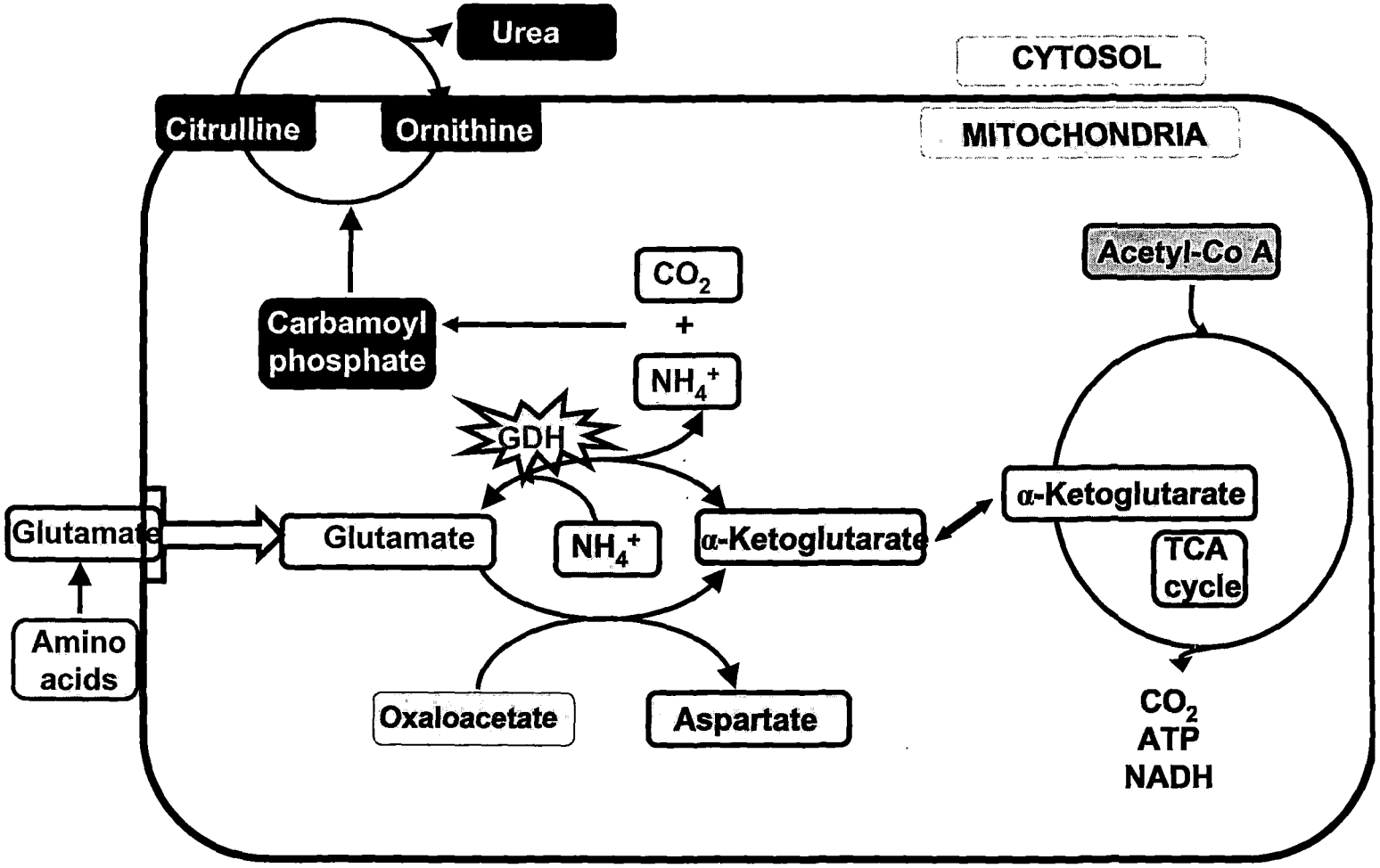


Figure: The role of glutamate dehydrogenase in the assimilation of nitrogen in the mitochondria

mitochondria and that the mitochondria GDH co-migrated in a native polyacrylamide gel with GDH3 isolated from developing seeds, thus suggesting that the seed GDH 3 was associated with mitochondria. Other researchers have also demonstrated mitochondrial GDH activities in soybean cotyledons (King & Yung-Fan Wu, 1971). McKenzie and Lees (1981) demonstrated that GDH2 was associated with the chloroplast while Shailendra and Shargool (1991) have reported the existence of plastid GDH in soybean. The number and relative proportions of the different GDH isoforms have been reported to vary in plant tissues, depending on developmental and environmental conditions (Turano *et al.,* 1996).

This enzyme is widely distributed in mammals where it is located in the mitochondrial matrix and microsomes and is abundant in the liver, heart and the kidneys (Kawajiri *et al.,* 1977). The mammalian forms of GDH constituted a more or less discrete class in sharing the ability to use either NAD(H) or NADP(H) (transhydrogenase) as co-enzymes. GDH occupies a central position in mammalian nitrogen metabolism since the reaction which it catalyze provides the major pathway by which ammonia become bound to the α-carbon atom of an α-ketoacid to generate glutamate (Harvey, 1985).

The reaction catalyzed by GDH can be used clinically in determination of ammonia and, products which can be converted to ammonia in body fluids such as blood urea nitrogen (BUN) determination and also in the assay of sGOT and sGPT. GDH is also used as a reagent in staining mixtures to detect the isozyme of enzymes, which catalyzed the production of ammonia (Nelson *et al.,* 1997). The enzyme plays a significant role in deamination of amino acids especially in those organisms that excrete ammonia. Other amino acids can be formed only by subsequent transamination

reactions (Harvey, 1984; Harvey *et al.,* 1985), further this enzyme is known to couple Krebs' cycle intermediates with amino acid catabolism. It supplies ammonia for urea formation and α-ketoglutarate for citric cycle activity. Whenever a hepatocyte needs fuel for the citric acid cycle, GDH activity increases making α-ketoglutarate available for the citric acid cycle and releasing NH_4^+ for excretion (Lehninger *et al*, 1993). However, in the mitochondria of liver tumor, GDH deaminates glutamate to supply ATP. Though not part of the urea cycle, GDH is regarded as accessory to it since it can fix free ammonia to form glutamate, the amino group of which can be transferred to oxaloacetate by transamination to form aspartate. Aspartate in turn, as substrate for arginosuccinate synthetase donates its amino group to give rise to one of the nitrogen groups of urea (Balinsky *et al.*, 1970).

The mammalian enzyme is a homohexamer, consisting of six identical subunits and is strongly influenced by the positive modulator ADP and the negative modulator GTP. GDH from beef liver has a molecular weight of 280 kilodaltons and is synthesized as precursor larger than its mature counterpart by 6 kilo Daltons. The intracellular site of synthesis is known to be on the free polysomes (Katsuyoshi *et al.*, 1982). GDH is amongst the highly stable of enzymes (Bird *et al.,* 1997) and is physiologically active up to 12 hours after death in humans (Bhargava and Telang, 1986) with a half-life of about 5.5 days (Kawajiri *et al.*, 1977).

Studies have confirmed that two GDH isoenzymes of distinct genetic origin are expressed in human tissues (Mavrothalassitis *et al.,* 1988; Shashidharan *et al.*, 1994). The first encoded by *GLUD1* gene (Michaelides *et al.*, 1993), is expressed widely and is associated with housekeeping activities, whereas, the second, encoded by the *GLUD 2* gene, is specific for neural and testicular tissues. Further studies on

recombinant GLUD1 and GLUD2-derived GDH revealed that the nerve tissue specific isoenzyme is the heat labile form of human GDH (Shashidharan *et al.*, 1997). It has also been reported that the presence of four differently sized mRNAs and multiple gene copies for GDH occur in the human brain (Plaitakis *et al.,* 1993) and a novel cDNA encoded by an X-chromosome linked intronless gene has been isolated from human retina (Shashidharan *et al.,* 1994; 1997). Colon *et al* (1986) showed that rat brain contains two distinct GDH activities, and similar observations have been made recently for bovine brain (Cho, *et al.*, 1995). In addition, human brain contains four GDH isoproteins differing in molecular mass and isoelectric point (Hussain *et al.,* 1989). Immunocytochemical studies on rat brain revealed that GDH is expressed predominantly in astrocytes (Aoki *et al*, 1987; Wenthold *et al.*, 1987; Rothe *et al*,. 1990, 1994). Further, it has been observed that there is a considerable regional variation in GDH immunoreactivity in rat brain, with the enzyme being particularly enriched in regions that received dense glutaminergic innervation (Aoki *et al.,* 1987). These reports provided indirect evidence that GDH is involved in the metabolism of transmitted glutamate as suggested by Plaitakis *et al* (1982). GDH isozyme has also been reported in the nucleus of beef liver and differs by about 10% in amino acid sequence from that of mitochondrial GDH (McDaniel, 1995).

In mammals, the activity of GDH is primarily regulated by guanosine triphosphate (GTP) through allosteric inhibition. Bicarbonate at physiological levels is also known to increase GDH activity in rat liver mitochondria (Wanders *et al.,* 1983). Stanley *et al.,* (1998), reported that mutation in the GDH gene at exon 12 within the GTP-binding site, i.e. at positions corresponding to amino acids between Ser-445 and His-454 leads to a loss of the GTP-binding site. Enzymatic characterization of the mutant GDH revealed normal basal activity accompanied by diminished allosteric

inhibition by GTP. This loss of GTP inhibition of the enzyme leads to *Hyperinsulinism-hyperammonemia* syndrome (HHS), a condition characterized by elevated levels of insulin and ammonia. The suggested mechanism of HHS is that this activating mutation of the GDH gene resulted in elevated intracellular level of α-ketoglutarate and ammonia accompanied by a relative decrease in the level of N-acetylglutamate. On the one hand, elevated intracellular α-ketoglutarate leads to an increase of the ATP levels within pancreatic β-cells and thus to closure of the K-ATP channels resulting to secretion of insulin. On the other hand, a reduction in intracellular N-acetylglutamate could lead to a reduced activity of the urea cycle enzyme; carbamylphosphate synthetase, which then causes an accumulation of ammonia, leading to *ammonemia*. Similar findings have been reported on HHS, with mutation occurring outside the GTP-binding site, but nonetheless the enzyme exhibited elevated activity with a normal response to the allosteric modulators ADP and GTP (Cho *et al.*, 1996; Yorifuji *et al.*, 1999).

Glutamate

Glutamate, the main substrate in oxidative deamination reaction catalyzed by GDH, is an acidic amino acid that plays a central role in the catabolism of ammonia and amino groups in amino acid oxidation. Glutamate also forms the source of amino groups for most other amino acids, through transamination reactions. Glutamate is one of the most abundant free amino acids in the central nervous system and is thought to serve as a major excitatory neurotransmitter (Mukhin *et al.*, 1996). In the cerebellum, glutamate is thought to be the transmitter in at least granule cells, at the synapse between parallel fibers and Purkinje cells (Hudson *et al.*, 1976; Ito, 1984). L-glutamate has also been implicated in neurodegenerative changes following insults

such as hypoglycemia (Wieloch, 1985) or ischemia (Rothman, 1984) and is also implicated in the regulation of NF-κB (Guerrini *et al.,* 1995) an important nuclear transcription factor implicated with neurodegenerative diseases including Alzhiemer (Ferrer *et al.,* 1998), Parkinson's (Hunoi *et al.,* 1997), cancer (Kim *et al.,* 2006) and is also associated with a variety of aggressive tumor types (Jackson-Bernistas *et al.,* 2007). When applied in vitro onto cerebellar slices, glutamate or its analogs induce neuronal cell death (Garthwaite & Garthwaite, 1986; Hajos *et al.,* 1986). Therefore, Duvoisin and Plaitakis (1984) postulated that GDH may be necessary to protect areas having active glutamate transmission against the cytotoxicity of this amino acid.

Furthermore, it has been showed that parentally administered mono sodium glutamate produced an acute degenerative lesion in the inner retina of normal neonatal mice and subcutaneous injections of monosodium glutamate induce acute neuronal necrosis in several regions of developing brain in newborn mice (Olney, 1971). Although glutamate does not readily cross the brain barrier (Bradbury, 1979), doses of monosodium glutamate in the range of those sometimes fed unwittingly to human young as a food additive destroy central nervous system neurons when administered orally to immature animals (Olney, 1984). Most glutamate degrading enzymes have much lower affinities for glutamate, but a higher capacity for glutamate elimination. The known glutamate degrading enzymes such as GS, GDH and glutamate pyruvate transaminase have already been shown to have neuroprotective value in models of glutamate excitotoxicity (Matthews *et al.,* 2000). Further, it has been shown that prolong exposures to monosodium glutamate may be one of the factors involved in the control of GDH expression in brain (Yoon *et al.,* 2002).

The oxidative deamination reaction catalyzed by GDH is the major route for the entrance of glutamate carbon skeleton (α-ketoglutarate) into the TCA cycle (Sonnewald *et al.,* 1997). Glutamate is known to enhance the binding of reduced coenzyme (Bell *et al.,* 1984).

With this background in consideration, the role and involvement of glutamate dehydrogenase during development is included in the study. The study is divided under the following sections.

1. Endogenous level of the enzyme GDH in different tissues of mice at the various postnatal ages: (day 1, 10, 30, 60 and 90) in order to find out the changes if any, in the activity level of this enzyme in the various tissues namely the heart, brain kidney and liver at different postnatal ages which may predict age or tissue-specific involvement of the enzyme in the course of development of mice.

2. Effects of hormones such as glucocorticoid, thyroid, and testosterone and their combinations on the activity of GDH in the various tissues of mice at two age groups 10-and 90-day old mice.

3. Purification and physico-chemical characterization of liver GDH from the two age groups 10- and 90-day old mice to determine if any, the alteration in the physico-chemical and kinetic properties of this enzyme as the animal ages.

CHAPTER - II

MATERIALS

Biochemicals and reagents

α-ketoglutarate, nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide phosphate, reduced (NADPH), sucrose, ammonium acetate, ethylenediaminetetraacetic acid-disodium salt (EDTA), adenine diphosphate-disodium salt (ADP), guanosine triphosphate (GTP), sodium azide, guanidine hydrochloride (Gdn.HCl) and immidazole were obtained from Hi-Media. Acrylamide, bis-acrylamide, ammonium persulphate, N,N,N'N' tetramethylethylene diamine (TEMED) and glycine were obtained from Sisco Research Laboratories, Mumbai. Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FICA), goat anti-rabbit IgG-ALP conjugated and ALP substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) were purchased from Genei, Bangalore.

Bovine glutamic acid dehydrogenase, tris(hyhdroxymethyl)aminomethane (tris buffer), testosterone, dexamethasone, triiodothyronine (T_3), phenylmethyl sulphonyl fluoride (PMSF), Sepharose 6B, Sephadex G-50, diethylaminoethyl (DEAE)-cellulose, 2-mercaptoethanol, Dithiothreitol (DTT) were obtained from Sigma Chemicals, USA. Trypsin TPCK was obtained from Cooper Biomedical, USA.

Instrumentation

Cary 50 Bio UV-visible Spectrophotometer (Varian, USA), cooling centrifuge (Sigma 3K USA), homogenizer, cyclomixer, magnetic stirrer (Remi

motors, India), lyophilyzer (Heto lyolab, Switzerland) and ultra freezer (Heto, Switzerland), pH meter (Cyberscan 510), dialysis tubing (Sigma, USA), electrophoresis unit, (Bangalore Genei, India), peristaltic pump (Pharmacia, Sweden).

pH measurements

A CyberScan digital pH meter model 510 was routinely used for all pH measurements at room temperature and calibration was done using standard buffer tablets of different pH.

Homogenization

Homogenization of tissues were carried out using a Remi motor type RQ-127 A, HP 8, rpm 8000 (1.1 Amps, 220/230 V) homogenizer fitted with teflon pestle. Borosil glass homogenizing tubes (5-50 ml) were used for the purpose.

Centrifugation

All centrifugations were carried out either in Sigma refrigerated centrifuge model 3K30 or Beckman J2 21 refrigerated centrifuge, at 4° C.

Absorbance measurements

A Varian model Cary 50 Bio UV-visible spectrophotometer was used for all absorbance measurements in the UV and visible region using glass and quartz cuvettes of 1 cm path length, respectively.

Lyophylizer

Sample lyophilizations were carried out in a Heto lyophilizer model Lyolab 3000 at -57° C.

Miscellaneous

Sartorius balance model 2434 (0.01-160 mg), homogenizer, cyclomixer, magnetic stirrer (Remi motors, India), syringes (Dispovan, India), Whatman filter paper (India), dialysis tubing (Sigma, USA), electrophoresis unit, (Bangalore Genei, India). Mini trans-blot electrophoretic transfer cell (Biorad, U.S.A), Chromatography columns (Pharmacia, Sweden) and programmable fraction collector (Waters, USA).

Animals

Male Swiss albino, Balb C strain mice purchased from Pasteur Institute, Shillong. They were housed in laboratory animal room with temperature maintained at 25°C. The mice were fed with mice pellets containing the usual supplements of carbohydrates, proteins, minerals and antibiotics and water was given *ad libitum.*

METHODS

Preparation of tissue extract

Male Swiss-albino mice of the various postnatal ages were killed by cervical dislocation and the organs were quickly removed and washed in 0.09% saline solution. A 20% (w/v) homogenate of the minced tissue in 25 mM Sucrose tris-HCl, pH 7.4 (isolation medium; IM), was prepared by homogenizing in a borosil homogenizing tube with a motor driven pestle in cold. The homogenates were then centrifuged at 800-1000 x g for 15 min at 4°C. The supernatant obtained was further fractionated twice at 14,000 x g for 20 min at 2°C. The mitochondrial pellets obtained were then suspended in a 25 mM Tris-HCl, pH 7.4 containing Triton X-100 (0.05% final concentration) and used for enzyme assay.

Enzyme Assay

GDH activity was assayed according to the procedure of Wrzeszyzynski & Colman (1994) with slight modifications. The change in absorbance per minute (ΔE) was monitored spectrophotometically in the direction of reductive amination of α-ketoglutarate in a medium containing the following final concentrations; 84.7 mM immidazole buffer (2.5 ml) pH 7.9, 217 mM ammonium acetate (50 µl), 0.12 mM NADH (30 µl), 0.9 mM EDTA (100 µl), and 1.7 mM ADP (50 µl). 20 µl of enzyme extract was added to the reaction mixture and the reaction was initiated by the addition of 200 µl of α-ketoglutarate to a final concentration of 13.6 mM. A unit of GDH is defined as the amount required to oxidise 1µmol of NADH/min at 25°C.

Protein estimation

Protein concentrations were 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 reagent A and B and the resulting solution was stored in amber bottle at 4°C for future use.

Working reagent was prepared fresh by diluting the Bradford reagent to 15% in Millipore distilled water and the solution was 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 and 8.5% (w/v) phosphoric acid.

Protein samples were appropriately diluted to 1.0 ml with distilled water to which 5 ml of Bradford working solution was added and mixed thoroughly with the help of a cyclomixer and incubated at room temperature for 10 min. The intensity of the colour was measured at the wavelength of 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 10-100 μg.

Hormonal studies

The effects of triiodothyronine (T_3), dexamethasone and testosterone on the enzyme activity of both the age groups (10- and 90-day) of mice were investigated.

Mice were injected according to the procedure of Katyare *et al., (*1994) and Prieur *et al.,* (1998) with slight modifications. Three sets in duplicate, containing 4-5 animals per set were used. One set was used as normal without any injections given, the second set was injected with saline (0.09%) or solvents used for suspending the hormones. The third set was administered with the respective hormone or hormone combinations. Care was also taken to avoid fluctuations which may result due to avoid clashes with the cicardian rhythms by injecting animals only at fixed time of the day (09.00 h).

Single hormone treatment

Triiodothyronine (T_3)

T_3 (0.5 μg/g bw) suspended in 0.1 ml of normal saline containing 0.1mM NaOH was injected intraperitoneally to mice of 10-and 90- day old. The dose was repeated after 24 h and the animals were sacrificed 6 hr later. The controls were injected with 0.1ml of normal saline containing 0.1 mM NaOH. The tissues were subsequently removed, washed and stored at -20°C for future enzyme assay.

Dexamethasone

Dexamethasone in a final dose of 10 μg/g bw dissolved in 0.1 ml of saline solution containing 10% ethanol was administered (i.p.) in the same way as described for T_3. The controls were injected with 0.1ml of normal saline containing 10% ethanol. The animals were sacrificed 6 hr after second injection. The tissues were removed and stored at -20°C, and later assayed.

Testosterone

Testosterone (10 μg/g bw) suspended in 0.1 ml sesame oil was injected subcutaneously to mice of 10- and 90-day. Other conditions are the same as for other hormones. The controls were injected with 0.1ml of sesame oil. They were then sacrificed 6 hr later and tissues similarly removed and stored at -20°C for enzyme assay.

Combined Hormone treatment

A set of two hormone combinations namely, dexamethasone and T_3 mixture and testosterone and T_3 mixture were used to see if they act in synergistic and/or antagonistic manner to influence GDH activity, for both ages of male mice (10- and 90-day) used for the study.

Dexamethasone and T_3 mixture

Dexamethasone and T_3 mixture containing an equivalent of 10 μg and 0.5 μg per gram body weight body weight, respectively were administered to the test animals intraperitoneally in a final volume of 0.1 ml suspension mediums. Other conditions remained the same as for single treatment. Controls were administered only with the respective suspension medium. The animals were sacrificed 6 h after the second injection. The different tissues were removed, washed, blotted dry and stored at -20 °C and later processed for GDH activity.

Testosterone and T_3 mixtures

Testosterone and T_3 mixture containing an equivalent of 10 μg and 0.5 μg per gram body weight, respectively were administered to the test animals intraperitoneally

in a final volume of 0.1 ml suspension mediums. Other conditions remained the same as for single treatment. Tissues were removed and stored at -20°C.

Isolation and purification of mitochondrial GDH

Mitochondrial glutamate dehydrogenase was purified from immature (10-day) and matured (90-day) mice with modification of the method used by Minambres *et al.*, (2000). All the steps in purification were carried out in a cold chamber which was maintained at 2-4°C. Minced mice liver (30g) of both the ages were separately homogenized in ten volumes (w/v) of ice cold 25 mM sucrose-Tris-HCl pH 7.4, using a borosil glass tube immersed in ice, and a Teflon pestle. The homogenates were centrifuge at 800-1000 x g for 10min, at 4° C. The resultant supernatants were further centrifuged at 14,000 x g for 20 min with the temperature maintained at 4°C. The pellet containing both the heavy and light mitochondria was washed with the isolation buffer and re-centrifuged at 14,000 x g for 20 min at 4° C. The pellet obtained was dissolved in 10 ml each of the isolation medium and then subjected to sonication of 10 sec pulse at <40% amplitude in ice with regular spacing of 30 sec. The sonicate was then subjected to centrifugation at 20,000 x g for 20 min at 4° C. The clear supernatant obtained was stored at -20° C for subsequent uses.

