REGULATION OF ADENOSINE DEAMINASE DURING POSTNATAL DEVELOPMENT OF CHICKEN

Abstract

By

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Thesis

Development is a continuous process by which organisms grow. The process of development onsets as soon as the fertilized egg begins to grow. It may be divided into two main periods, prenatal and postnatal. The prenatal development involves differentiation and encompasses three main stages- ovum, embryonic and fetal stages. Postnatal development begins with birth and continues into neonatal, infancy, childhood, adolescence and adulthood. Differentiation and development are programmed processes which occur due to sequential activation and repression of genes causing alterations in the levels of enzymes. The activities of several enzymes decrease and of several others increase as a function of age of an organism. Age leads to alterations in the levels of enzymes and their inducibility by certain hormones. The rate of synthesis of enzymes usually changes in response to changes in the extracellular environment. The regulatory mechanisms which control the level of enzymes at the biochemical level involve a change in the rate of synthesis and/or degradation and post-translational modifications. A number of hormones and growth factors exert an intricate but judicious control on the process of development in aves. The divergent morphology and physiology observed during development with many distinct complex and coordinated processes involve the control of the programmed circuits of gene expression. Organisms have certain genes which control specific events during development. Gene expression in organisms is regulated at essentially all possible levels- transcription, pre-mRNA processing, mRNA transport, mRNA stability, translation, and post-translational protein processing. The regulatory mechanisms with largest effects on phenotype have been shown to act at the levels of transcription and mRNA processing.

Adenosine deaminase (ADA; EC 3.5.4.4), a key enzyme participating in the purine salvage pathway, catalyzes the irreversible hydrolytic deamination of the substrates adenosine and 2'-deoxyadenosine to yield the products inosine and 2'-deoxyinosine, respectively. ADA is a well-characterized enzyme involved in the depletion of adenosine. It is essentially required for lymphocyte proliferation and differentiation. The physiological function of ADA is critical for controlling the levels of adenosine and 2'-deoxyadenosine in immunological, neurological and cardiovascular systems. ADA activity is widely distributed in human tissues and is highest in lymphoid tissues. In humans, rats and mice, the highest level of enzyme activity is found in

thymus, spleen, placenta, and in organs comprising the gastrointestinal tract, whereas low activity is found in muscle, lung and kidneys.

A correlation has been drawn with the failure of both B- and T-lymphocytes mediated functions due to deficiency of ADA because of different missense mutations in exon 4 of its gene. The absence of ADA in humans results in severe combined immunodeficiency (SCID), which is characterized by hypoplastic thymus, T lymphocyte depletion and autoimmunity. Abnormalities of this enzyme have also been reported in other diseases of immune system including AIDS, lymphomas, leukemias, anemia and several other unrelated disorders like short-limbed dwarfism, hepatitis and jaundice. Increased serum ADA activity has been reported in patients with liver diseases like chronic hepatitis and liver cirrhosis.

Various avian species provide models of evolution, development and differentiation, behaviour and ecology. Avian genomes appear to show relatively high levels of conservation, and the genome sequence and related information that are available for chicken provide added benefits for the genetic analysis of all wild and domestic birds. The immune system of chicken has the thymus, a paired lobulated gland along the neck of the chicken which is considered to be the source of T cells or small lymphocytes which mediate the rejection of hemografts, graft-virus-host reaction and delayed hypersensitivity. They also have the bursa of fabricus, an organ situated dorsally to the cloaca which is considered to be the source of B cells which form plasma cells and produce antibody. In chicken, the gene for ADA is located on chromosome 20 and is approximately 64 kb in length. Sp1 protein is essential for both enhancer-mediated and basal activation of ADA promoter.

Keeping in mind the role of ADA in the regulation of a wide array of physiological processes, the present work embodied in this thesis has been directed towards the following:

- To determine the normal endogenous activity level of ADA in different tissues of GIT (esophagus, crop, proventriculus, small intestine) and spleen at various postnatal ages (day 1, 10, 30, 60 and 90) of male chicken.