Ammonium sulphate precipitation

The supernatant obtained after centrifugation was subjected to ammonium sulphate fractionation. The protein fraction, precipitated between 30-60% ammonium sulphate saturation was collected after centrifugation at 14,000 x g for 30 min at 4 ° C and dissolved in minimum amount of chilled isolation medium (IM; containing 25 mM sucrose-tris-HCl buffer, pH 7.4 containing 1 mM 2-mercaptoethanol, 1mM

phenyl methyl sulfonyl fluoride; PMSF). This preparation was then used in the subsequent purification steps.

Desalting

A sephadex G-50 column (100 x 2.6 cm) was set up according to Pharmacia prescribed bulletin and the preparation obtained after ammonium sulphate was then loaded onto this column desaltation. The protein fractions obtained were monitored spectrophotometrically at wavelength of 280 nm and then concentrated by lyophilization.

Sepharose 6B gel chromatography

The concentrated desalted protein sample was then loaded onto a Sepharose 6B column (90 x 1.6 cm) equilibrated with 25 mM sucrose Tris-HCl, pH 7.4 containing 1 mM 2-mercaptoethanol and 1mM PMSF. The sample volume was kept below the prescribed 5% of total bed volume for optimal separation. Fractions of 5 ml per tube were collected at a flow rate of 20 ml/hr, using automatic programmable Waters fraction collector. The sample was eluted, and the active sample fractions collected were pooled, lyophilized and used for further purification.

DEAE-cellulose ion exchange chromatography

Prior to use, DEAE-cellulose was activated by equilibriating with 5x its volume of 0.1 N HCl. The acid was drained and followed by washing with 0.1 N NaOH solution. The charged gel was then loaded onto the column (1.6 x 14 cm) equilibrated with 25 mM sucrose tris-HCl, pH 7.4 at a flow rate of 20 ml/hr. Active fractions collected from Sepharose 6B column chromatography was loaded onto this

column. On application of the sample, the column was washed twice with 25 mM sucrose tris-HCl buffer, pH 7.4. containing 1 mM 2-mercaptoethanol and 1mM PMSF, approximately up to 5 column volumes. Subsequently, a linear gradient of 200 ml of the eluent and 200 ml of the same buffer but containing 0.5 M NaCl was applied with the help of a gradient mixer to elute the bound fractions. Active fractions were collected, pooled and concentrated by lyophilization and used for further purification.

Determination of native molecular weight

Active fractions collected from DEAE-cellulose ion exchange chromatography were further reloaded onto the Sepharose 6B chromatography column which was equilibrated with the isolation medium and calibrated with molecular markers that includes phosphorylase b hexamer (584.4 kDa), phosphorylase b pentamer (487 kDa) β-amylase (200 kDa) and alcohol dehydrogenase (150 kDa). The void and total volume was determined by passing blue dextran and potassium ferricyanide respectively. The distribution coefficient (K_d) is derived through the following relation.

$K_d = (V_e - V_o)/V_i$

$K_{av} = (V_e - V_o)/(V_t - V_o)$

Where, V_t (total volume) = $\pi r^2 l$

r and l, being the radius and length of the column respectively;

V_i is the internal volume of the gel and is given by

$V_i = V_p - V_o$

Electrophoretic mobility studies using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE.

SDS-PAGE (7.5%) was performed according to the procedure of Laemmli (1970) with the following composition of reagents.

1. Acrylamide stock:

   Consist of a monomer acrylamide (30%) and a cross-linking agent N, N' methylene bis acrylamide (0.8%) solution.

2. Electrode buffer, pH 8.3

   Prepared by taking 25 mM tris-buffer and 192mM glycine and 0.1% SDS in a total volume of 1 litre distilled water

3. Staining solution:

   Prepared by taking 0.2% coomassie brilliant blue (R-250) in methanol, acetic acid and water in the ratio of 4:1:5

4. Sample buffer:

   Comprises of 10 mM tris-HCl buffer, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol and 20% glycerol and 0.006% bromophenol blue

5. Destaining solution:

   This solution contains methanol, acetic acid and water in the ratio of 4:1:5

6. Stacking gel buffer:

   Contains 1.5 M tris-HCl buffer, pH 6.8

7. 10% SDS aqueous solution

8. N, N, N'N'-tetramethylenediamine (TEMED)

Gel casting

7.5% gels were prepared by taking a mixture of 7.5ml of acrylamide stock, 3.75 ml of resolving gel buffer, 17.25 ml of water, 0.3 ml of 10% SDS, 15 µl of TEMED in a conical flask, stirred, and degassed for 15 minutes. 15 µl of freshly prepared 10% APS was then added and the solution was then casted into a slab gel apparatus and allowed to polymerize.

Sample containing approximately 50-75 µg of protein was boiled in equal volume of sample buffer in presence or absence of 2-mercaptoethanol for 2 minutes and applied in a total volume of 50 µl per well. Along with the sample, molecular weight markers which consist of BSA (66 kDa), bovine GDH (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and cyt C (12.4 kDa) were loaded into the wells. Samples were allowed to run for approximately 2hrs at a fixed potential of 100 volts.

The relative mobilities (Rm) of the samples were determining according to the method as described by Laemmli (1970) using the expression:

$$R_m = \frac{\text{Distance traversed by the sample (cm)}}{\text{Distance traversed by the dye (cm)}}$$

Native polyacrylamide gel electrophoresis (PAGE)

Native polyacrylamide gel electrophoresis (PAGE) composition was similar as that for SDS-PAGE except for the omission of sodium dodecyl sulphate and the reducing agent 2-mercaptoethanol, the rest of the conditions remain as described for SDS-PAGE.

Physico-chemical characterization of GDH

Physico-chemical characterization of the enzyme was carried out using dialysed sample preparations and or the purified enzyme. Crude GDH was made free of endogenous substrate and metal ions, which might otherwise interfere during analysis, by dialyzing against the isolation medium containing 1 mM 2-mercaptoethanol and 1mM PMSF. Dialysed sample was then used to assess the effects of various effects of substrates, metal ions and other inhibitors.

The mitochondrial extract from the liver of both the ages (10- and 90-day) were dialysed in the cold (4° C) against 25 mM sucrose tris-HCl buffer, pH 7.4 containing 1 mM 2-mercaptoethanol and 1 mM phenylmethyl sulphonyl fluoride (PMSF). Subsequently, suitably diluted enzyme preparations were used for kinetic analysis.

Determination of optimum ionic strength

Varying ionic strength (10-200 mM) of immidazole buffer was used for determining the optimal buffer ionic strength for GDH from the liver 10- and 90-day old. The other conditions of assay remained as described under method section.

Determination of pH optima

The pH optima was determined using 25 mM sucrose tris HCl and or immidazole buffer pH (7.0-9.0) with buffering capacities in the alkaline range of same ionic strength and sodium acetate buffer of the same ionic strength, covering the lower pH range. The other conditions of assay remained as described earlier. The pH optima for GDH of the two age groups were determined.

Inhibition Studies

Various concentrations of GTP (0-50 μM) were incubated with the enzyme in the presence and or absence of ADP for one hour in the cold. This was followed by normal assay of the preparations to assess the degree of inhibition of GDH by this compound. The result is expressed in per cent activity.

Effects of reducing agents

The enzyme preparations from the two age groups (10- and 90-day) were separately incubated in assay buffer containing varying concentrations of DTT (0-10 mM) and 2-mercaptoethanol (0-10 mM) for one hour in the cold followed by normal enzyme assay. The results are expressed in per cent activity.

Inactivation studies

Using various concentrations (0-5M) of guanidine hydrochloride prepared in assay buffer, the enzyme preparations from the two age groups (10- and 90-day) were incubated in separated test tubes for one hour in the cold followed by normal enzyme assay. The results are expressed in terms of percentage activity.

Determination of the K_m and V_{max} and K_{cat} the effects of other co-substrates on the activity of the enzyme

Varying concentrations of α-ketoglutarate (0.1-6.5 mM), were use to determine the kinetic characteristics of purified liver GDH from the two ages. The effects of other co-enzymes NADH (25-150 μM), NADPH (5-150 μM) and the allosteric modulator ADP (0.5-2.0 mM) on the activity of the enzyme from the two

ages was also determined. In each case only one parameter is varied, other conditions being as in normal assay.

Immunological studies

Polyclonal antibodies production

Polyclonal antibodies were raised against purified liver GDH from either age (10- or 90 day) in young healthy rabbit. For the priming immunization the lyophilized antigen, purified liver GDH was dissolved in phosphate buffered saline (PBS), pH 7.4 at a concentration of 2 mg ml^{-1}. An emulsion of the antigen was prepared with equal volume of Freund's complete adjuvant (FCA) by homogeneous mixing. The resulting thick, stable emulsion of the antigen and FCA in aliquots of 500 µl was administered subcutaneously at multiple sites of a shaved and sterilized area of the rabbit. Booster dose was administered 4 weeks later with a lesser amount (1mg ml^{-1}) of the antigen emulsified in Freund's incomplete adjuvant.

Bleeding

Blood was collected in a clean glass tube washed with 0.9% normal saline from the lateral ear veins by making gentle diagonal incision, 7 days after the first booster. Repeated bleeding was done on the 7^{th} day after every booster immunisation.

The blood containing tube was allowed to stand in a slightly inclined position overnight at room temperature for clotting to occur. The serum was then pipetted out into a clean centrifuged tube and centrifuged at 10,000 x g for 10 min at 4°C to remove any remaining traces of debris.

Ouchterlony immunodiffusion

1% agarose gel in 0.9% saline solution containing 0.02% sodium azide was gently heated to a boil. About 5 ml of the molten gel was spread on clean glass slides and allowed to solidify at room temperature. Three gelation wells were punched less than a cm apart from each other. The central well was loaded with rabbit serum containing the desired anti-mouse GDH IgG (polyclonal) and the outer wells were loaded with 1mg BSA per ml of normal saline and 1 mg purified liver GDH per ml of normal saline. The slides were then placed in moist chamber in the cold for about 36 h. On appearance of the precipitin line, the slide was immediately photographed.

Western blotting

Western blot analysis of the enzyme from either age was done according to the procedure of Towbin *et al.*, (1979) with slight modifications. The method involves running the samples in PAGE and transferring the protein bands from the gel to a nitrocellulose (NC) membrane and further processing was carried out as detailed below.

Materials

For PAGE: As previously described.

Towbin buffer: prepared by mixing 192 mM glycine and 2 5mM tris in a final volume of 1 litre, pH 8.3

Tris buffered saline (TBS): Prepared by dissolving 500 mM NaCl in 20 mM Tris, pH 7.5.

Tween-tris buffered saline (TTBS): Prepared by dissolving 0.05% tween-20 in TBS, pH 7.5

Blocking solution (BS): Prepared by taking 5% non-fat dry milk (NFDM) in TBS

Serum containing primary antibody (polyclonal).

Goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (ALP-IgG)

ALP-substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT)

Procedure

About 50 µg of liver GDH from both the age groups (10- and 90-day) was separately loaded onto PAGE and electrophoresced at 100 V for 1hr as described earlier.

⇩

The protein (GDH) from PAGE was then electroblotted onto a nitrocellulose membrane (NC) in chilled Towbin buffer medium for 1hr

⇩

Western-blotted NC membrane was blocked with 15 ml non-fat dry milk solution for 2-3 hr at room temperature (RT) with occasional shaking

⇩

Blocking solution was decanted and NC membrane washed twice with TTBS at RT

⇩

The NC membrane was then incubated with primary IgG (polyclonal IgG against GDH) solution at RT overnight

⇩

The NC membrane was washed with TTBS for 15 min at RT

⇩

The NC membrane was then incubated with the ALP-conjugated secondary Ab solution for 2-3 hrs at RT

⇩

The NC membrane was again washed with TTBS for 15 min at RT with gentle shaking

⇩

Transferred to a solution containing BCIP/NBT substrate and incubated at 37°C till optimum bluish colour developed

⇩

The NC membrane was washed with Millipore distilled water for 10 min at RT, dried and scanned.

CHAPTER - III

The results for the various experiments conducted are presented in the form of tables and figures. The table contents are represented by means which are a result of 4-5 separate experiments; standard error mean (SEM±), significance level and percentage (%) increase (+) or decrease (-) values. For all purposes, glutamate dehydrogenase used is extracted from the mitochondria and activities are expressed in terms of specific activity i.e. the amount of enzyme that can catalyse 1 µmol NADH per minute at 25°C and percentage activity with the highest taken as 100%.

Normal endogenous activity of GDH

Heart:

There was a significant increase (509%) in the specificity activity of the enzyme at day 10 which was followed by a sharp decline (25%) at day 30 and no significant changes in GDH activity was observed thereafter in this tissue (Table: 1 & fig: 1).

Brain

A significant increase (405%) in GDH activity (1.7U/mg) of the enzyme was observed 10 days after birth. The activity further peaked to a specific activity of 2.63 U/mg an 115% increase at 90-day (Table :1 & Fig: 1).

Kidney

In the kidneys the enzyme showed a significant increase (64%) in activity (4.57 U/mg) at day 10compared to 1 day old (2.78 U/mg) which was further followed by a 70% decrease in the specific activity at day 30. Thereafter, no significant change was observed (Table: 2 & Fig: 2).

Liver

In this tissue, there was a significant increase (52%) in GDH activity (5.57 U/mg) at day 10 compared to day 1 (3.65U/mg). This was followed by a sharp decline (61%) at day 30 (2.15 U/mg) and no further significant changes was observed thereafter (Table 2 & Fig: 2).

A comparison of the specific activity (U/mg) of GDH for the various tissues of 10- and 90-day showed that liver (5.57 U/mg) and kidney (4.57 U/mg) has maximum activity at day 10: at 90-day, the activities were comparable although the heart was lower (2 U/mg) than other tissues (Table 3 & Figure 3).

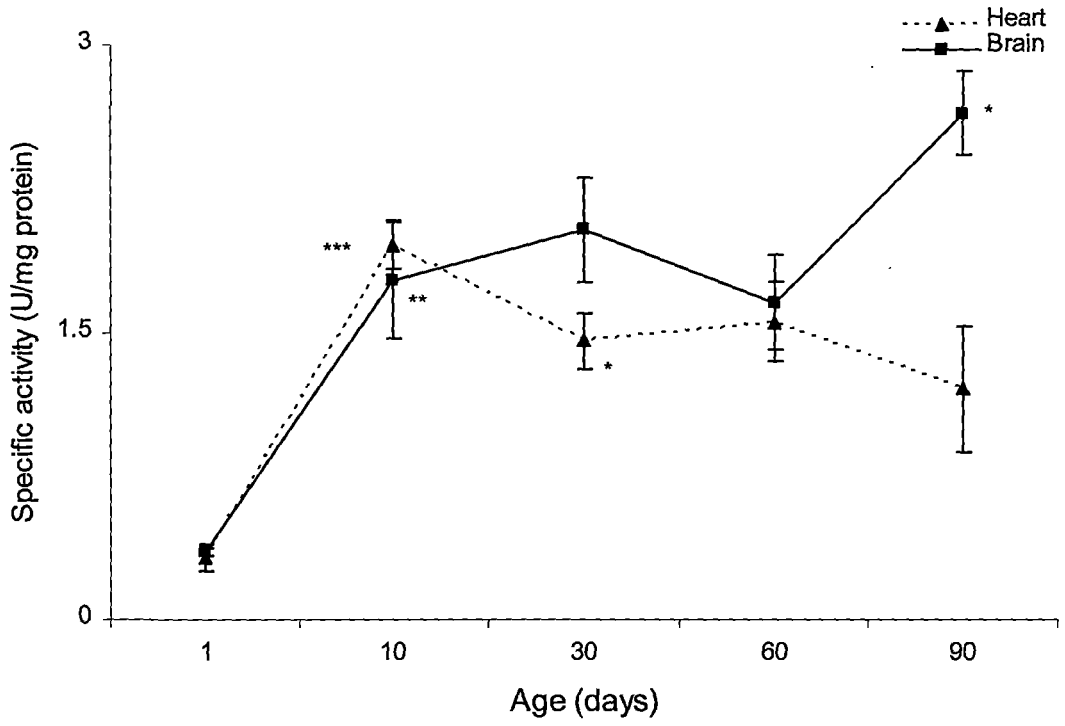


Fig 1: Specific activity of mitochondrial GDH in the **brain** and **heart** of mice at various postnatal ages Results are means of 5 animals each, bars represents SEM (±) and *, **, *** are the levels of significance at $p<0.05$, 0.01 and 0.001, respectively.

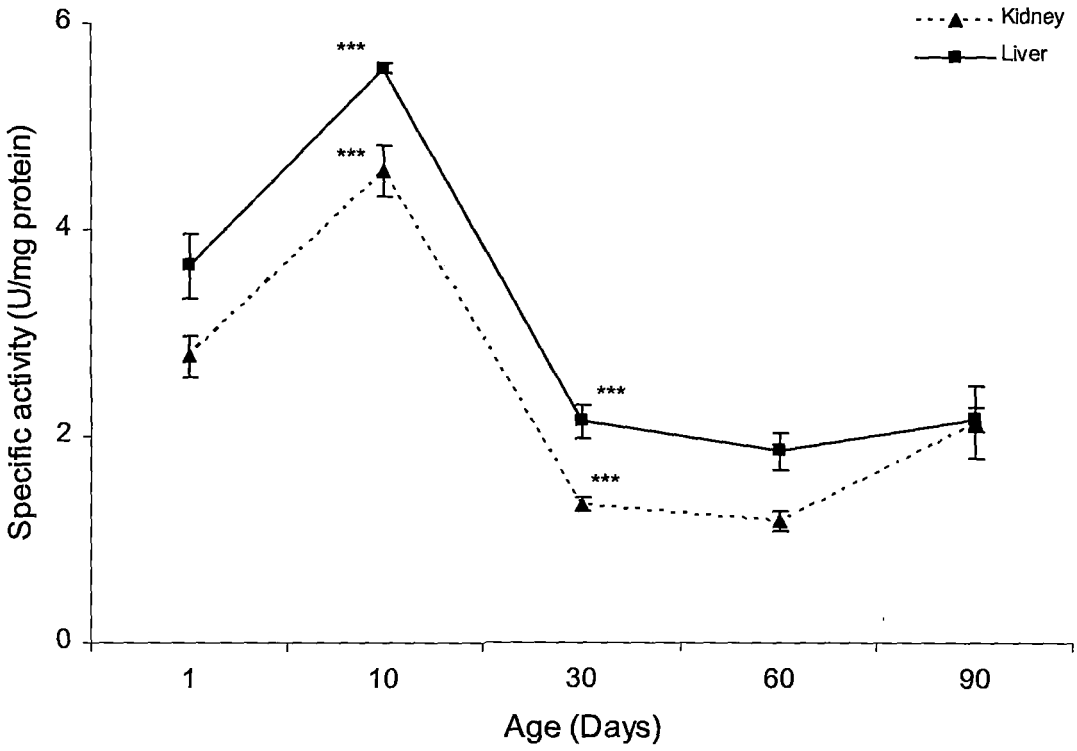


Fig 2: Specific activity of mitochondrial GDH in the **kidney** and **liver** of mice at various postnatal ages. Results are means of 5 animals each, bars represents SEM (±) and *, **, *** are the levels of significance at $p<0.05$, 0.01 and 0.001, respectively.

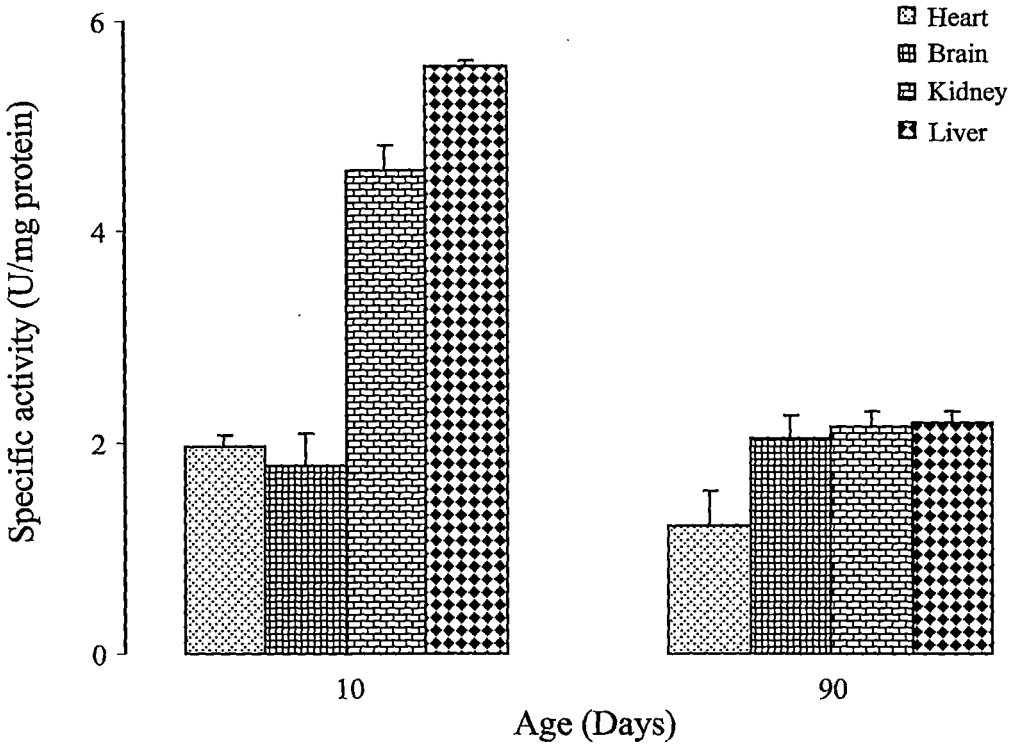


Fig 3: Comparison of specific activity (U/mg) of GDH from the various tissues of 10- and 90-day old mice. Results are means of 4-5 animals and bars represent SEM (±).

Table 1: Specific activity (U/mg) of glutamate dehydrogenase in the **brain** and **heart** of mice at the various postnatal ages.

	Brain				**Heart**			
Age (Days)	Mean	SEM	p	%	Mean	SEM	p	%
1	0.35	0.02			0.32	0.07		
			0.01	+405.7			0.00	+509.3
10	1.77	0.31			1.95	0.12	1	
			NS	-				-25.6
30	2.03	0.27			1.45	0.10	0.01	
			NS	-				-
60	1.65	0.25			1.55	0.21	NS	
			0.05	+115.0				-
90	2.63	0.22			1.20	0.33	NS	

Means are the result of 5 independent experiments; SEM (±) is the standard error mean; p, level of significance; % (±), percent increase or decrease; NS, not significant.

Table 2: Specific activity (U/mg) of glutamate dehydrogenase in the **kidney** and **liver** of mice at the various postnatal ages.

	Kidney				**Liver**			
Age (Days)	Mean	SEM	p	%	Mean	SEM	p	%
1	2.78	0.20			3.65	0.31		
10	4.57	0.25	0.001	+64.4	5.57	0.04	0.001	+52.6
30	1.34	0.07	0.001	-70.6	2.15	0.16	0.01	-61.4
60	1.18	0.09	NS	-	1.86	0.18	NS	-
90	2.14	0.35	NS	-	2.17	0.12	NS	-

Means are the result of 5 independent experiments; SEM (±) is the standard error mean; p, level of significance; % (±), percent increase or decrease; NS, not significant.

Table 3: Comparison of the specific activity of GDH for various tissues of 10- and 90- day.

Tissues	**10 day**		**90 day**	
	Specific activity (U/mg)	SEM	Specific activity (U/mg)	SEM
Heart	1.95	0.12	1.20	0.33
Brain	1.77	0.31	2.63	0.22
Kidney	4.57	0.25	2.14	0.35
Liver	5.57	0.04	2.17	0.12

Means are the result of 5 independent experiments; SEM (±) is the standard error mean; p, level of significance; % (±), percent increase or decrease; NS, not significant.

HORMONE TREATMENTS

Single hormone treatment

The effect of hormones triiodothyronine (T_3), dexamethasone and testosterone have been studied to see if there are any comparative changes in the specific activity (U/mg protein) of the enzyme in the different selected tissues (heart, brain, kidney and liver) and between the two age groups (10- and 90-day).

Heart

Specific activity of GDH resulted in a significant 2.5 fold increase (159%) in the 10-day old, when treated with T_3 but no significant changes was observed at 90-day. The 10-day old mice showed no response to dexamethasone treatment, however a significant 2 fold increase (99%) in GDH activity was observed in the adult mice (90-day). Testosterone treatment showed no alteration in GDH activity in both 10- and 90-day old mice (Table: 4 & Fig: 4).

Brain

The brain tissue exhibited significant response to T_3 administration injection with the 10-day old mice showing a 4 fold increase in activity (297%) whereas at 90-day a comparatively smaller 2 fold enhancement (139%) was observed. Dexamethasone administration resulted in a significant 3 fold increase (224%) of GDH activity only in the 90-day old mice. There was no significant response in GDH activity to testosterone treatment in brain tissue in both the age groups (10- and 90-day) studied (Table: 5 & Fig: 5).

Kidney

T_3 administration exerted a significant increase in the specific activity of GDH for both the ages. A 1.5 fold increase (67%) in GDH activity was observed in the 10-day old, while the 90-day old showed a comparatively higher (110%) response to T_3 treatment. Dexamethasone treatment also resulted in a significant 1.7 fold increase (76%) in GDH activity in both the ages (10- and 90-day) studied. No significant change in GDH activity (U/mg) was observed in the tissue of both ages (10- and 90-day) when treated with testosterone (Table: 6 & Fig: 6).

Liver

Treatment with T_3 hormone resulted in a significant 2 fold increase in GDH activity (109%) at 10 day. A higher 4 fold increase (306%) was observed at 90 day. Dexamethasone administration also exerted a significant increase of the enzyme activity in the liver tissue of both the age groups with a 116% and 120% increase in the 10- and 90-day old mice respectively. Testosterone treatment did not exert any significant alteration in GDH activity of this tissue in both the age groups studied (Table: 7 & Figure: 7).

Table 4: Effect of treatment of hormones triiodothyronine (T_3), dexamethasone and testosterone on the specific activity (U/mg protein) of GDH in the ***heart*** of 10- and 90- day old mice.

	10 day				**90 day**			
Hormones	Mean	SEM	p	% (±)	Mean	SEM	p	% (±)
Control	1.79	0.36			1.29	0.16		
T_3	4.64	0.46	0.01	+159.2	1.50	0.49	NS	-
Dexamethasone	1.83	0.07	NS	-	2.57	0.55	0.05	+99.2
Testosterone	1.20	0.06	NS	-	2.41	0.68	NS	-

Means are obtained from 4-5 mice; SEM (±)-standard error mean, p-level of significance and %(±) - the percentage stimulation or inhibition, NS- not significant

Table 5: Effect of treatment of hormones triiodothyronine (T_3), dexamethasone and testosterone on the specific activity (U/mg protein) of GDH in the ***brain*** of 10- and 90-day old mice.

	10 day				**90 day**			
Hormones	Mean	SEM	p	% (±)	Mean	SEM	p	% (±)
Control	1.53	0.15			1.99	0.39		
T_3	6.08	0.33	0.001	+297.3	4.77	0.71	0.01	+139.6
Dexamethasone	2.78	0.55	NS	-	6.46	0.49	0.001	+224.6
Testosterone	1.27	0.27	NS	-	2.71	0.51	NS	-

Means are obtained from 4-5 mice; SEM (±)-standard error mean, p-level of significance and %(±) - the percentage stimulation or inhibition, NS- not significant

Table 6: Effect of treatment of hormones triiodothyronine (T_3), dexamethasone and testosterone on the specific activity (U/mg protein) of GDH in the ***kidney*** of 10- and 90-day old mice.

	10 day				**90 day**			
Hormones	Mean	SEM	p	% (±)	Mean	SEM	p	% (±)
Control	4.66	0.26			2.32	0.39		
T_3	6.97	0.33	0.001	+67.5	4.88	0.45	0.01	+110.3
Dexamethasone	8.23	1.05	0.01	+76.6	4.10	0.20	0.01	+76.7
Testosterone	3.32	0.41	NS	-	3.56	1.08	NS	-

Means are obtained from 4-5 mice; SEM (±)-standard error mean, p-level of significance and %(±) - the percentage stimulation or inhibition, NS- not significant

Table 7: Effect of treatment of hormones triiodothyronine (T_3), dexamethasone and testosterone on the specific activity (U/mg protein) of GDH in the ***liver*** of 10- and 90-day old mice.

	10 day				**90 day**			
Hormones	Mean	SEM	p	% (±)	Mean	SEM	p	% (±)
Control	5.62	0.40			2.10	0.43		
T_3	11.75	0.51	0.001	+109.0	8.53	0.91	0.001	+306.9
Dexamethasone	12.18	1.03	0.001	+116.7	4.63	0.54	0.01	+120.4
Testosterone	5.32	0.41	NS	-	4.26	1.67	NS	-

Means are obtained from 4-5 mice; SEM (±)-standard error mean, p-level of significance and %(±) - the percentage stimulation or inhibition, NS- not significant

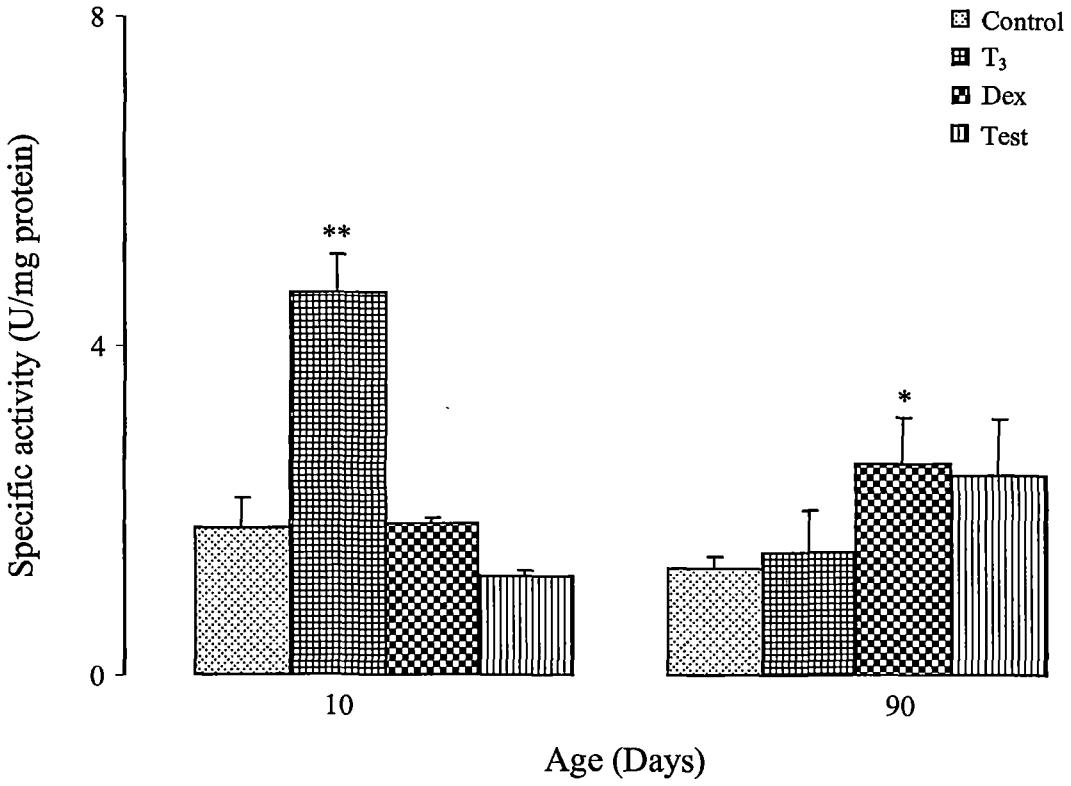


Fig 4: Effect of T_3, dexamethasone and testosterone on the activity of GDH in the **heart** of 10- and 90-day old mice. Experimental details are as described in methods section. Results are means of 5 animals with SEM (±); *, **, *** represent values that are significantly different from the control at $p<0.05$, 0.01 and 0.001, respectively

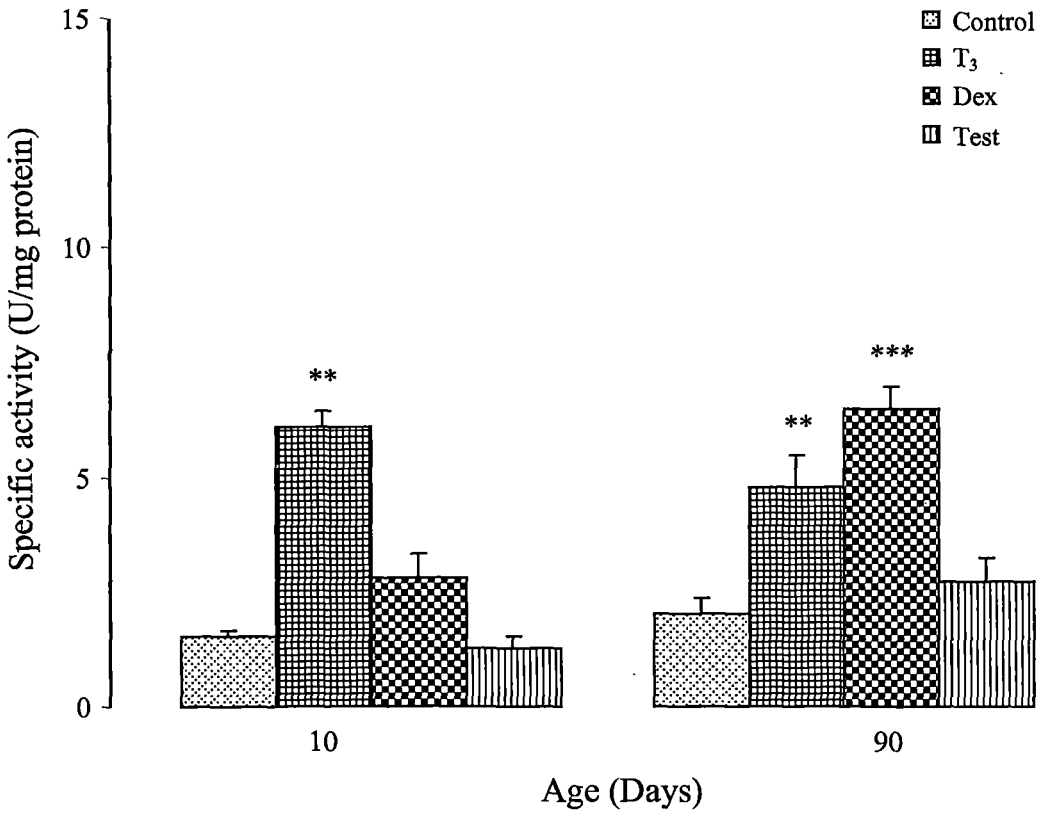


Fig 5: Effect of T3, dexamethasone and testosterone on the activity of GDH in the **brain** of 10- and 90-day old mice. Experimental details are as described in methods section. Results are means of 5 animals with SEM (±); *, **, *** represent values that are significantly different from the control at $p<0.05$, 0.01 and 0.001, respectively

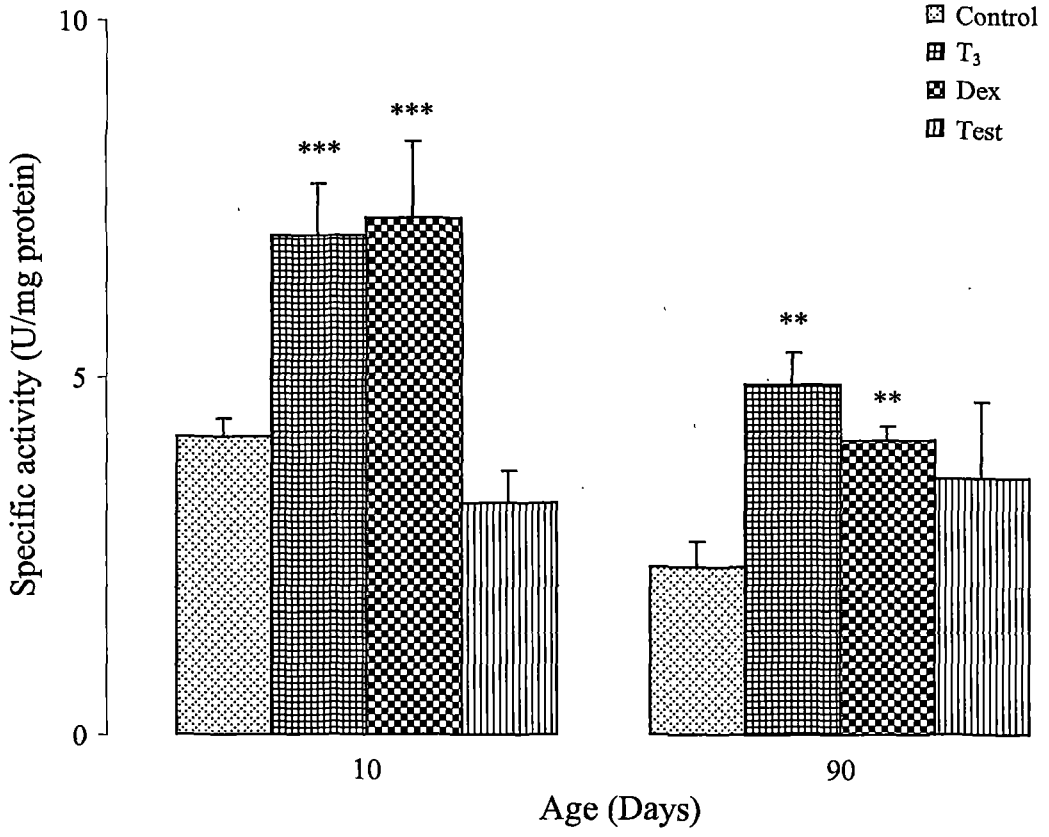


Fig 6: Effect of T_3, dexamethasone and testosterone on the activity of GDH in the **kidney** of 10- and 90-day old mice. Experimental details are as described in methods section. Results are means of 5 animals with SEM (±); *, **, *** represent values that are significantly different from the control at $p<0.05$, 0.01 and 0.001, respectively

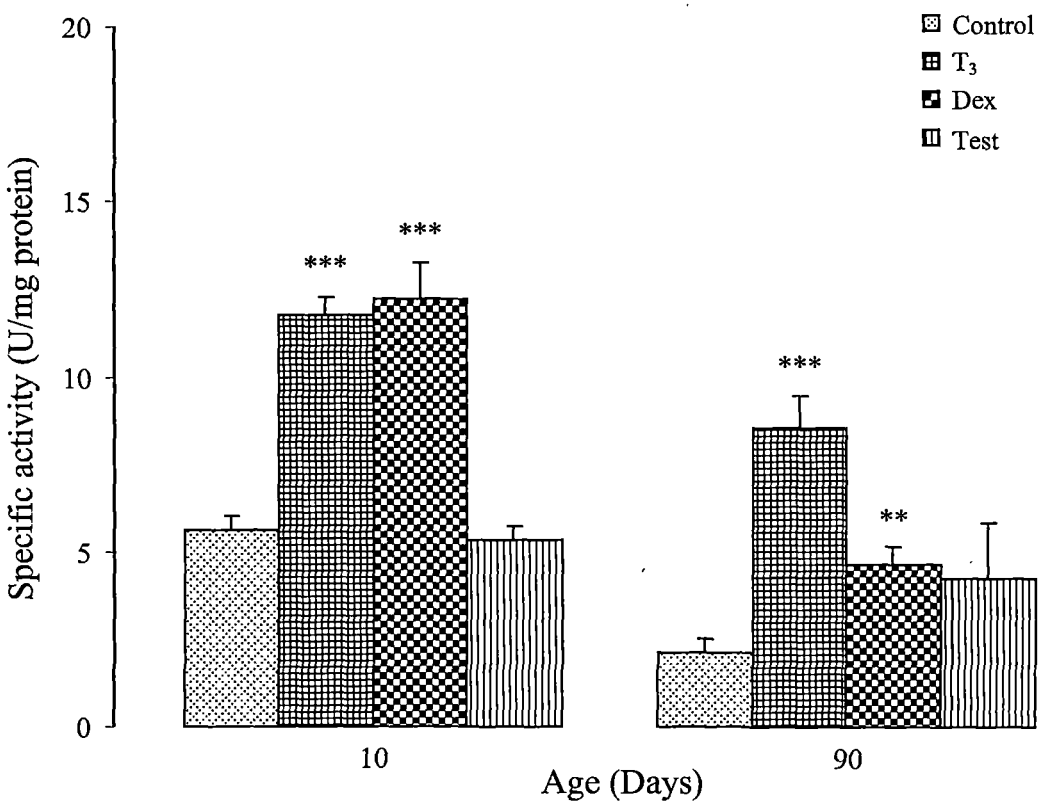


Fig 7: Effect of T_3, dexamethasone and testosterone on the activity of GDH in the **liver** of 10- and 90-day old mice. Experimental details are as described in methods section. Results are means of 5 animals with SEM (±); *, **, *** represent values that are significantly different from the control at $p<0.05$, 0.01 and 0.001, respectively

Hormone combination treatment

Two hormone combinations which include dexamethasone-triiodothyronine (dex-T_3) and testosterone-triiodothyronine (test-T_3) were used to study the synergistic or antagonistic effects, if any, on the GDH activity of the various selected tissues (heart, brain, kidney and liver) and at two ages- 10 and 90-day old mice.

Heart

a) Dex-T_3 administration exerted a significant 4.4 fold elevation in GDH activity (339%) of the 10-day old mice only. The induction by this hormone combination is more compared to that exerted by the individual (T_3 or dexamethasone) hormones

b) Test-T3 treatment showed no marked changes in the enzyme activity of heart tissue in both the ages (10- and 90- day) studied (Table: 8 & Fig: 8).

Brain

There was a significant increase in GDH activity in the brain tissue when treated with both hormone combinations.

a) Dex-T_3 combination resulted in a 5 fold increase (394%) in GDH activity and therefore more potent compared to single hormone administration of T_3 which resulted in 297% (4 fold) increase at the young age (10-day). In the older mice (90-day), in contrast, the combination was apparently antagonistic, showing only 66% increase with dex-T_3 combination, compared to T_3 alone (139%).

b) The brain tissue was also responsive to test-T_3 hormone treatment. While the 10- day old exhibited a 167% increase, the 90-day old showed a 147% increase in GDH activity, which was much higher than either single hormone treatment (Table: 9 & Fig: 9).

Kidney

a) Dex-T_3 hormone combination treatment induced a small yet significant 1.7 fold increase (72%) in GDH activity of 10-day and marked 3.4 fold increase (246%) in the 90-day old mice. However, in 10-day old no enhancement over single hormone treatment was observed with dex-T_3 combination. In contrast, the older mice (90-day) showed a significant enhancement of GDH activity with dex-T_3, which was elevated to 246%, this was more pronounced enhancement of activity compared to T_3 (110%) and dex (76%) individual treatment alone.

b) The specific activity of GDH showed a significant 1.6 fold increase (60%) in response to Test-T_3 combination in the adult (90-day) mice. The specific activity of GDH however did not exceed beyond what was observed in the kidney of 10-day old mice (Table: 10 & Fig: 10:).

Liver

a) A significant 2.8 fold increase (184%) in the specific activity of liver GDH in the 10-day old mice was observed with dex-T_3 combination which was comparatively more effective than - T_3 (109%) or dex (116%) individual treatment alone. A similar enhancement (370%) was seen in older mice (90-day) with this hormone combination, compared to single hormone treatment; dex (120%) and T_3 (306%) alone

b) Test- T_3 combination induced a significant 4.6 fold increase (362%) in GDH activity in the adult (90-day) mice which was slightly higher compared to the individual effect (T_3; 306%). No significant change was observed in GDH activity in the young (10-day) mice (Table: 11 & Fig: 11).

Table 8: Effect of hormones dexamethasone-T_3 (dex-T_3) and testosterone- T_3 (test-T_3) combination treatment on the specific activity (U/mg protein) of GDH in the ***heart*** of 10- and 90- day old mice.

	10 day				**90 day**			
Hormones combination	Mean	SEM	p	% (±)	Mean	SEM	p	% (±)
Control	1.79	0.36			1.29	0.16		
Dex-T_3	7.86	0.54	0.001	+339.1	1.95	0.53	NS	-
Test-T_3	1.03	0.51	NS	-	1.49	0.27	NS	-

Means are obtained from 4-5 mice; SEM (±) -standard error mean, p-level of significance and % (±) - the percentage stimulation or inhibition, NS- not significant

Table 9: Effect of hormones dexamethasone-T_3 (dex-T_3) and testosterone- T_3 (test-T_3) combination treatment on the specific activity (U/mg protein) of GDH in the ***brain*** of 10- and 90-day old mice.

	10 day				**90 day**			
Hormones combination	Mean	SEM	p	% (±)	Mean	SEM	p	% (±)
Control	1.53	0.15			1.99	0.39		
Dex-T_3	7.56	1.68	0.01	+394.1	3.32	0.34	0.01	+66.8
Test-T_3	4.10	0.70	0.01	+167.9	4.93	0.93	0.01	+147.7

Means are obtained from 4-5 mice; SEM (±)-standard error mean, p-level of significance and % (±) - the percentage stimulation or inhibition, NS- not significant

Table 10: Effect of hormones dexamethasone-T_3 (dex-T_3) and testosterone- T_3 (test-T_3) combination treatment on the specific activity (U/mg protein) of GDH in the ***kidney*** of 10- and 90-day old mice.

	10 day				90 day			
Hormones combination	Mean	SEM	p	% (±)	Mean	SEM	p	% (±)
Control	4.66	0.26			2.32	0.39		
Dex-T_3	8.04	0.91	0.01	+72.5	8.05	1.07	0.001	+246.9
Test-T_3	4.93	0.70	NS	-	3.72	0.50	0.05	+60.3

Means are obtained from 4-5 mice; SEM (±)-standard error mean, p-level of significance and % (±) - the percentage stimulation or inhibition, NS- not significant

Table 11: Effect of hormones dexamethasone-T_3 (dex-T_3) and testosterone-T_3 (test-T_3) combination treatment on the specific activity (U/mg protein) of GDH in the ***liver*** of 10- and 90-day old mice.