- To assess the role of hormones and their analogues like corticosterone (adrenalcortical hormone), testosterone (sex hormone), triiodothyronine (thyroid hormone) and a membrane permeable analog of cAMP, dibutyryl cAMP, on the activity of adenosine deaminase in different tissues of chicken at two specific postnatal ages (day 10 and 60) ascertained, and to postulate the effect of these hormones, if any, in modulation of ADA in a tissue- and age- specific pattern.

- To purify ADA from small intestine of two select age groups (day 1 and 90) of chicken and characterize its physicochemical and kinetic properties, with a view to analyze the alterations, if any, in these parameters of the enzyme during the course of development.

NORMAL ENDOGENOUS LEVEL OF ADA

ADA activity in the male chicken exhibits tissue specificity as well as age-related changes. The normal endogenous level of ADA activity (U/mg protein) has been ascertained for esophagus, crop, proventriculus, small intestine and spleen at various postnatal ages. The data indicate the highest level of activity in small intestine, followed by proventriculus, esophagus, crop and low activity of ADA in spleen. Our findings reveal that the normal endogenous level of ADA in the GIT of chicken is highest on the day of hatching. Amongst the regions of the GIT studied, the highest level of ADA activity is found in the small intestine followed by the esophagus, proventriculus and crop. In the esophagus, the activity is highest in day 1 and is seen to decrease significantly at day 10 and thereafter shows a slight decrease. In the crop, ADA activity is highest in day 1 showing a decrease at day 10 and then remains constant. The proventriculus is also seen to have a very high level of ADA activity at day 1 which shows a significant decline at day 30 and 90. Region specific studies indicate that in the small intestine, the level of activity is highest in day 1 followed by a sharp decrease at day 10 and then remains almost constant. Unlike all the tissues of GIT, the activity of ADA in the spleen is seen to increase maximally at day 30 after which there was a decline. In all the studies, our interest was to find out if there was a change in the level of protein of the enzyme for which the slot and Western blots were done for the two ages where differences were

observed in almost all cases. Representative data of the blots show that the level of the protein of ADA is indeed seen to change or remain constant for the ages studied. The avian gastrointestinal mucosal immune system has evolved with specialized features that reflect their role as the first line of defense on mucosal surfaces. High level of ADA on the day of hatching may ensure lower adenosine and better survival of lymphoid cells. Thus, a high ADA activity at early ages of chicken probably helps the tissues to cope with the increasing need for immunological competence, thus decreasing adenosine, which could otherwise exert unwanted physiologic effects.

HORMONAL REGULATION OF ADA

Our studies also show that corticosterone significantly inhibits the ADA activity in all the regions of GIT except proventriculus, in an age- and region- specific manner. In the spleen, corticosterone decreases the activity of ADA at both day 10 and day 60. The magnitude of inhibition is more pronounced at the later stage of chicken development (60-day) compared to a very young age (10-day). The findings of age- and tissue-specific inhibition may be correlated to the differential adaptive role and maturation of corticosterone action mechanism, its receptor and post-receptor events. Since corticosterone is immunosuppressive, it may be acting through the inhibitory action of ADA activity, leading to an accumulation of adenosine and 2'-deoxyadenosine, producing lymphotoxicity, leading to an immunosuppressive action. The inhibition of ADA activity level by corticosterone was also ascertained using slot and Western blot analyses which confirmed the inhibition of ADA activity at protein expression level. The immunosuppressive actions of corticosterone may thus control the host's immune response to a great extent. Pronounced inhibition at a later stage may be attributed to a greater maturation of corticosterone receptors and post-receptor events, thus facilitating greater binding of the hormone to its receptor and/or hormone-receptor binding to ADA gene promoter leading to inhibition of cognate gene expression.