	10 day				**90 day**			
Hormones combination	Mean	SEM	p	% (±)	Mean	SEM	p	% (±)
Control	5.62	0.40			2.10	0.43		
Dex-T_3	15.97	1.73	0.001	+184.1	9.89	0.24	0.001	+370.9
Test-T_3	4.71	0.28	NS	-	9.72	1.51	0.001	+362.8

Means are obtained from 4-5 mice; SEM (±)-standard error mean, p-level of significance and % (±) - the percentage stimulation or inhibition, NS- not significant

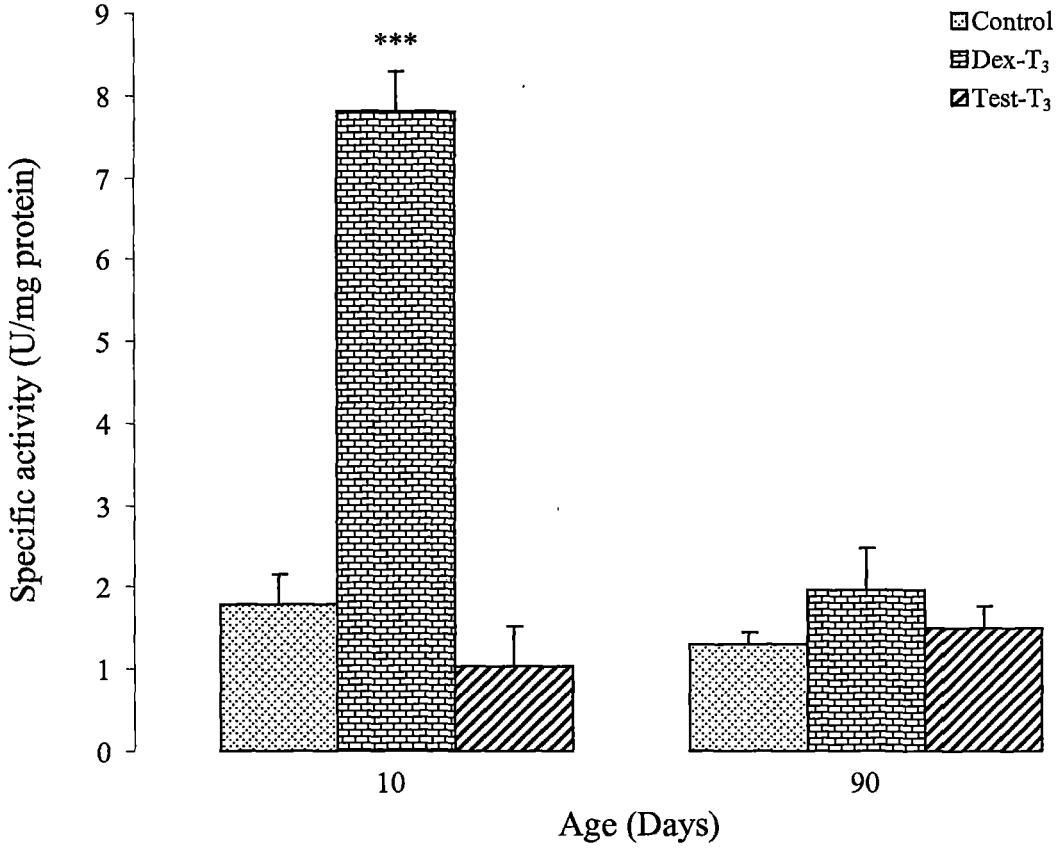


Fig 8: Effect of the various combinations of testosterone, dexamethasone and triiodothyronine (T_3) on the activity of GDH in the **heart** of 10- and 90-day old mice. Details are as mentioned in materials and method section. Results shown are means of 5 animals with SEM (±); *, **, *** represent values that are significantly different from the control at $p<0.05$, 0.01 & 0.001, respectively.

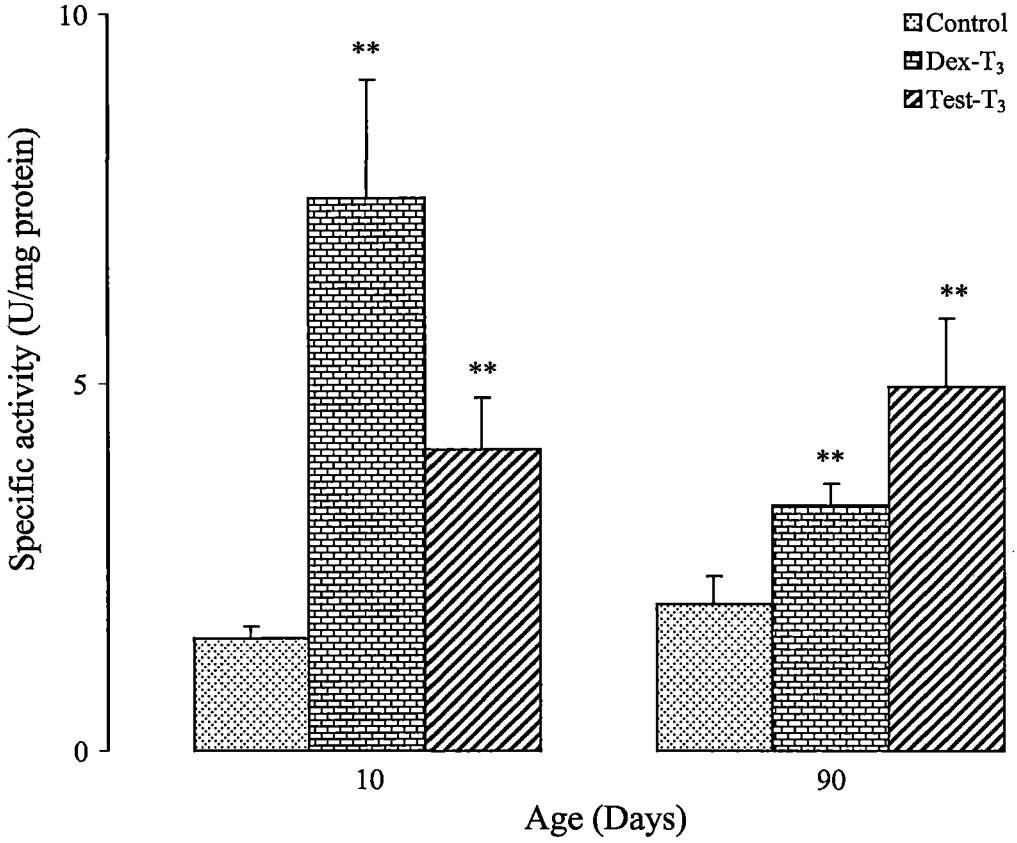


Fig 9: Effect of the various combinations of testosterone, dexamethasone and triiodothyronine (T_3) on the activity of GDH in the **brain** of 10- and 90- day old mice. Details are as mentioned in materials and method section. Results shown are means of 5 animals with SEM (±); *, **, *** represent values that are significantly different from the control at $p<0.05$, 0.01 & 0.001, respectively.

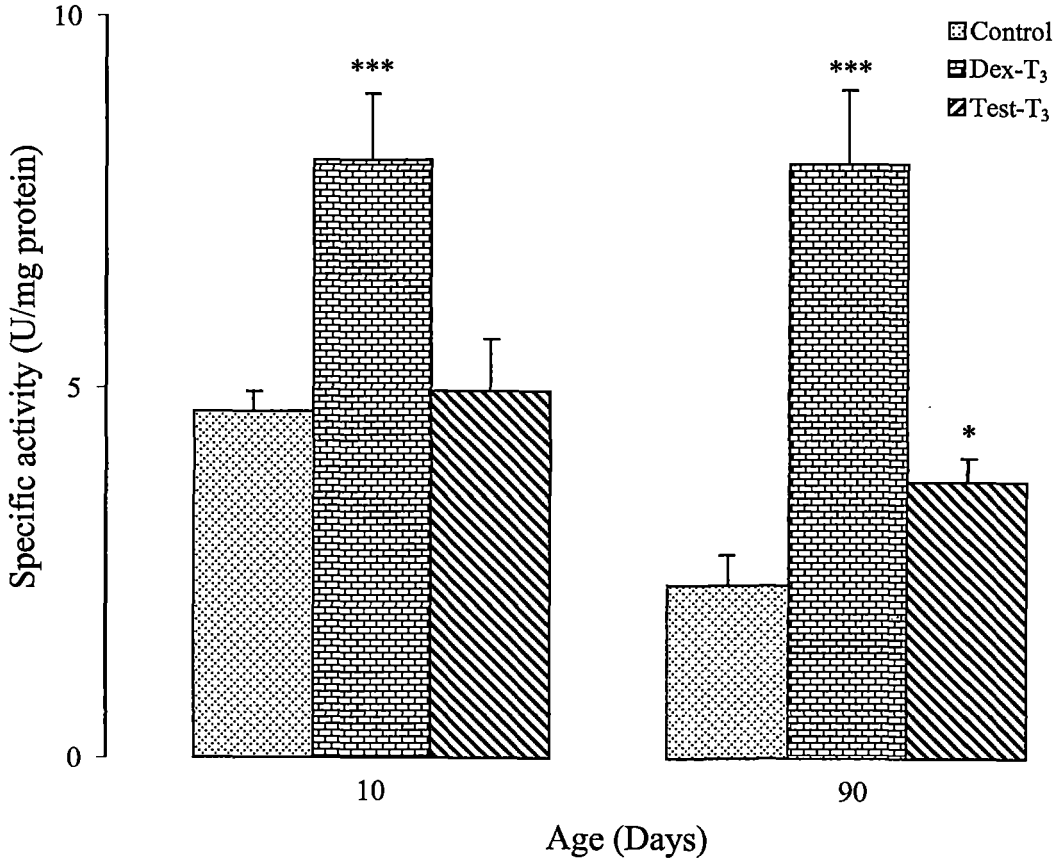


Fig 10: Effect of the various combinations of testosterone, dexamethasone and triiodothyronine (T_3) on the activity of GDH in the **kidney** of 10- and 90- day old mice. Details are as mentioned in materials and method section. Results shown are means of 5 animals with SEM (±); *, **, *** represent values that are significantly different from the control at $p<0.05$, 0.01 & 0.001, respectively.

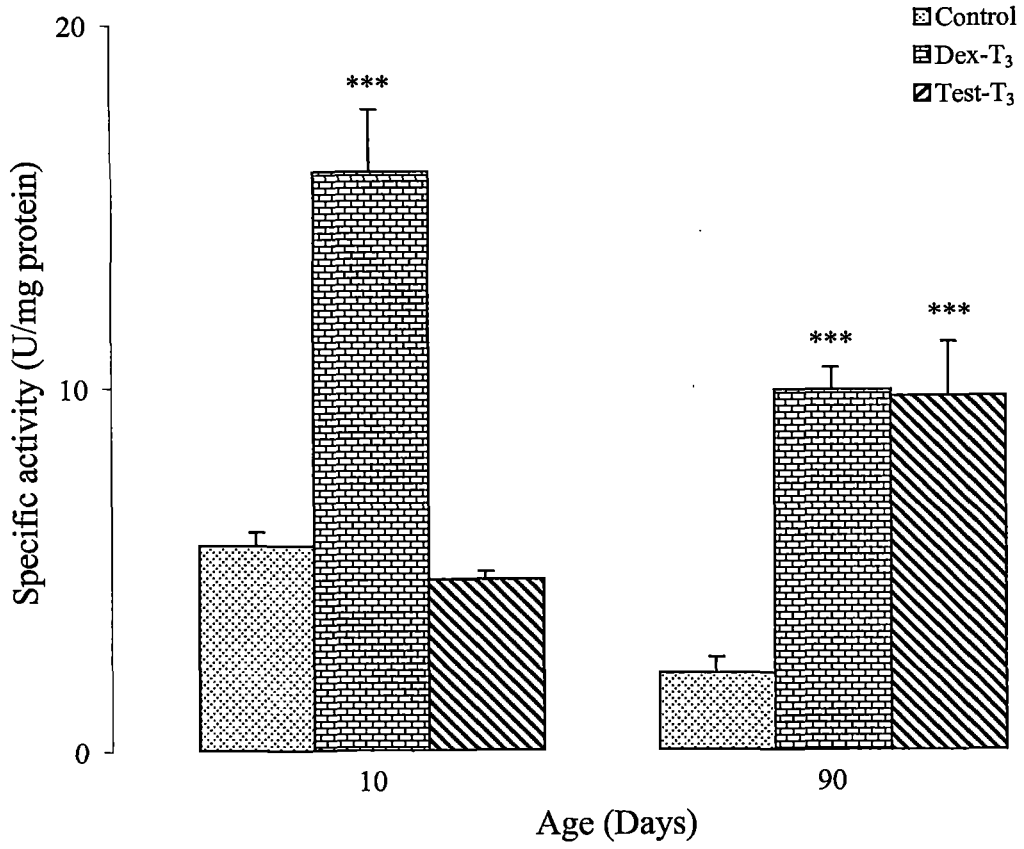


Fig 11. Effect of the various combinations of testosterone, dexamethasone and triiodothyronine (T3) on the activity of GDH in the **liver** of 10- and 90-day old mice. Details are as mentioned in materials and method section. Results shown are means of 5 animals with SEM (±); *, **, *** represent values that are significantly different from the control at $p<0.05$, 0.01 & 0.001, respectively.

PHYSICO-CHEMICAL CHARCTERIZATION

Effect of ionic strength on GDH

Dialysed crude GDH preparations from both the ages were assayed at varying concentration (10-200 mM) of immidazole, pH 7.9 as described in materials and method section. In both the ages the enzyme activity, expressed as percentage activity from the maximum, showed identical molar requirement with the maximum activity at 175 mM of immidazole (Fig: 12).

pH stability of GDH

The pH profile of both the age group was determined at various pH range with sodium acetate buffer in the acidic range (pH 4-6.5) and immidazole buffer in the alkaline range (pH 7-10) as described in material and methods section. In both the cases, the pattern was similar and the optimum pH observed was 8.0 (Fig: 13).

Effect of temperature on the stability of GDH

Dialysed enzyme preparation from 10- and 90-day old mice were pre-incubated in assay buffer at various temperatures for 15 min and assayed for activity as described earlier. GDH from both the ages showed a typical temperature profile with optimal activity expressed as % activity from the maximum observed at 25°C and decreases to about 4% and 3% in 10 and 90- day respectively at 65°C (Fig: 14).

Effects of reducing agents

The thiol-containing reducing agents, 2-mercaptoethanol and dithiothreitol (DTT) in varying concentrations (0-10 mM) were separately incubated in the assay buffer containing approximately 100 μg of dialyzed GDH for 1 hr in the cold and is

followed by routine enzyme assay. Enzymes from either age exhibited similar pattern of activity under the influence of either reducing agents. DTT was only slightly better than 2-mercaptoethanol (Fig: 15 A & B).

Effect of guanidine hydrochloride on GDH

The dialyzed enzyme form both age groups showed similar susceptibility to Gdn. HCl. More than 50% inactivation of enzyme activity by Gdn.HCl at 0.1M was observed and become almost completely inactivated at 1.25 M with activity falling below 10% from the control (Fig: 16).

Effect of inhibitor guanosine triphosphate (GTP)

The effect of GTP on the activity of liver GDH was compared for enzyme inhibition from the two age groups (10- and 90-day). Varying concentrations (0-50 μM) of GTP were separately incubated with dialyzed GDH preparations in assay buffer for 1 hr in the absence of ADP and followed by normal GDH assay. Result showed a 60% reduction in the activity of the enzyme at 5 μM concentration and at 15 μM and beyond, the activity was suppressed to a less than 10% for both age groups (Fig: 17 A).

GDH from the liver of 10-and 90 day was also separately incubated with GTP, but in the presence of 1mM of adenosine diphosphate (ADP), a positive modulator of the enzyme. Results showed inhibition of GDH activity by GTP was slightly disrupted especially with the adult mice due to the presence of ADP (Fig: 17 B). In both the age groups an 80% inhibition could be achieved only at 50 μM.

Effect of other co-substrates

Adenosine diphosphate (ADP): Crude dialyzed samples of GDH from the liver of 10- and 90-day old mice were separately assayed for activity with varying concentrations (0-2 mM) of ADP. GDH activity for the immature mice exhibited maximum activity at 1mM ADP which reduces to 55% from that of maximum at 2 mM. In the adult mice maximal activity was observed at 1.5 mM. The activity of the enzyme remained by and large constant at higher concentration of ADP (Fig: 18).

Nicotinamide adeninedinucleotide, reduced (NADH): Various concentrations (5-200 μmol) of NADH were added separately into the enzyme assay mixture and the activity recorded. The optimal concentration of NADH for the young mice was found to be around 125 μM whereas, 150 μM provided maximum activity for the 90 day old mice (Fig: 19).

Nicotinamide adeninedinucleotide phosphate, reduced (NADPH): The effect of NADPH on GDH activity was also determined for both 10- and 90- day old mice. NADPH in the range of 5-150 μM was separately added to the assay mixture in place of NADH. In both the age groups, maximum activity was observed only at 150 μM concentration (Fig: 20).

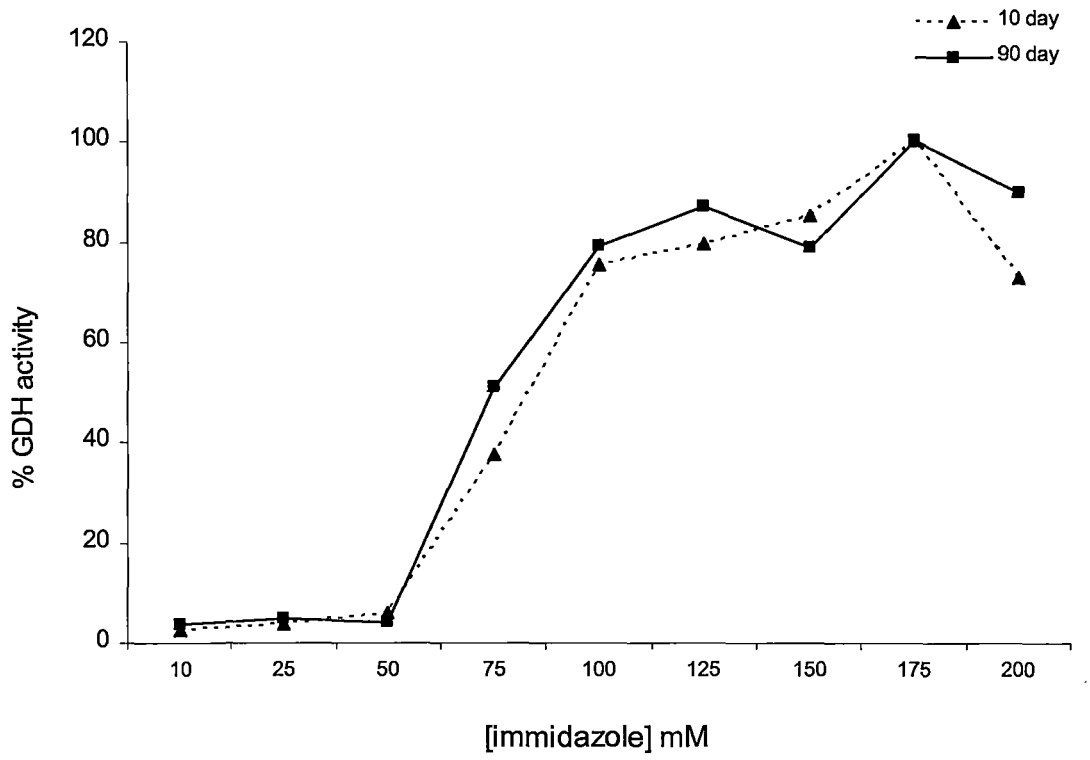


Fig 12: Effect of immidazole concentration on the activity of liver GDH in the two age groups. Details of the experiment are described in method section. Results are mean of 2-3 independent experiments.

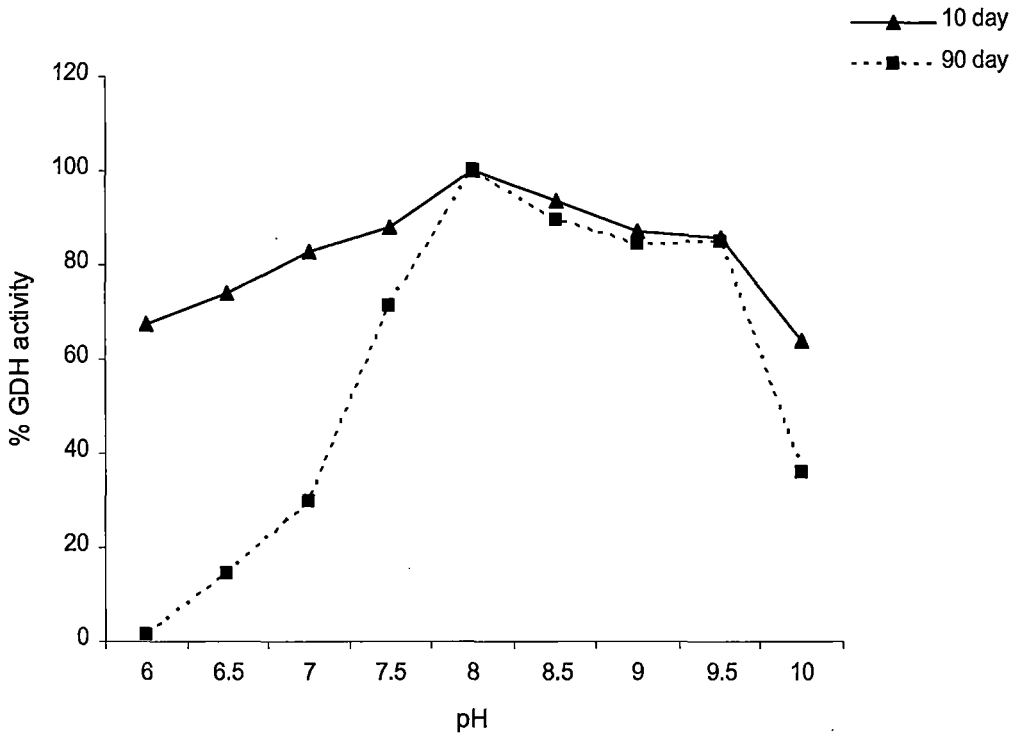


Fig 13: Effect of pH on the stability of purified liver GDH from 10- and 90- day old mice. Details of the experiment are described in method section. Results are means of 2-3 independent experiments.

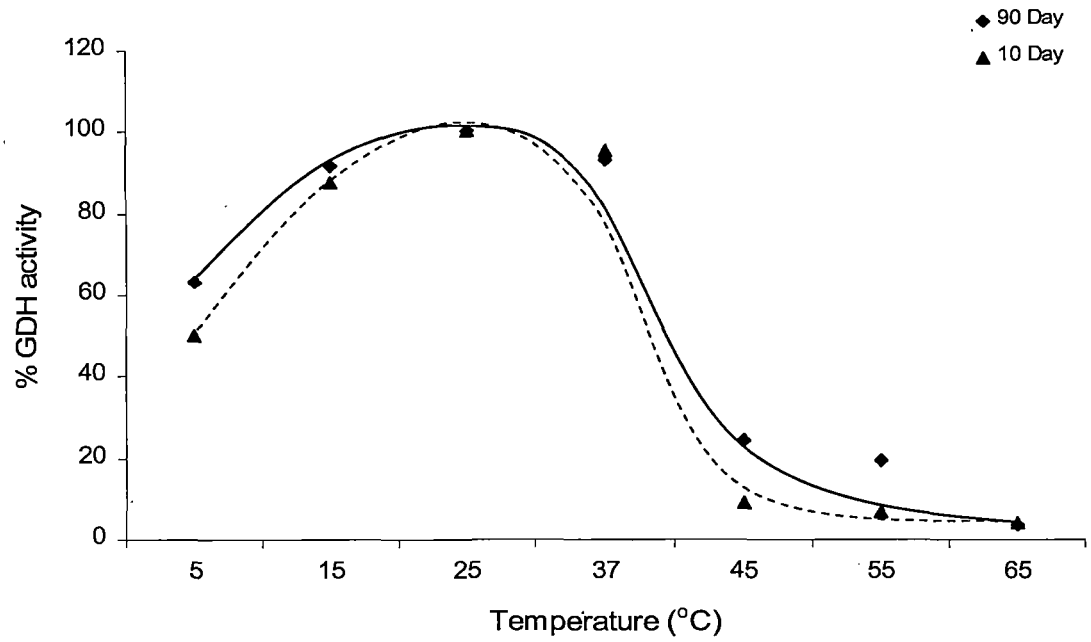


Fig 14: Effect of temperature on the activity of liver GDH in the two age groups. Details of the experiment are described in method section. Results are mean of 2-3 independent experiments

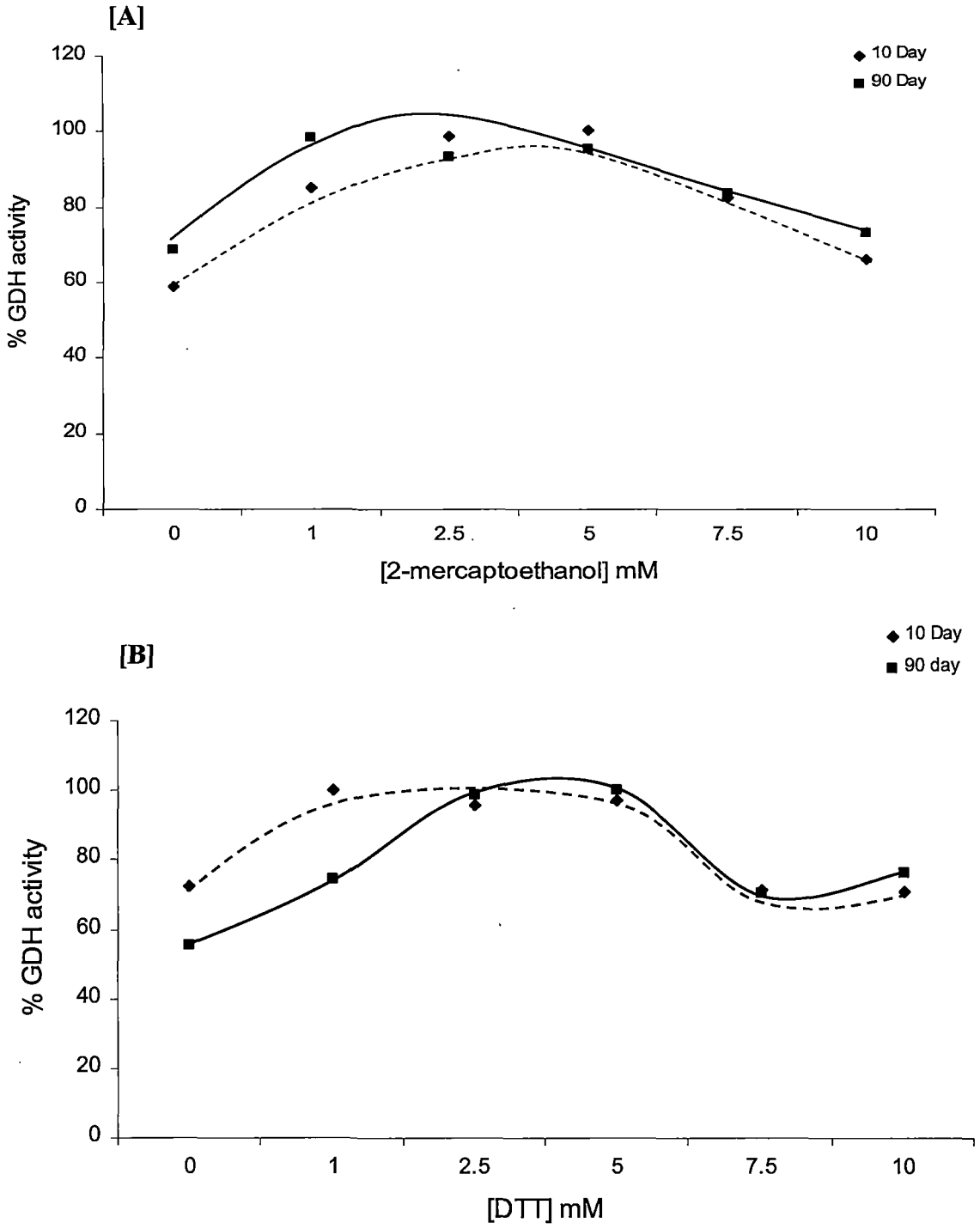


Fig 15: Effect of [A] 2- mercaptoehanol and [B] dithiothreitol (DTT) concentration on the activity of liver GDH in the two age groups. Details of the experiment are described in method section. Results are mean of 2-3 independent experiments

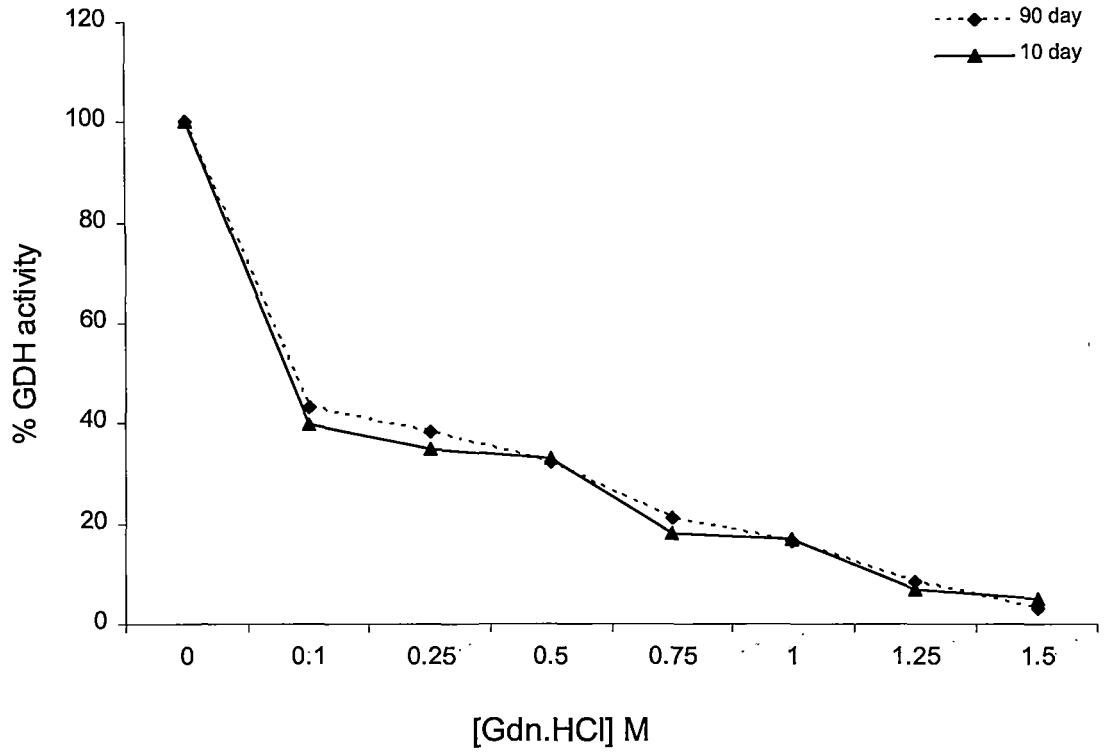


Fig 16: Inactivation profile of GDH from the liver of 10- and 90-day old mice by guanidine hydrochloride (Gdn.Hcl). Details of the experiment are described in method section. Results are means of 2-3 independent experiments.

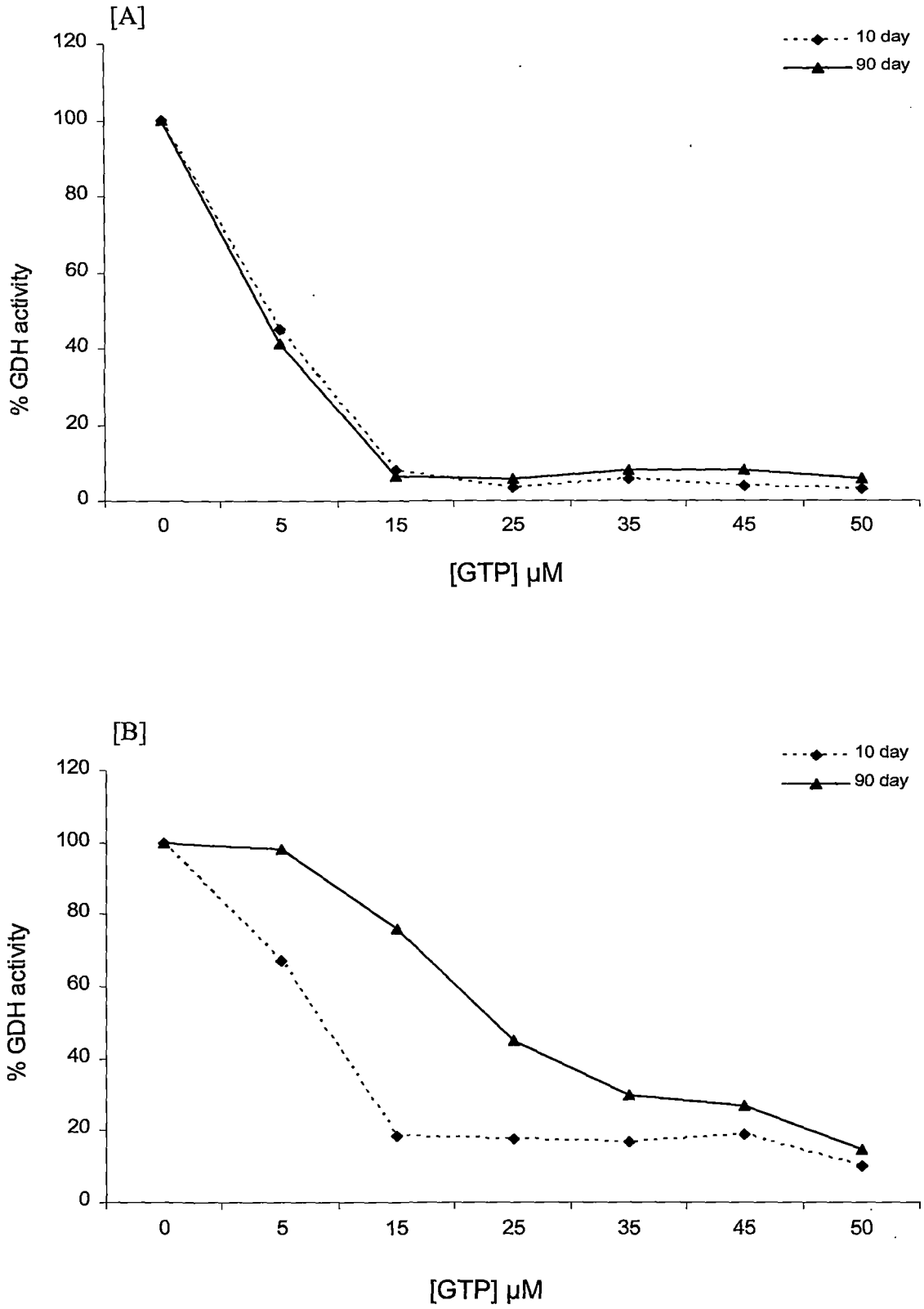


Fig17: Effect of GTP concentration on the activity of liver GDH from the two age groups in absence of ADP [A] and in presence of 1 mM ADP [B]. Details of the experiment are described in method section. Results are mean of 2-3 independent experiments.

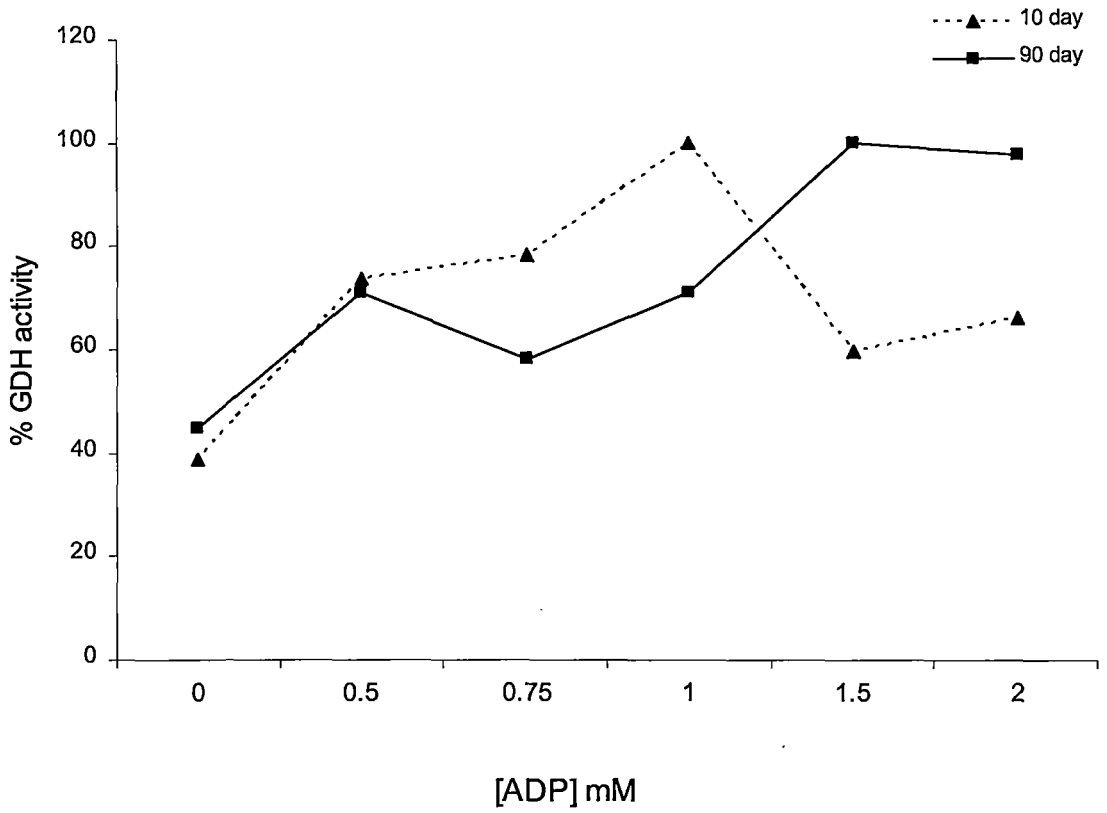


Fig 18: Effect of ADP concentration on the activity of liver GDH in the two age groups. Details of the experiment are described in method section. Results are mean of 2-3 independent experiments

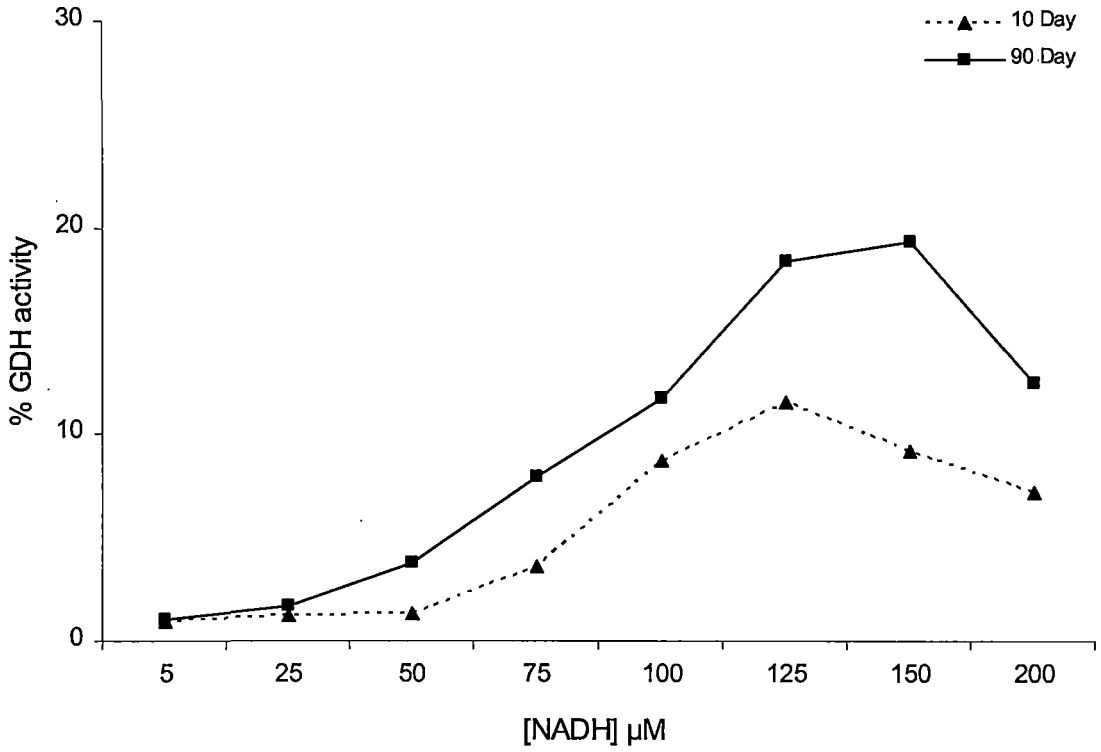


Fig 19: Effect of NADH concentration on the activity of liver GDH in the two age groups. Details of the experiment are described in method section. Results are mean of 2-3 independent experiments.

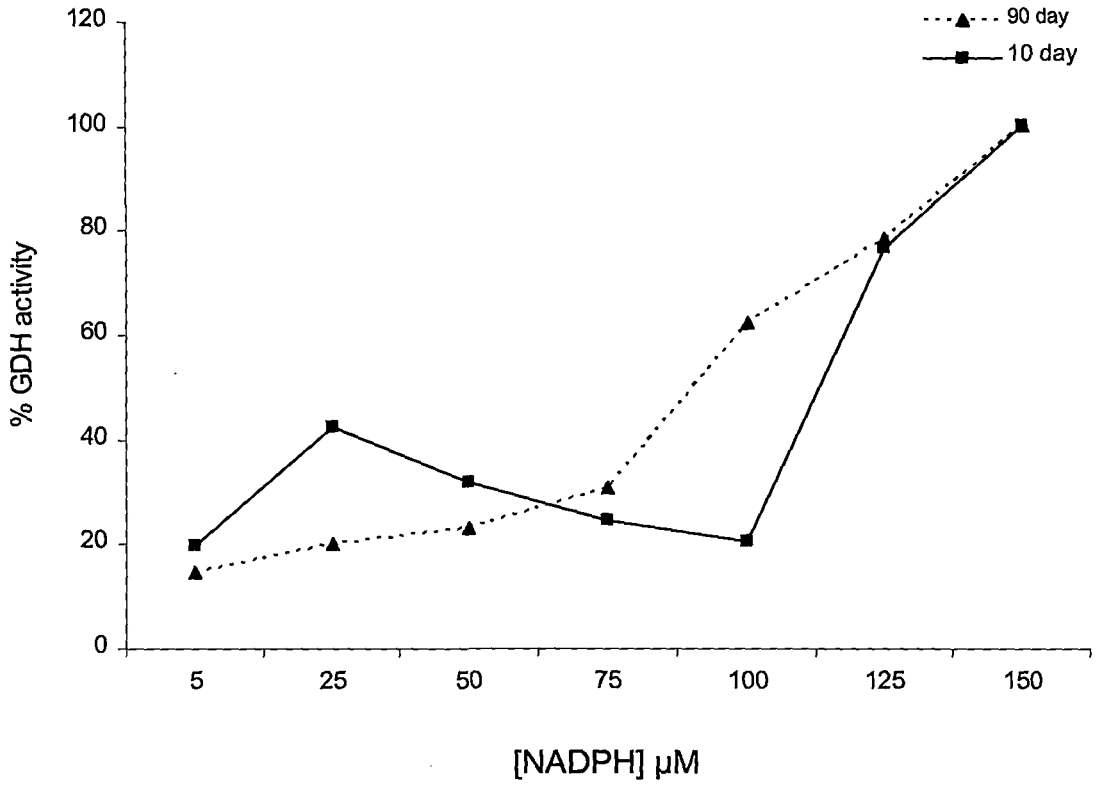


Fig 20: Effect of NADPH concentration on the activity of liver GDH in the two age groups. Details of the experiment are described in method section. Results are mean of 2-3 independent experiments

Extraction and purification of liver GDH

Glutamate dehydrogenase from the liver of 10- and 90-day old mice was purified according to the procedure described in materials and method section. Approximately 25g of liver from the two age groups was taken and subjected to various steps of the isolation procedure as outlined in table 12 & 13 and all steps were carried in the cold chamber whose temperature was maintained at 0-4°C. Concentrated protein obtained from ammonium sulfate fractionation (30-60%) was desalted using Sephadex-G-50 column and the protein fractions were further concentrated by lyophilization. The desalted proteins were then loaded onto a Sepharose 6-B column (1.6 x 80 cm) which was equilibrated with 25 mM sucrose tris-HCl buffer, pH 7.4 and adjusted to a flow rate of 20 ml/h. The sample was eluted by passing 25 mM sucrose tris-Hcl buffer pH 7.5 and the active fractions, indicated by a horizontal bar were pooled together and lyophilized (Fig: 21 & 22). The lyophilized sample was dissolved in minimum amount of 25mM sucrose tris-HCl buffer and further purification was carried out by loading the lyophilized sample onto an anion exchanger DEAE-cellulose column, where the sample was eluted with a linear gradient of 0-0.5 M NaCl solution as described in the method section. GDH from 10- and 90-day eluted at approximately 0.15 M. The elution profile is shown in figures 23 & 24. The active fractions, represented by a horizontal bar in the figure were further dialyzed, concentrated and used for further characterizations. The percentage yield and purification fold of GDH from both the ages is given in table 12 & 13.

Molecular weight determination

Determination of the native molecular weight was done by subjecting the protein through Sepharose 6B column chromatography (1.6 x 80 cm) which was precalibrated with various protein molecular markers (Fig: 25). The other parameters

were determined by passing blue dextran and potassium ferricyanide to obtain the values for V_o and V_p respectively, where V_o is the void volume of the column and V_p; the total volume of the gel.

Using the relations described under material and methods section, the molecular weight of GDH from the liver of the two age groups have been determined by plotting V_e/V_o versus the logarithm of molecular weight (log M) as shown in figure 26. The molecular weight determined for both the 10- and 90- day old mice GDH was approximately 330 kDa.