Bt_2cAMP, which is a membrane permeable analog of cAMP, is found to increase the activity of ADA in all regions of GIT studied except crop. Like the tissues of GIT, in the spleen

too, Bt_2cAMP increases the activity of ADA at day 60 but unlike the tissues of GIT, there was no effect at day 10. This stimulation is seen to be age and region specific. The substrate adenosine influences the intracellular concentration of cAMP. Deficiency of ADA leads to an accumulation of adenosine and 2'-deoxyadenosine which are reported to be lyphotoxic. Thus, cAMP may increase the immune responses by stimulating ADA activity, lowering the level of adenosine and 2'-deoxyadenosine, to ensure better survival of lymphocytes. The immunoinducing role of cAMP may be because of enhancing the activity of ADA, thus decreasing the intracellular concentration of adenosine, which ensures an environment better suited for lymphocyte proliferation. In the GIT, the activity level of ADA was found to be greatly enhanced at a later age (day 60) of chicken development when compared to the younger age (day 10). This may be because of the differential expression of secondary messenger cascade at the later stage of GIT development. Such induction of ADA activity level by Bt_2cAMP was also reaffirmed using slot and Western blot analyses confirming that ADA activity is indeed induced at the protein expression level.

Thyroid hormones are known to decrease the activity of ADA in humans. However, in our course of study, there is no significant effect of thyroid hormone on the activity of adenosine deaminase in the tissues studied. The slot/Western blots also do not show any change in the level of the enzyme. This may be because of the difference in the physiology of humans and chicken, thyroid hormone receptors and post-receptor events or the lack of the regulatory elements of thyroid hormone in the chicken ADA gene.

Testosterone is also seen to decrease the activity of human ADA. But, no significant decrease in the activity level of ADA is seen in the tissues studied at the two postnatal ages, upon administration of testosterone. It could be due to a difference in the testosterone receptor and/or post-receptor events in human and chicken. There could also be a possibility of lack of testosterone/androgen regulatory elements (AREs) in the ADA gene of chicken. The slot and Western blot analyses performed also indicate no change in the activity at the protein expression level.

PURIFICATION AND PHYSICOCHEMICAL CHARACTERISATION OF ADA

Intestinal ADA from immature (1-day) and mature (90-day) chicken was purified using identical procedures. ADA preparations from both the ages were passed through sephadex G-100 gel filtration and DEAE-cellulose columns. The elution profiles indicated that both ADA, from immature and mature chicken, have similar molecular weights and ionic net charges. Gel filtration, PAGE and SDS-PAGE analyses indicate that both immature and mature ADA have similar molecular weight of 100 kDa, a similar overall charge and consist of a single molecular form. From the Michaelis-Menten equation and Lineweaver-Burk transformation, both the ADA show similar K_m for adenosine of 33.3 μM and 34.2 μM, respectively, for 1-day and 90-day old chicken. The computed K_m values of the immature and mature small intestinal ADA for 2'-deoxyadenosine are 14.3 μM and 14.3 μM, respectively. Analyses of the data indicate that there is no age-related difference in the affinity of ADA for adenosine as well as 2'-deoxyadenosine. However, both ADA from immature and mature chicken show more affinity towards 2'-deoxyadenosine than adenosine, indicating that a lower concentration of 2'-deoxyadenosine is required to reach a similar reaction velocity. Analysis of data indicates no significant difference between K_m, V_{max} and K_{cat} values of the enzyme for both substrates in the two age groups. There is also no significant difference observed in the K_i of immature and mature ADA for purine riboside. Hence, analyses of the data indicate that purine riboside is a strong competitive inhibitor of both ADA with a similar K_i of 6 μM and 7 μM, respectively. The pH stability studies indicate that ADA from immature and mature chicken is most stable in the broad range of pH from 5.5 to 8.0, after which slight instability starts in both the ADA. Thus, it implies that the salt bridge contributes to both immature and mature ADA by the same degree. The inactivation in the alkaline or in the urea solution can be derived from a slight deformation of the active site which cannot hold the entire activity due to a change in the molecular form at sites distant from the active site, whereas, acidic pH is capable of producing a direct change in or around the active site. When purified ADA from immature and mature chicken is assayed at different temperatures, the enzyme is stable till 45°C, after which a drop in the activity is seen in both the ages, with almost all activity lost at 70°C. This is because the increase in temperature after this does not increase the kinetic energy of the enzyme but instead