The purity of the preparation was confirmed on non-denaturing PAGE, which showed a single band from each age group (fig:27).

The subunit mass composition of GDH from the two age categories have also been determined using SDS-PAGE, as shown in figure 28. A plot of the relative mobility (R_m) against log M of the enzyme GDH along with various protein markers gave the approximate subunit molecular mass of 55 kDa indicating that the subunit mass were equal in size, as shown in figure 29.

Determination of V_{max}, K_m and K_{cat} of liver GDH

The kinetic characteristics of purified liver GDH from 10- and 90-day old mice for the substrate α-ketoglutarate and at various concentrations have been determined. Data obtained from the experiment were computed using Sigma Enzfitter program (Perella, 1988). The figures were drawn using the Michealis-Menten equation and the insets to these figures were drawn using the Eadie-Hofstee transformation. The maximum velocity, V_{max} for the immature mice using α-ketoglutarate as substrate was 4.6 μM/min and the matured mice showed a value of 4.7 μM/min, K_m obtained was 5.2 and 6.1 mM for the 10- and 90-day old liver enzyme, respectively. The catalytic constant (K_{cat}) calculated for the immature mice is 294 sec^{-1} and 297 sec^{-1} in case of the matured mice (Table 14: & Fig 30 & 31).

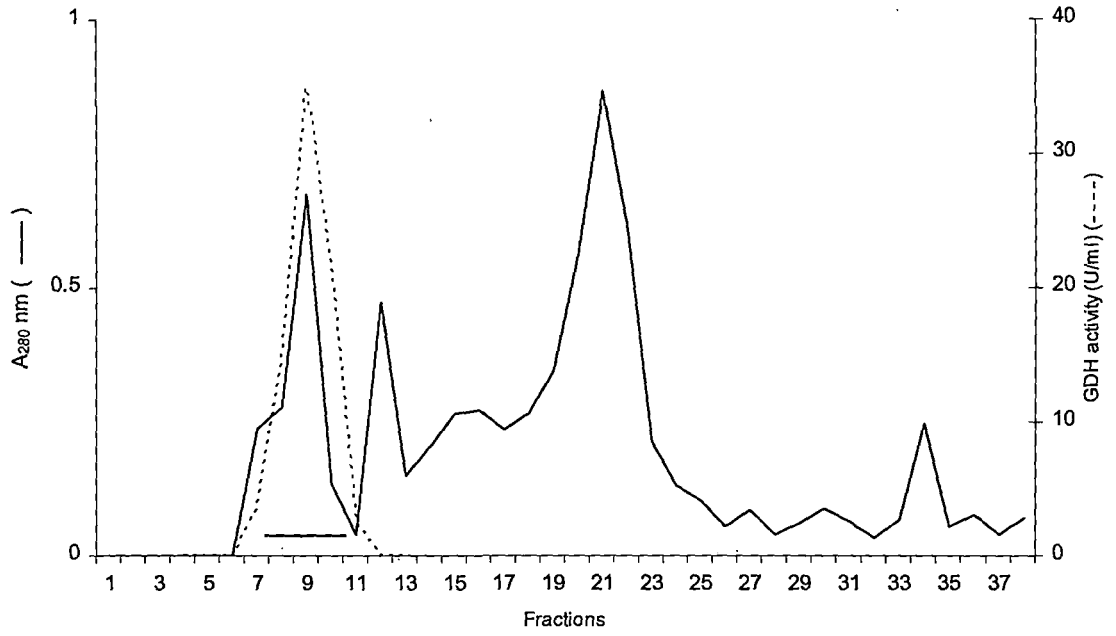


Fig 21: Elution profile of liver GDH of immature mice (10-day) on Sepharose 6B. Dotted line represents the activity (U/ml) plot of the enzyme. Horizontal bar indicates active fractions. Absorbances were taken at wavelength 280 nm and 340 nm for protein and activity determinations respectively.

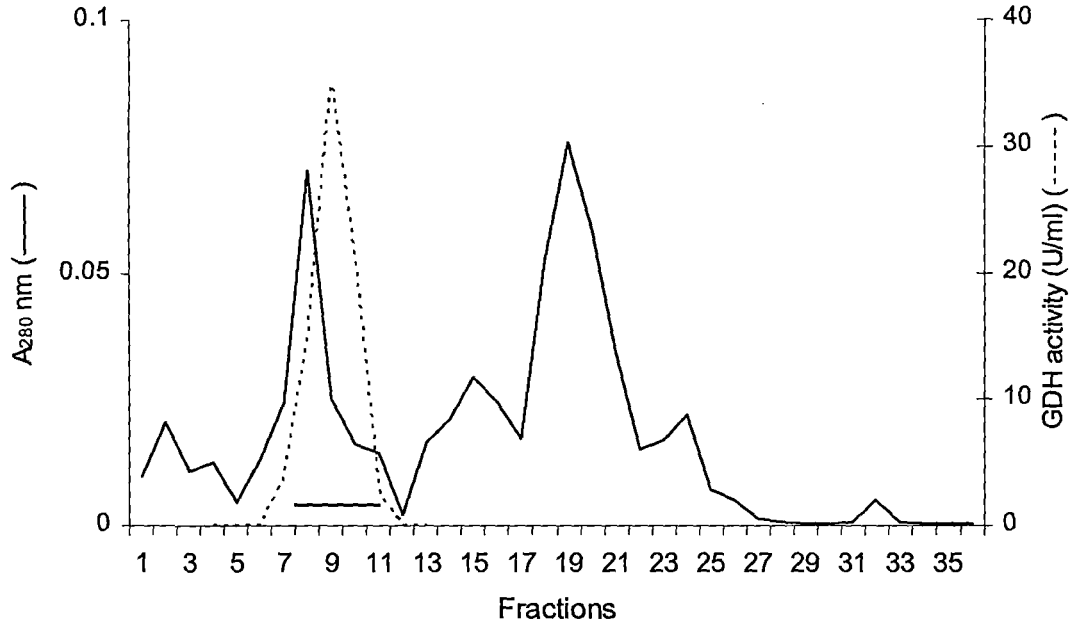


Fig 22: Elution profile of liver GDH of matured mice (90-day) on Sepharose 6B. Dotted line represents the activity (U/ml) plot of the enzyme. Horizontal bar indicates active fractions. Absorbances were taken at wavelength 280 nm and 340 nm for protein and activity determinations respectively.

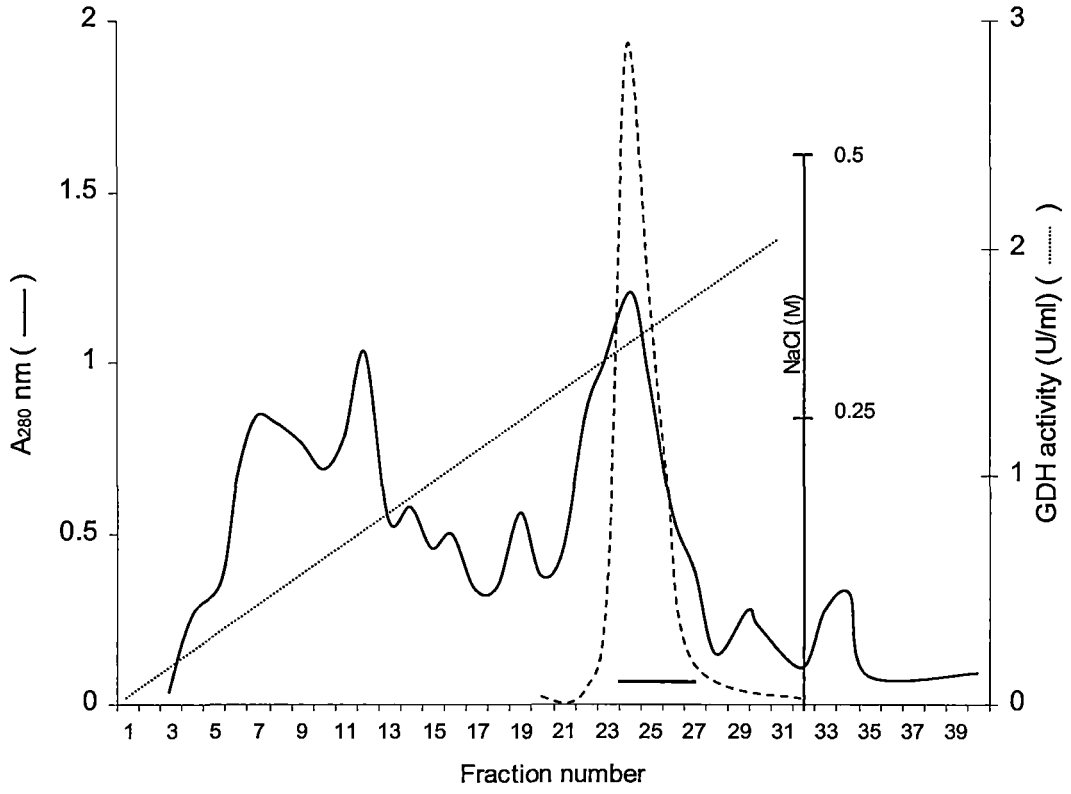


Fig 23: Elution profile of GDH from the liver of 10-day old mice through DEAE-Cellulose. The details of the experiment are described in detail in materials and methods section. GDH was eluted by a linear gradient of 0-0.5M of NaCl and proteins eluted were monitored spectrophotometrically at wavelength 280nm and GDH activity was assayed at 340 nm.

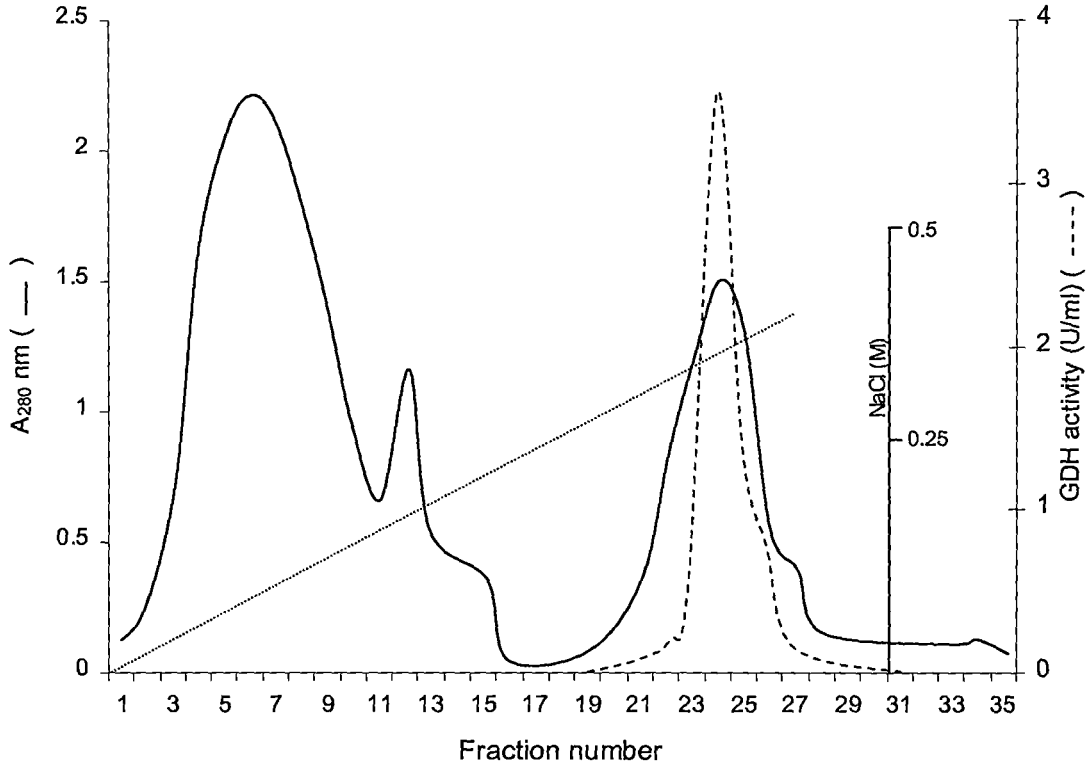


Fig 24: Elution profile of GDH from the liver of 90-day old mice through DEAE-Cellulose. The details of the experiment are described in detail in materials and methods section. GDH was eluted by a linear gradient of 0-500mM of NaCl and proteins eluted were monitored spectrophotometrically at wavelength 280nm and GDH activity assayed at 340 nm.

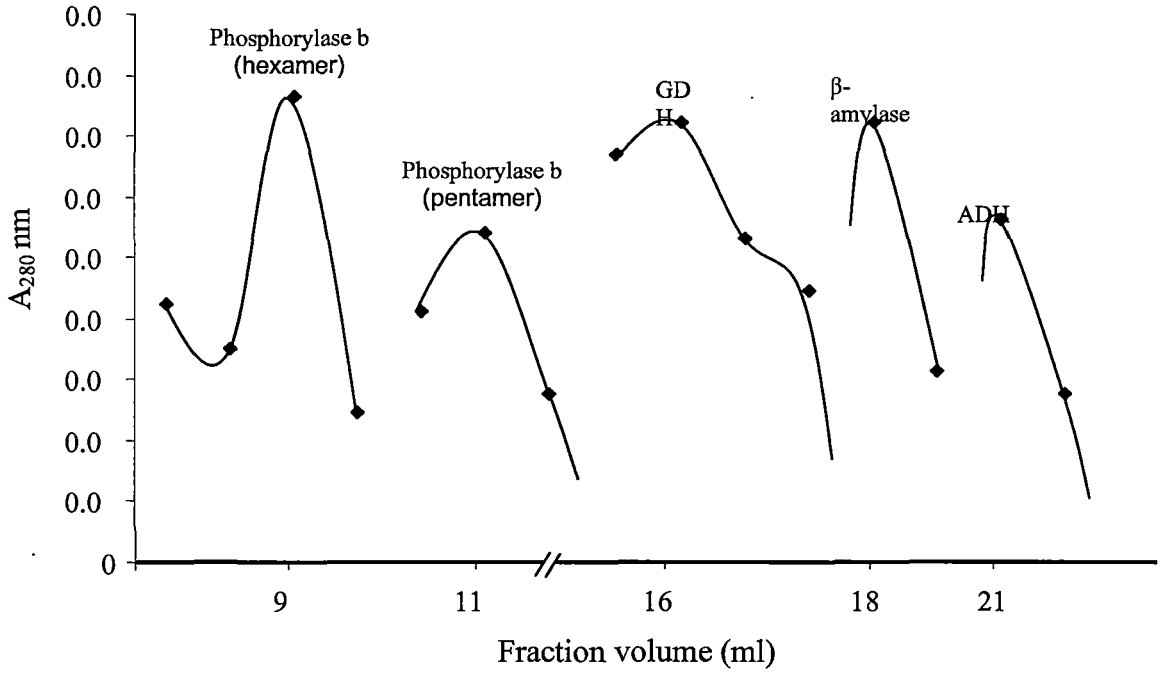


Fig 25: Calibration of Sepharose 6B column using various marker proteins. The protein molecular markers are- phosphorylase b hexamer (584 kDa), phosphorylase b pentamer (487 kDa), β-amylase (200 kDa) and alcohol dehydrogenase (150 kDa).V_0 andV_p are the void and total volume determined by passing blue dextran and $K_3[Fe(CN)_6]$, respectively.

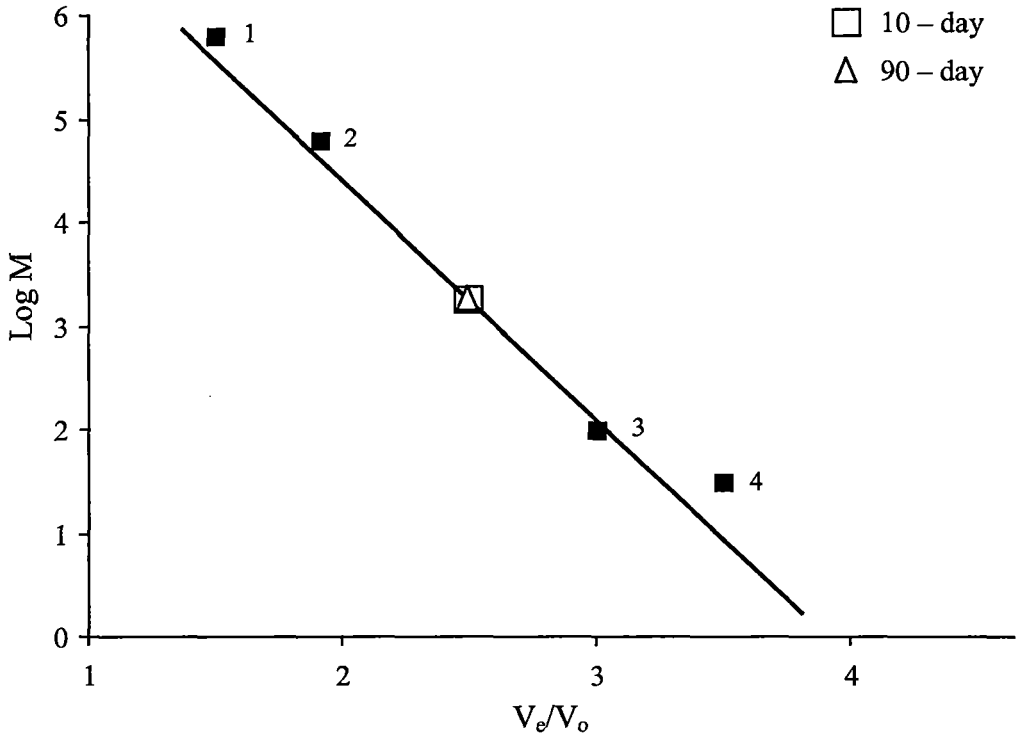


Fig 26: Plot of elution volume/void volume (V_e/V_o) versus logarithm of molecular weight (log M). The protein molecular markers are-phosphorylase b hexamer (584 kDa), phosphorylase b pentamer (487 kDa), β-amylase (200 kDa) and alcohol dehydrogenase (150 kDa).Straight line was computed and drawn using regressed equation of Sigma Enzfitter programme.

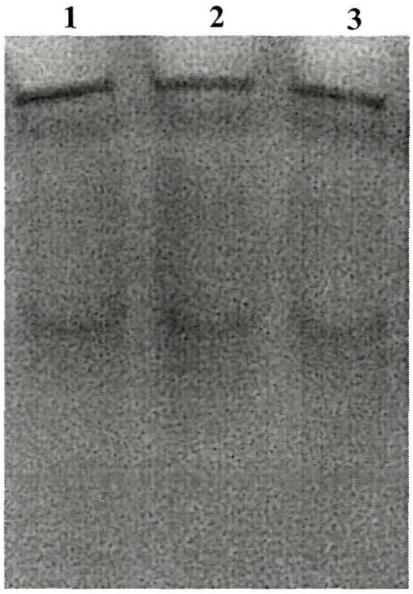


Fig. 27: Polyacrylamide gel electrophoresis of GDH from the liver of 10- and 90-day old mice. Lanes 1 and 2 was loaded with GDH purified from 10- and 90-day old mice and lane 3, bovine GDH.

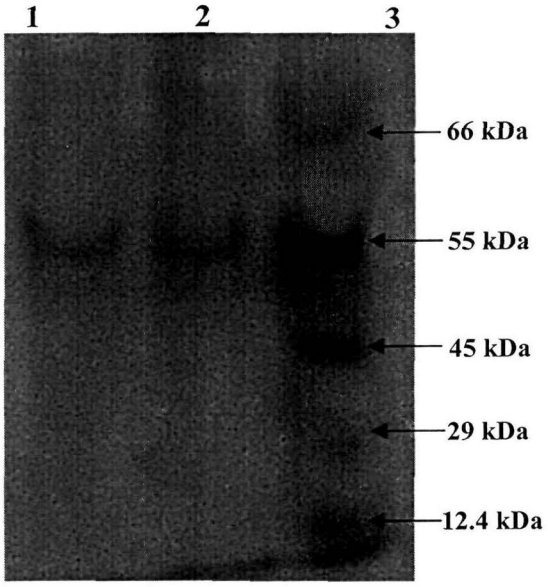


Fig 28: SDS-PAGE of GDH from 10-(lane1) and 90-(lane2). The standard markers are BSA (66 kDa), bovine GDH (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa).

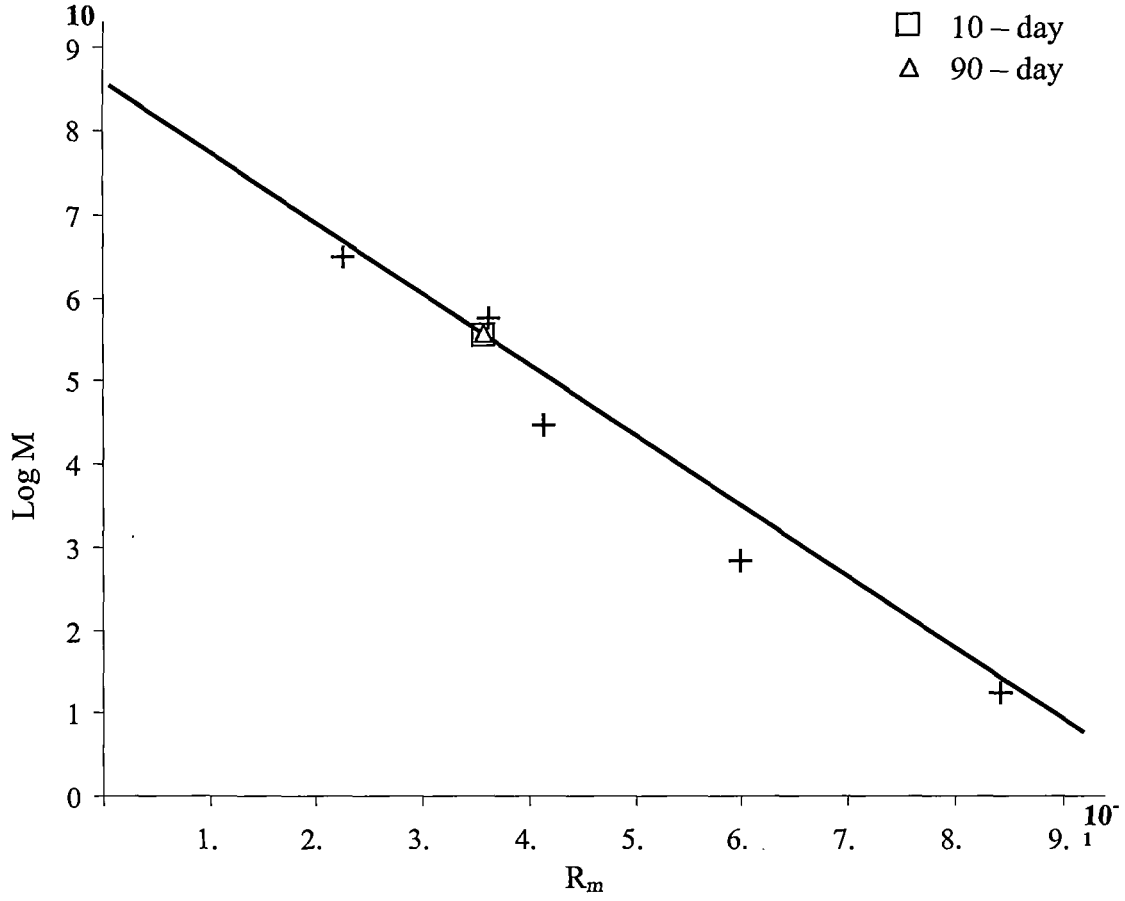


Fig 29: Plot of log M versus relative mobility (Rm) for the determination of subunit mass of liver GDH from 10- and 90- day old mice. Marker proteins include BSA (66 kDa), bovine liver GDH (55 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and Cyt C (12.4 kDa). Plot was computed using linear regression of Sigma Enzfitter programme.

Table 12: Purification table for GDH from the liver of 10-day old mice

	Protein recovery (mg)	Activity recovery (U)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Mitochondrial extract	1745	9213	5.28	1	100
$(NH_4)_2SO_4$ fractionation (30-60%)	141	6486	46	8.7	70
Sepharose 6-B eluate	17	2244	132	25	24
DEAE-cellulose eluate	3	1962	654	124	21

Details of the experiments are described in materials and method section

Table 13: Purification table for GDH from the liver of 90-day old mice.

	Protein recovery (mg)	Activity recovery (U)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Mitochondrial extract	2945	6626	2.25	1	100
$(NH_4)_2SO_4$ fractionation (30-60%)	244	4664	19.1	8.5	70.4
Sepharose 6-B eluate	27	1523	56	25	23
DEAE-cellulose eluate	4.9	1391	280	124.8	20.9

Details of the experiments are described in materials and method section

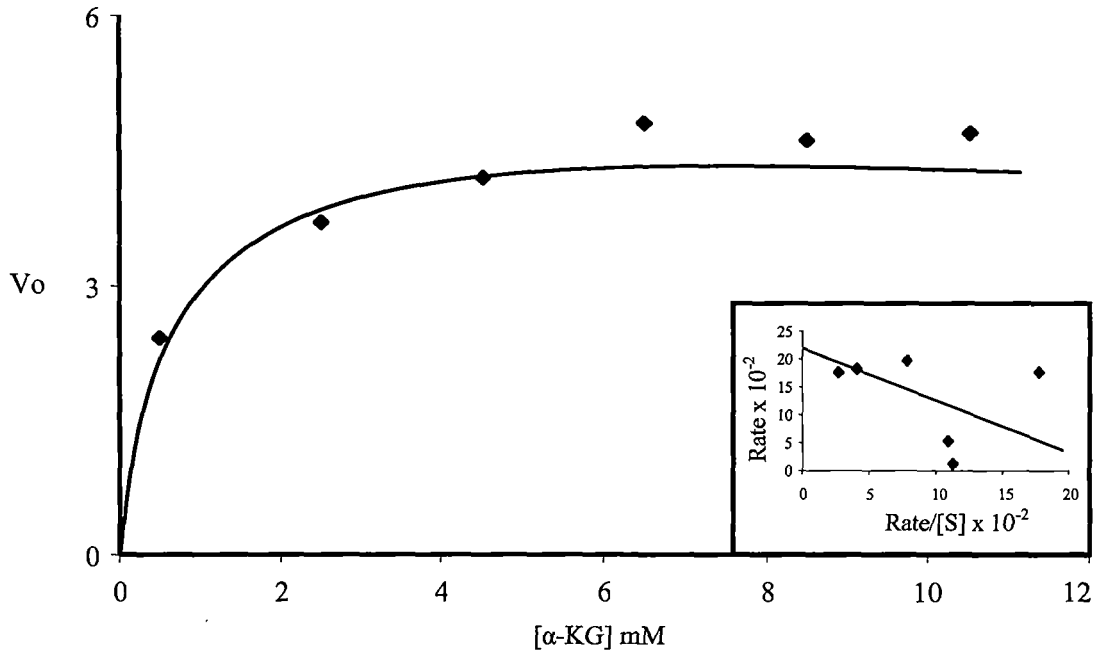


Fig 30: Plot of V versus [S] gives the Michaelis-Menten curve computed and drawn using the Enzfitter programme of Sigma. Inset to figure is the Eadie-Hofstee transformation. The plot yields the K_m, K_{cat} and V_{max} values for α-ketoglutarate of liver GDH purified from 10-day old mice.

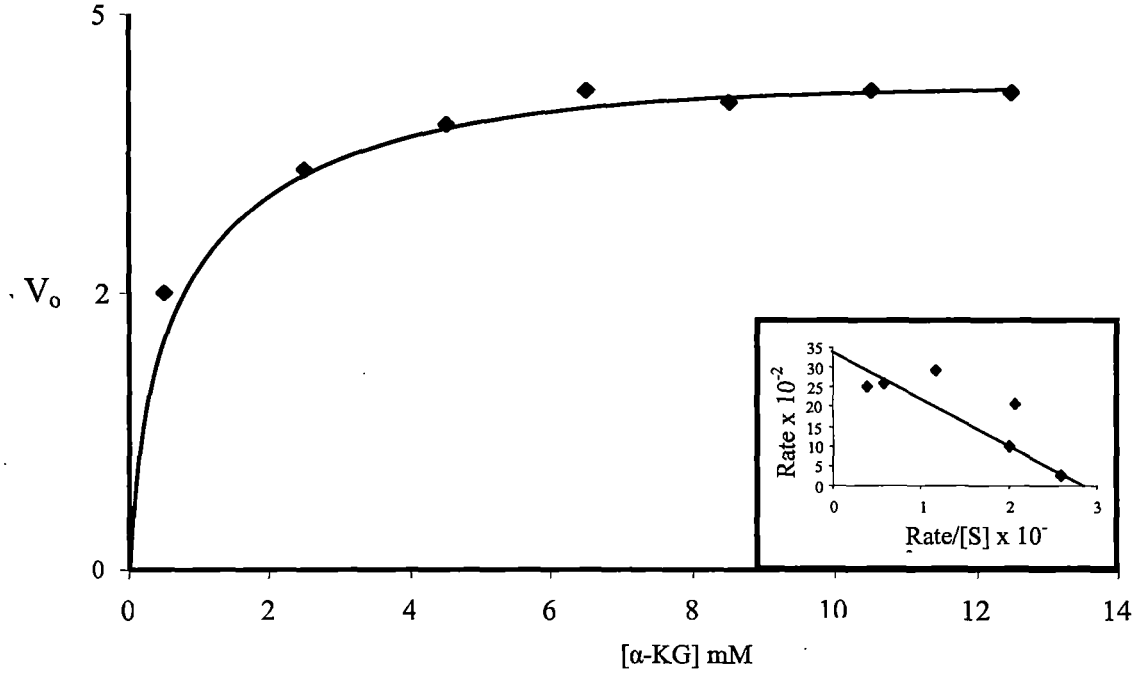


Fig 31: Plot of V versus [S] gives the Michaelis-Menten curve computed and drawn using the Enzfitter programme of Sigma. Inset to figure is the Eadie-Hofstee transformation. The plot yields the K_m, K_{cat} and V_{max} values for liver GDH purified from 90-day old mice.

Table 14: Michaelis-Menten kinetics of GDH using α- ketoglutarate as substrate

Parameters	10 Days	90 Days
K_m (mM)	5.2	6.1
K_{cat} (sec^{-1})	294	297
V_{max} (μM/min)	4.6	4.7

Immunological studies

The immunogenicity of the antigen (purified liver GDH from either age) against the antiserum (raised in rabbit) was ascertained by Ouchterlony immunodiffusion assay. As shown in figure 32, a qualitative precipitin line was formed between purified liver GDH (Ag) and the polyclonal antibody (Ab) of the serum, but not with BSA, a test protein. The precipitin line formed in the equivalence zone confirmed the immunogenicity between the antiserum and the purified liver GDH.

Quantitative determination of the purified liver enzyme from the two ages was done through Western blotting. The purified antigen from the immature and matured mice was subjected to PAGE and the bands formed were then electrophoretically transferred onto a nitrocellulose membrane (blot). The blot was incubated in the presence of primary antibody (polyclonal) followed by another in presence of conjugated secondary antibody and processed as detailed in material and method section. As shown in figure 33 the intensity of the band was more in the immature (10-day) compared to that of the matured (90-day) mice. The difference in the intensity of the bands between the two ages possibly reflects the level of abundance of mitochondrial GDH in the liver of the 10-day compared to 90-day old mice.

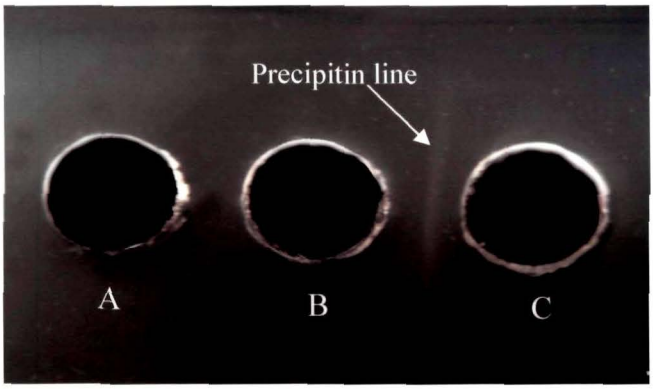


Fig 32: Formation of precipitin line by Ouchterlony immunodiffusion on 1% agarose between rabbit serum (containing Ab raised for mice GDH) and the antigen (purified liver GDH). The three wells A, B, and C contain BSA; rabbit serum and purified mice liver GDH respectively.

Fig 33: Western blot analysis of liver GDH from the two age groups- 10-day (band 1) and 90day (band 2) old mice

CHAPTER - IV

DISCUSSION

The earliest work on changes in enzyme activities during development established that enzymes were synthesized at discrete times rather than being gradually accumulated throughout the period of development (Walker, 1983). These enzymes were clustered or group according to the time at which they were first synthesized. These includes late fetal, neonatal and late suckling with 15-20 enzymes being organized in each group with definite patterns of development (Greengard, 1971).

Glutamate dehydrogenase has been studied intensively because of its importance in nitrogen and carbon metabolism (Fisher, 1985; Koppe *et al.*, 1966; Timmerman *et al.*, 2000) as well as its role in neurodegenerative disorders (Duvoisin & Plaitakis, 1984) and hyperinsulinism-hyperammonemia (Stanley *et al.*, 1998; Yorifuji *et al.*, 1999). Further, the activity of this enzyme is considered to be of major importance in the development of catabolic conditions leading to gluconeogenesis prior to birth (Timmerman *et al.*, 2002). Much evidence has shown that the enzyme is widely distributed in various tissues, but that its expression levels and activity vary. Thus, RNA blotting analysis (Mavrothalassistis *et al.*, 1988) have shown that the GLUD-related transcripts are ubiquitous in many of the mammalian tissues with liver and brain being the major sites of GLUD expression. However there are species differences; the intestine and heart are the major sites of GLUD-related mRNAs in rabbit but are moderate or minor sites in monkey and human. Further it was shown through immunoblotting analyses that GDH expression in the brain, heart, liver, stomach and muscle was higher compared to those in the lung and testes, an

indication that GDH expression is tissue-specific (Jang *et al.*, 2003). These tissue-dependent differences may be indicative of fine regulatory adjustments.

Metabolic fluxes through pathways depend not only on cellular concentrations of enzymes but also on substrates, co-factors and other regulatory factors. Glutamate the main substrate of GDH is present in fairly high concentration in the liver (Boon *et al.*, 1991) and brain, where it serve as an important neurotransmitter (Fonnum, 1984). Glutamate serves as an important carbon source for gluconeogenesis especially in the fetal/neonatal liver, where increased α-ketoglutarate production from glutamate oxidation by GDH is directed into the TCA cycle (Timmerman *et al.,* 2002). It has also been reported that glutamate; a metabolite produced during the course of gluconeogenesis, upon administration to adrenalectomized rat, mimics cortisone in eliciting stimulated purine nucleotide biosynthesis in liver (Feigelson & Feigelson, 1966) suggesting that the gluconeogenic intermediate; glutamate, possibly mediate certain other aspects of hormone function at other target sites.

Tissue distribution

The specific activity of GDH exhibited an age- and tissue specific pattern of distribution with the highest activity observed in the liver, followed by the kidney, brain and heart.

The high activity of GDH in the *liver* of the immature compared to the matured mice is possibly due to the role played by this enzyme in nitrogen and carbon metabolism especially in carbon supply for gluconeogenesis during the first 10-days after birth. Furthermore, the liver metabolic functions are not only dictated by local needs but also directed towards the control of other systems. This increase in liver

GDH activity in this study coincides with the reported increase in mitochondrial DNA (mtDNA) content of the developing liver during the early postnatal period, indicating the increased metabolic activity at these ages (Prieur, *et al.,* 1998). In the rat, a relatively low activity of hepatic GDH activity has been reported with activity increasing just prior to birth (Koppe *et al.*, 1960; Barnes *et al.,* 1978) coinciding with a rise in glucocorticoid level at that stage. Further it has been shown that GDH mRNA expression levels increased two days prior to birth, followed by a decline at prebirth, and then rise again to peak levels at 3-4 postnatal weeks. The capacity of liver to synthesize urea has been shown to reach adult levels during the few days after birth as indicated by the level of arginine synthetase which rises to 55% at 10 day postnatally (Kretchner *et al.*, 1966; Raiha & Suihkhonen, 1968). The level of GDH activity, which is also considered as accessory enzyme to the urea cycle, also coincides to the rise of activity of arginine synthetase, which showed maximum activity at 10 day postnatally, this may serve as a possible indication of the role played by GDH in urea synthesis at the immature stages. Reports using histochemical analysis in developing marsupial liver have shown that by the 9th postnatal day large accumulations of glycogen were present in the majority of hepatic cells under observation and remained abundant for another 6 days or more (Krause *et al.*, 1975).

The level of activity of GDH in the *kidney* exhibited a significant increased during the first 10 days after birth which decreases significantly at day 30. The pattern of enzyme distribution was similar in both liver and kidney, with a rise in activity during the first 10-days followed by a significant decline to adult level thereafter, though GDH activity was comparatively higher in the liver. The lower activity observed in the kidney compared to liver may be correlated to the role of GDH in gluconeogenesis in this tissue; where it was reported that the capacity for

gluconeogenesis in the mammalian kidney is less than that in the liver (Krebs, 1964). In developing rat kidney, an active process of cortical cell proliferation and differentiation occurs as late as day 20. The medulla was the most immature zone at birth and displays the greatest morphological changes during this period (Márquez *et al.*, 2002). Cell proliferation and differentiation demands a lot of biosynthetic activity and possibly involves active participation of GDH as well as other enzymes. In the rat kidney, several functions such as glucose and sodium reabsorption or gluconeogenesis appear at the end of the fetal life and increases rapidly after birth (Geloso, *et al,* 1974; Lelievre-Pegorier, *et al,* 1980; Delaval, *et al,* 1979). Na, K-ATPase, the major ATP consumer in the adult kidney, exhibits a marked increase in activity between days 19 and 20 day of gestation and another one during the first 24 h of the extrauterine life (Prieur *et al.*, 1995), therefore, increasing amount of energy is required for perinatal kidney differentiation. At the end of the fetal life the mitochondrial oxidative properties are low, but reach 80% of the adult values one day after birth (Delaval, *et al,* 1990). The increased GDH activity at immature ages may correlate with the role played by this enzyme at these stages especially in terms of energy metabolism; linking amino acid catabolism to the Krebs cycle. It may also be mentioned that mammalian kidney become fully operational during the early stages after birth as indicated by LDH level in mice which showed maximum activity during the first few weeks after birth (Rugh, 1968).

The *brain* tissue exhibited an overall increase in GDH activity with the maximum activity observed at day 90. This result is in agreement with earlier findings of Rothe *et al.*, (1983) who showed that a steep rise was observed in the activity profile of aspartate aminotransferase (AAT) and glutamate dehydrogenase in rat brain which was discussed as being a consequence of the maturation of preferably

glutamatergic structures. These results points to a quantitatively significant participation of glutamate transmitter metabolism in the CNS. The GDH activity profile in the brain also coincides with the earlier reports, that glutamate transporter (GLT1) and high affinity glutamate transporters (GLAST) expression and GDH activity in astrocytes is associated with the formation of glutamatergic synapses, neuronal structures involving glutamate as transmitter, in the astrocytes of developing rat hippocampus (Schmitt & Kugler, 1999). It may be mentioned that the brain and spinal cord is the most highly differentiated tissue of the vertebrate's body with no residue of undifferentiated cells functioning as growth centre. The result is that the brain and spinal cord throughout life grow more slowly than other parts of the body (Balinsky, 1981). Studies have also shown that cerebral oxygen consumption and the activity of the associated enzymes are low in fetal life and at birth, and then rise rapidly during the period of cerebral growth and development, and reached a maximal level at about the time maturation is completed (Timiras, *et al.*, 1973; Clarke & Sokoloff, 1999). Whereas other studies have shown that in the CNS neurons, GDH expression was observed to be significant only at the mRNA level but not at protein level, suggesting that under normal conditions GDH in neurons is involved in the general ammonia metabolism rather than metabolism of transmitter glutamate (Schmitt & Kugler, 1999).

The *heart* tissue exhibited a significant increase in the specific activity during the first 10 days after birth and no significant change was observed at later stages of development. It has been reported that mitochondrial content and enzyme activities are low in early fetal heart, and although enzyme content is similar in the late fetus and adult, mitochondrial enzyme activities increase two fold postnatally, indicating that fetal heart mitochondria become completely developed only during the postnatal

stages (Rolph, 1982). GDH activity in this tissue also correlates with the observed transhydrogenase activity that undergoes two steep increases of activity, one at birth and another between 15 and 25 days postnatally (Andres *et al.*, 1983). Several enzymes have been reported to increase their activity in this tissue which commensurate with their role in energy metabolism at these ages. For instance it has been reported that, NADPH generating enzyme like the "malic" enzyme (Andres *et al.*, 1980) and cytoplasmic thioredoxin undergoes increase activity at birth (Andres *et al.*, 1983) which possibly coincide with the higher activity observed for GDH at this age, since this enzyme also generates NADPH during oxidative reactions. It has also been reported that the distinction between the left and right ventricular activities of lactate dehydrogenase, which is visually clear in adult guinea pigs, was observed to be absent in the fetus and appeared only during postnatal development, other examples include the palmitylcarnitine transferase, an enzyme involved in fatty acid transportation in the mitochondria, the activity was reported to be low in the fetal heart continued to increase substantially during the first 2 wk after birth (Barrie and Harris, 1977). The observed higher activity at the early postnatal age may be correlated with the increased energy demand as well as the biosynthetic activities in this tissue during the initial periods after birth.

Hormonal studies

Hormones and receptors levels also play an important role in the development and/or aging of an organism. In the past there were a number of attempts to study the role of hormones in enzyme inductions during the course of lifespan. In many of these studies the induction by hormones indicated an age- (Singhal 1976a, Singhal, *et al*,

1969) and tissue- specific responses (Chainy & Kanungo 1976, 1978a; Sharma & Patnaik, 1983, 1984).

Developmental changes in relation to the effect of dexamethasone on amino acid transport and metabolism have been previously noted. In newborn babies and in new born rats, dexamethasone, infusion causes a marked increase in plasma concentration of several amino acids, including glutamine and alanine (Girard *et al.*, 1976; Ng *et al.,* 1992; Williams & Jones, 1992) whereas in adult men, it causes no significant concentration changes (Wise *et al.*, 1973). It has also been shown that cortisol increases the activity of hepatic glucogenic enzymes and hepatic glycogen storage (Barnes *et al.,* 1978) and diversion of hepatic glutamate carbon to flux into the citric acid cycle involves participation of dexamethasone. On the other hand, it has been reported that, in sheep, dexamethasone infusion in the fetus decrease the hepatic uptake of glucogenic amino acids and does not induce hepatic glucose output (Timmerman *et al.*, 2000). Sex steroids for instance, testosterone and 17β-estradiol have been reported to exert age- and sex-dependent synthesis and phosphorylation of brain cortex proteins (Mukherjee *et al.*, 1999) and their action have been reported to change as a function of age (Thakur, 1988). Testosterone is also implicated with modification of brain androgen receptor gene in an age- and tissue-specific manner, resulting in genetic instability in the brain of aged female mice (Thakur *et al.*, 2000).

Single hormone treatment

The hormones used in this study included the effect of thyroid hormone triiodothyronine, dexamethasone and testosterone injection on selected tissues, namely the brain, heart, liver and kidney from 10- and 90-day old mice.

In the *heart*, T_3 administration showed an age-specific response with a 2.5 fold increase in the immature mice. In the adult mice, GDH activity was slightly above basal level, indicating that the hormone may be more involved in homeostatic regulation at this age. In general T_3 is known to play a role in the alteration of the DNA and RNA content of heart and liver mitochondria (Di Meo *et al.,* 1992; Mutvei *et al.*, 1988). The *brain* tissue exhibited a 4 fold increase in GDH activity of the immature mice whereas; a 2 fold increase was observed in this tissue of the matured animal. T_3, have been reported to exert differential response in terms of age- and tissue-specificity. It has been shown to increase respiratory activity in the brain of neonatal and 11-day old euthyroid and hypothyroid rats (Rajan & Katyare, 1982), the age-specific influence of thyroid hormone on up-regulation of β-adrenoceptors in mouse brain cortex has also been reported (Viticchi *et al.*, 1990). This possibly correlates to the increasing involvement of GDH in energy production as well as in the metabolism of the neurotransmitter glutamate in response to T_3 treatment. The *kidney* also exhibited a marked enhancement in GDH activity with the matured mice showing higher response compared to its immature counterpart. T_3 is also implicated with the tissue-specific enhancement of mitochondrial oxidative respiration in the rat kidney (Satav & Katyare, 1991), its role in amphibians during metamorphosis has also been reported, implying the importance of T_3 during development (Balinsky, 1979). In the *liver*, which showed maximum increase (306%) of GDH activity, the pattern of induction by T_3 hormone was similar to that of the kidney, but with the matured mice exhibiting higher enzyme activity compared to that of the immature mice. The liver tissue in general assume greater role in metabolism. GDH being central to carbon and nitrogen flux is expected to show higher activity in liver which commensurate well with the role in this tissue. Further, the tissue-specific action of thyroid hormones can

also be explained partly on the basis of the number of nuclear binding sites present in the responsive tissues (Oppenheimer, 1979, 1983). Presence of specific binding site for thyroid hormones in the mitochondria from various organs i.e. liver, kidney, intestine, heart, lung and skeletal muscle has also been well described (Oppenheimer, 1979; Hashizhume & Ichikawa, 1982; Muller & Seitz, 1984; Nunez, 1988). T_3 is known to have a significant effect on the respiration rates (state 3 and state 4) in the brain, kidney, heart, liver and other tissues (Satav & Katyare, 1991; Katyare *et al.*, 1994). It has also been shown that in the liver of thyroidectomized rats the level of GDH activity was significantly increased by T3 administration (Yaroni & Balinsky, 1984). Further, thyroid hormones are also known to modify the level of mitochondrial DNA content in heart and mitochondrial RNA content in heart and liver (Di Meo, *et al.*, 1992; Mutvei, *et al.*, 1988). The influence of thyroid hormones on the mitochondrial respiration could serve as a possible link with the hormone-induced increased GDH activity. The increased GDH activity along with other dehydrogenases may be required to sustain the high respiratory rates resulting from thyroid hormone treatment especially in the brain (Katyare *et al.*, 1994).

Dexamethasone (dex) treatment also resulted in a significant increase in the specific activity of GDH in the brain, heart, kidney and liver. In the *heart*, GDH induction by dex was rather age specific. The immature mice showed no significant response to dex treatment, but a 2 fold increase in GDH activity was observed in the matured mice, indicating that this hormone may be associated with the energy metabolism in this tissue at this age. In the *brain,* only the matured mice responded significantly to dex treatment with a 3 fold increase in GDH activity observed. This possibly indicates the age specific action of this hormone in this tissue. In *kidney*, the response to dex administration was similar in both ages with a 1.7 fold increase in

GDH activity observed in the kidney of immature and matured mice. This may imply the homeostatic role of this hormone in regulating carbon and nitrogen metabolism especially in gluconeogenesis and ammoniagenesis in kidney tissue of both the ages. The *liver* exhibited a similar pattern of induction with a 2 fold increase in GDH activity in both the ages was observed. Earlier studies have shown that in the rat, a relatively low activity of hepatic GDH was observed with the activity increasing just prior to birth, coinciding with a rise in glucocorticoid level at that stage (Koppe *et al.,* 1966; Barnes *et al.,* 1978). It has also been shown that glucocorticoid administration result in enhanced glutamate formation which, in turn influences purine nucleotide and protein biosynthesis. The hepatically generated glutamate may serve as an amplifying mechanism by which glucocorticoids influence metabolic processes in various target organs. It may be mentioned that, among the earliest responses to glucocorticoid administration is rapid augmentation in liver and inhibition in thymus and spleen in the rate of glycine-2-^{14}C incorporation of acid-soluble adenine nucleotide (Feigelson & Feigelson, 1996) whereby, glutamate serve as an important nitrogen source. It has also been demonstrated that dexamethasone infusion caused a significant increases in GDH gene expression accompanied by an increase in mitochondrial GDH activity of the liver (Regnault, 2003). Further it has been shown that fetal dexamethasone infusion caused a marked reduction in the net release of glutamate from the fetal liver whereby, glutamine carbon flux was redirected into the citric acid cycle, suggesting increased placental GDH under these conditions (Timmerman, *et al.*, 2002) and is considered to be of major importance in the development of catabolic conditions leading to gluconeogenesis. Earlier studies have reported that adrenalectomized rats showed decreased level of the enzyme GDH, and this decrease in the level of the enzyme can be restored to a normal level by a single

injection of physiological doses of cortisol (Yaroni & Balinsky, 1984). Thus, it appears from these studies that the maintenance of normal level of GDH in adult animals is dependent on continued induction by glucocorticoid. Further, the inhibition of this response by actinomycin D and cycloheximide confirm the involvement of RNA and protein synthesis in this induction (Yaroni & Balinsky, 1984a, 1984b; McLean & Gurney, 1963). These reports along with our findings, signifies the role of glucocorticoids in inducing gluconeogenesis through gluconeogenic enzymes including phosphoenolpyruvate carboxykinase and GDH in the fetal and neonatal animals, and a general increase in the turnover of other metabolites. Furthermore, steroid hormones are known to increase the mtDNA content of developing liver during the postnatal period. Such influence was not reported in the developing heart tissue, suggesting a tissue-specific regulation of mitochondrial replication and transcription by adrenal steroid hormones in the developing organs (Preiur, *et al.*, 1998).

Testosterone administration alone did not evoke significance responses in any of the tissues of the two ages (10- and 90-day) studied. However, there were some responses when given in combination with T_3 hormone. This lack of response may be attributed to low levels or down regulation of receptors in the immature and mature mice, respectively. It has been reported that in primary cultures of rat hepatocytes, no influence of testosterone or estrogen on glutamine synthetase (GS) activity was observed, even after stimulation of glutamine synthetase activity with dexamethasone and growth hormone (Sirma *et al.*, 1996). This effect on glutamine synthetase may be extended to the observed lack of effect of testosterone on GDH, since both of these enzymes are involved in glutamate metabolism. Testosterone levels have been reported to be already at high levels in adult males (Hansen & Jungermann,

1987) which may account for the absence of effect of the hormone vis-à-vis GDH in mature mice. However, in young mice the lack of response may be accounted by low receptor. It may also be mentioned that an inverse relationship (Thakur, 2004) between androgen receptors and testosterone could be responsible for the lack of response. Similar observations have been reported with other dehydrogenases utilizing NAD/NADH where no changes was observed in activities of the enzymes glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malate dehydrogenase of male rat hepatocytes culture when treated with testosterone (Hansen & Jungermann, 1987).

Hormone combination treatment

The hormone combination used (dex-T_3 and test-T_3) showed an age- and tissue- specific response in the various organs of immature and matured mice.

Dex-T_3 administration exerted synergistic effect, with a significant 4 fold induction in the *heart* of immature mice, in contrast to the individual effects of either dex or T_3 hormone. Single T_3 administration as stated above, exerted significant response only in the young mice while dex showed no effect at the young ages but caused an increase in GDH activity in the heart tissue of adult mice. This response to these combination possibly imply that in the immature mice GDH plays an important role in nitrogen and carbon metabolism with a concomitant increase in energy demand coinciding with the pre-weaning period. In adult mice these hormones may be involved in homeostatic regulation only. The lack in response in adult may be attributed to the fact that the basal ATP production has already reached its maximum, since heart has more energy demand than other organs (McDaniel *et al.*, 1986). Besides, in the heart, GDH is known to exists in two different forms or isoenzymes which showed more activity when glutamate is oxidized in presence of pyruvate and

octanoic acid. Because of the functional differences it is reasonable to expect differences in the regulation of GDH activity in heart (McDaniel *et al.*, 1986).

Test- T_3 administration resulted in no significant change in GDH activity in the *heart* tissue of both immature and matured mice. The lack of response to this combination in both the ages may indicate that testosterone act as an antagonist to T_3 action which alone exerted a 2.5 fold increase in GDH activity in this tissue in immature mice.

In the *brain* Dex- T_3 combination exerted a significant 5 fold increase from that of the control especially in the immature animal compared to the matured which showed a rather antagonistic effect. This is possibly because of the role played by GDH in neurotransmission which may be amplified by concerted synergistic action of these hormones during developmental stages. The observed decrease in the effect of this hormone combination in this tissue of the adult may correlate to the lesser sensitivity of the tissue to these hormones especially steroids which has been reported to show decrease responsiveness in target tissues during senescence (Thakur, 1988).

In the *brain,* Test- T_3 combination treatment produce an effect that was rather age specific in which the immature mice showed an apparent antagonistic effect (167%) when compared to single T_3 injection (297%). In the adult mice however this tissue exhibited a moderate synergistic increase over single T3 administration. Thus, it may be inferred that this hormone combination exert a rather differential response in this tissue with respect to age.

In the *kidney,* GDH activity was significantly enhanced in this tissue with synergistic response observed in the adult mice following dex-T_3 administration as

compared to the induction by the individual hormones. The higher response in adult mice may indicate the abundance of matured receptors in this tissue. It has also been suggested that dexamethasone exerts a permissive action of growth hormone (GH) in inducing glutamine synthetase in the adult liver, an enzyme that is involved in glutamate metabolism (Gebhardt, 1979). Likewise dex may play a similar role in this tissue at this age by complimenting to T_3 induction of GDH. Such synergistic effects between hormonal actions have also been reviewed for several enzymes (Sharma, 1993).

Test- T_3 combination treatment appeared to play an antagonistic role in the *kidney* of both immature and matured mice, while T_3 single administration resulted in a significant increase in GDH activity in both the age group of mice. The antagonistic effect was more pronounced in the immature compared to matured mice. As earlier suggested, it may be possible that, T_3 regulation of sex steroid receptors is less effective at matured ages in this tissue (Thakur & Sharma, 2006).

Dex-T_3 injection showed an apparent synergistic action in the *liver* tissue which resulted in a significant increase in the GDH activity in both the ages as compared to the individual effect of the dex and T_3 hormones. Liver is the most responsive tissue to this hormone combination possibly because of the role of this tissue in carbon and nitrogen metabolism and energy production which caters not only locally but to the peripheral tissues as well. The permissive action of dex on T_3 action may also holds true for this tissue as discussed earlier (Gebhardt & Mecke, 1979).

Test-T_3 combination treatment showed antagonistic effect in the *liver* of immature animals; in contrast, the matured showed an apparent synergistic effect. This finding may suggest the differential role of these hormones with regards to age,

sex, and tissue in the developing and matured animal especially on the nitrogen and carbon metabolism and energy status of the tissue.

The observed enhancement of GDH activity by these hormone combinations in some of the tissues studied is largely unclear, in part, it may be a sum of the individual contribution of the two hormones used, or a possible interaction or cross-talk in between the signal transduction pathway, past the hormone receptors (Sharma, 1993).

Study of GDH physico-chemical properties

The activity and stability of GDH is known to be influenced by a number of factors which includes the pH, ionic strength, types of substrates, inhibitors and their concentration, and reducing agents (Neumann *et al.,* 1976; Gafni & Yuh, 1989b; Smith and Piszkiewicz, 1973). In this study, we sought to compare the enzyme activity and/or stability of GDH from liver mitochondria of immature (10-day) and matured (90-day) mice, using dialysed preparations. Dialysis ensures removal of endogenous substrates and co-factors/ metal ions which might otherwise interfere with the physico-chemical assay.

Effect of Ionic strength

Using buffer of varying ionic strength, it was observed that GDH from the 10- and 90-day old mice exhibited a similar molar optima, indicating that there was no alteration in the conformation of the enzyme at these ages.

pH optima

The pH profile of GDH from both the ages (10- and 90-day) was apparently similar with optimum enzyme activity observed at pH 8.0, beyond which a decrease in enzyme activity was observed. Neumann *et al.,* (1976) have reported that the conformational transition of GDH from *Candida utilis* is pH- and temperature-dependent. The observed loss of activity at higher pH values may reflect deprotonation of cationic groups with pK values of 9 or higher. Such cationic groups might be those expected to interact with the carboxylic groups of the substrates, glutamate or α-ketoglutarate, or with the phosphate groups of pyridine nucleotide co-enzymes (Smith and Piszkiewicz, 1973).

Temperature stability

The effect of temperature on the stability of GDH from the two ages was also found to be similar. The enzyme activity was observed to be highest at 25°C for both the ages and looses more than 80% of enzyme activity above 55°C. In contrast, age related changes with respect to heat stability have been reported for many enzymes for example phosphoglycerate kinase, a glycolytic enzyme exhibited a marked increase in the protein's heat stability as a result of aging-related modification (Gafni & Yuh, 1989b).

Effect of reducing agents

Comparative study of the effect of reducing agents dithiothreitol (DTT) and 2-mercaptoethanol showed that, although the pattern of influence on the activity was similar in both the ages, DTT confer higher stability on liver GDH from both 10- and

90-day old mice. This signifies the role of the sulphydryl groups of the reagent in contributing to the stability of the enzyme, and the property did not change with age.

Inactivation studies

Dialysed GDH preparation from the liver of the immature and matured mice exhibited a similar response to guanidinium hydrochloride (Gdn.HCl) inactivation, with more than 50% decrease in enzyme activity observed at 0.1M for both ages. Sensitivity to this denaturant may provide an indication to the conformation of this enzyme. It was earlier suggested that, at concentrations of Gdn.HCl insufficient to cause denaturation, the hexamer dissociated into inactive trimers without any gross structural changes that could be detected by fluorescence or circular dichroism techniques (West & Price, 1988). Conformational changes including non-covalent modifications in proteins during aging have also been reported (Rothstein, 1979; 1985). Other studies shown that enolase, a glycolytic enzyme isolated from young nematodes, when subjected to denaturation by guanidine hydrochloride and allowed to refold, the folded protein showed a resemblance to the isomeric form with different catalytic property, present in the old nematode, signifying that the presence of young enolase in tissues of young animals attests to the presence in these tissues, of conditions that favour folding into this species (Sharma & Rothstein, 1978). Earlier biochemical works led to the finding that multiple forms of GDH are present in mammalian system and that the activities of these isotypes differ in their relative resistance to thermal inactivation, detergent extractability and allosteric regulation characteristics (Plaitakis *et al.*, 1984, 2000; Cho *et al.,* 1995; Shashidharan *et al.*, 1997). These forms have been designated as soluble and particulate GDH (Plaitakis *et al.*, 1984; Colon *et al.*, 1986; Hussain *et al.*, 1989). The GDH isoproteins are

differentially distributed in the two catalytically active isoforms of the enzyme (Colon *et al.*, 1986; Plaitakis *et al.*, 1993). The four different forms of GDH isoproteins were detected from the human cerebellum of normal subjects and patients with neurodegenerative disorders (Duvoisin *et al.*, 1983; Plaitakis *et al.*, 1984 Hussain *et al.*, 1989). Our studies however indicate that the liver enzyme conformation remains unchanged with age.

Inhibition studies

GDH is known to posses binding site for GTP, an allosteric modulator which negatively influence this enzyme. In the absence of ADP, about 60% inhibition of GDH activity in both ages was observed at 5 μM concentration; however, inhibition was minimized in the presence of the allosteric activator, ADP (1mM). GTP and ADP binds to distinct sites on the enzyme (Colman & Frieden, 1966) and, it may be mentioned that loss of GTP inhibition by mutation in the GDH gene carrying GTP binding domain leads to increase GDH activity that result in excess insulin and ammonia production (hyperinsulinism and hyperammonemia syndrome) and excess ammonia/glutamate is the possible causes of some neurodegenerative disorders (Yorifuji *et al.*, 1999; Cho *et al.*, 1996). From our studies, it is evident that the liver enzyme binding site for this modulator also remained unaltered with age.

Effect of co-substrates

Adenosine diphosphate

Adenine diphosphate (ADP), the allosteric activator of GDH is known to promote aggregation of the enzyme in solution thereby, activating it. Further, it was suggested that ADP activates GDH by facilitating the opening of the catalytic cleft

(Banerjee *et al.,* 2003). It may be mentioned that the binding site for ADP and NAD^+/NADH is identical and ADP increases the initial velocity of NADH oxidation by displacing the second inhibitory molecule of NADH, thereby, preventing the dissociation of GDH by NADH to its inactive form (Frieden, 1959b). In this study, ADP showed optimum enhancement at 1mM for the young and slightly more (1.5 mM) for the adult, indicating the sensitivity of GDH vis-à-vis the metabolic conditions and requirements at the two different ages. The reduced sensitivity in the adult mice to ADP activation has been attributed to a secondary modification of the GTP binding site, as the animal undergo development and/or aging (Jacobson & Colman, 1982). The ADP binding domain within two types of bovine brain GDH isoproteins (GDH I & GDH II) have been identified by photoaffinity labelling (Cho & Hye-Young, 1999). The functional areas and functional groups of bovine liver GDH have also been reported and it was suggested that the active sites and enzyme association sites are on different areas of the polypeptide chain (Hucho *et al.*, 1975). It has been shown that GDH undergoes aggregation and disaggregation under a variety of conditions including changes in concentration (Olson & Alfinsen, 1952) and with aggregation there are reciprocal changes in the rate of oxidation of glutamate and other substrates (Tomkins *et al.,* 1961). Other comparative studies using purified mitochondrial GDH from rabbit liver has shown that the mitochondrial enzyme showed greater stimulation by ADP when compared to its cytosolic counterpart (Kazarian *et al.,* 1985).

NADH and NADPH

Nicotinamide adenine dinucleotide, reduced (NADH), is the co-enzyme essential for GDH reductive amination reactions and functions by binding to a non-

active site of the enzyme molecule (Frieden, 1959b). The data obtained in this study showed that the optimum concentration of NADH was 125 μM and 150 μM for the young (10-day) and adult (90-day) mice respectively. Concentrations above these values result in an apparent inhibition of the GDH activity, probably because the enzyme dissociates into lower molecular weight molecule, an effect exerted by pyridine nucleotides when present at higher concentrations (Frieden, 1959a). On the other hand, the non-saturation or linear profile obtained with varying concentrations of NADPH in GDH activity from both the ages may correlate with the earlier findings (Frieden, 1959b), which demonstrated that NADPH cannot bind to the non-active site of the enzyme, as a result, it cannot cause dissociation of the enzyme nor can it displace ADP or ATP from the active site no matter how high the concentration.

Electrophoretic studies and mass composition

Analysis through electrophoretic mobility revealed no difference in the charge and/or electrical property between liver GDH enzymes from immature and matured (10- and 90-day) mice as a function of age. Further, this was strengthened and supported by ion exchange chromatography, which showed no difference in the degree of binding and molar elution of GDH from the two age groups.

GDH, isolated and purified from the liver of immature and matured mice was found to have identical mass and subunit size composition. The enzyme is a hexamer, consisting of six identical subunits, each having an approximate 55,000 Dalton, together totaling to approximately 330,000 Dalton, similar to that from the liver of its mammalian bovine cousin (Peterson *et al.*, 1997). It may thus be inferred that the enzyme from the two ages undergo no structural changes in terms of mass and subunit size.

Kinetic properties

GDH from the liver of immature and matured mice exhibits similar kinetic properties in terms of V_{max} and K_{cat} towards the substrate, α-ketoglutarate. However, they differ in their affinity for the substrate, with the enzyme from the immature mice showing higher affinity (low K_m) for the substrate compare to that of the matured mice. This is possibly a strong indication of the greater role of this enzyme in energy-related pathway during the developmental stages, in order to fulfill the intra- as well as extra-tissue demands. The kinetic data obtained in this study may reflect the ionization state of the enzyme as has been suggested that the V_{max} and K_m for the GDH oxidation of glutamate is dependent upon ionization of a probable cysteine residue with a pKa of 7.7 to 7.8 (Rogers, 1971). There are several enyme which have been reported to have altered kinetic property as a function of age (Sharma, 1988; Syiem & Sharma, 1996; Gafni, 1990).

Immunodetection

Immunodetection techniques using purified GDH from the liver of 10-and 90-day old mice provides an important insight into the antigenic property, as observed through immunodiffusion study, and quantitative (through Western blot) characteristics of GDH from immature and matured mice. The enzyme from both ages was observed to share similar antigenicity. It may be mentioned that recent reports have shown that isolated human brain GDH differed from other mammalian GDH in terms of immunogenicity and suggested the difference as a result of altered amino acid sequences or protein structure (Jang *et al.,* 2003). Western blot indicate the slightly higher levels of this enzyme in the liver of immature mice. Thus, implying that the level of expression at this age is slightly on the higher side compared to the

matured counterpart and possibly accounts for the higher activity concomitant with the role at the different developing postnatal period.

It may be concluded that the endogenous activity of GDH exhibited an age- and tissue specific pattern of distribution. The differential responses to hormonal treatment may indicate that this enzyme is under varying hormonal regulation especially at the early developing age. The physico-chemical properties of GDH from the liver of both age groups showed an overall similarity, except for the difference in terms of substrate affinity of GDH, which projects the role and commitment of this enzyme at the different stages of the animal life.

Summary

Glutamate dehydrogenase occupies a central role in nitrogen and carbon metabolism in practically all organisms. The reaction which it catalyses feeds the tricarboxylic acid cycle with carbon intermediate necessary for generating energy as well as precursors for other biosynthetic purposes such as in gluconeogenesis. GDH is also considered as an accessory enzyme to the urea cycle because of its involvement in NH_4^+ metabolism. It also plays an important role in the metabolism of the excitatory neurotransmitter glutamate in the central nervous system signifies the potential of this enzyme in influencing a wider aspect of metabolism in higher organisms, have gathered significant attention. However, its role in the development and/or aging of organism has not been adequately explored.

This study aims to determine the tissue distribution of GDH in mice at different postnatal ages and to gain insight into its regulation by various hormones at different postnatal ages. Further, this enzyme was purified from immature and matured ages to determine if any changes occur in its physico-chemical properties during the course of postnatal development.

Age and tissue distribution

GDH exhibited an age- and tissue-specific pattern of distribution in terms of activity with the highest activity observed in the liver, followed by the kidney, brain and heart. Except for the brain, which showed a gradual increase in activity with age, GDH from the liver, kidney and heart exhibited peak activity during the first 10 days after birth which declined to a more or less stable adult level after 30 days postnatally.

Hormonal responses

Administration of hormones to the immature and matured animals point towards an age- and tissue-specific pattern of response. Thus, it was observed that, T_3 administration resulted in 2.5 fold increase in GDH activity in the heart of the immature mice as compared to the mature mice which exhibited no response to this hormone. Whereas, dexamethasone treatment exerted response in the heart tissue of matured mice only. The brain tissue showed an overwhelming response to T_3 administration in terms of GDH activity with the immature mice exhibiting an excess of 2 fold over its matured counterpart and dexamethasone treatment evoked response only in the matured animal. T_3 administration also resulted in significant increase in GDH activity in the kidney and liver with the matured animals showing higher induction than the immature mice. Dexamethasone treatment however, induced similar responses in both the liver and kidney tissues with an approximately 2 fold increase in GDH activity for both the ages studied. Testosterone administration alone did not exert any significant changes in all of the tissues at both ages studied. Treatment with hormone combinations also resulted in differential response in the various tissues at the two ages. Thus, dexamethasone-triiodothyronine (dex-T_3) administration resulted in a significant 4 fold increase in GDH activity in the heart of the immature mice only with respect to the control, whereas testosterone-T_3 (test-T_3) treatment did not evoke any significant enhancement of GDH activity in this tissue at both the ages studied. In the brain, dex-T_3 administration resulted in a significant synergistic increase (5 fold) of GDH activity in the immature mice however; it was apparently antagonistic in the matured animal. Test-T_3 combination treatment exerted higher increase of GDH activity of the immature compared to the matured mice. In the kidney, dex-T_3 combination

treatment resulted in significant increase (3 fold) of GDH activity in the matured mice compared to the 1.7 fold increase in the immature mice. A moderate (1.6 fold) increase in GDH activity was observed in this tissue of the matured mice in response to test-T_3 administration. The liver tissue also exhibited a synergistic response to dex-T_3 treatment with a 2.8 fold increase in enzyme activity of the immature mice and 4 fold increases in the mature mice. Test-T_3 treatment also showed similar synergistic response vis-à-vis enzyme activity in the adult mice, however no significant response was observed in the immature mice in this tissue.

Physico-chemical comparison of GDH

The physico-chemical properties of dialysed GDH from immature and matured mice indicated that there was no alteration in terms of buffer ionic optima, pH and temperature stability. The enzyme from both the ages also showed an overall similar pattern of response to the effect of various co-substrates and co-enzymes. Inhibition and inactivation studies on the enzyme did not reveal any significant differences between the two ages and further comparison of the total and subunit mass also showed no differences. However, the only difference observed was in the kinetic behaviour of the enzyme towards its substrate; α-ketoglutarate with the matured mice showing higher K_m, compared to its immature counterpart. The antigenic property of glutamate dehydrogenase from the liver of immature and matured mice from both the ages remained unchanged.

In conclusion, this study has given some insight into the endogenous activity of GDH which was marked by age- and tissue-specific pattern of distribution and have also indicated the larger role played by hormones like T_3 and dexamethasone in the regulation of this enzyme.

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