disrupts the forces maintaining the shape of the molecule. Modulation studies on the activity of ADA show that DTT, β-mercaptoethanol, DTNB all inhibit the activity of purified ADA albeit to a similar degree in both the immature and mature ages. The extent of inhibition by these sulfhydryl modifying agents is in the range of 25-35%. Amongst the divalent cations, Ca^{2+} is found to be less inhibitory (15%) to ADA followed by Mg^{2+} (50%) and the Hg^{2+} (88%). In addition to these, caffeine is also found to be inhibitory to ADA activity to the extent of 45%.

From the findings embodied in this thesis, it is concluded that:

- ADA activity and its level expresses in a tissue- and age- specific manner during postnatal development of chicken to ensure its better suited physiological roles.

- Corticosterone inhibits ADA activity in a tissue- and age- specific pattern, indicating that ADA is under the tonic inhibition by circulating corticosterone. Age-related difference in the magnitude of ADA inhibition is correlated to a differental adaptive role and maturation of corticosterone action mechanism, its receptor and post-receptor events.

- Bt_2cAMP is found to be stimulatory to the activity of chicken ADA, thereby exhibiting an immunoinducing role of cAMP.

- T_3 and testosterone fail to produce any significant change in ADA activity of chicken in either tissues or ages studied.

- Physicochemical and kinetic properties of purified ADA from small intestine of chicken remain the same at both the immature and mature ages indicating that there is no alteration in these properties as a function of age in chicken during postnatal development.

The studies compiled in the present thesis provide an insight into the basic role of ADA during postnatal development of chicken and also pave the way for using corticosterone and Bt_2cAMP in inhibiting and inducing, respectively, the activity of ADA in various ADA-related diseases

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

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

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LIST OF COMMONLY USED ABBREVIATIONS

µ	Micro
ADA	Adenosine deaminase
ADA-BP	ADA-binding proteins
AK	Adenosine Kinase
ALP	Alkaline phosphatase
Asp	Aspartic acid
BCIP/NBT	5-Bromo-4-Chloro-3-Indolylphosphate/Nitroblue tetrazolium
BF	Basal forebrain
BSA	Bovine Serum Albumin
Bt_2cAMP	Dibutyryl-3'-5'-cyclic adenosine monophosphate
Ca^{2+}	Calcium
CAD	Cath. a differentiated
Camp	3'-5'-cyclic adenosine monophosphate
CBB	Coomassie Brilliant Blue
CBP	Core Binding Protein
cGMP	Cyclic guanosine monophosphate
c-MDH	Cytosolic malate dehydrogenase
CNS	Central Nervous system
CTCF	CCCTC-binding factor
Cys	Cysteine
DAG	1,2- Diacylglycerol
DCF	2'-deoxycoformycin
dCF	Deoxycoformycin
ddH_2O	Double distilled water
DEAE	Diethylaminoethyl
DPP	Dipeptidyl peptidase
DR	Dietary restriction
dsRNA	Double-stranded RNA
DTNB	5,5'-Dithiobis(2 nitrobenzoic acid)
EDTA	Ethylene Diaminetetraacetic Acid
EHNA	Erythro-9-(2-hydroxy-3-nonyl) adenine
EMSA	Electrophoretic Mobility Shift Assay
FACS	Fluorescence-activated cell sorting
FMF	Familial Mediterranean Fever
FP	Footprinting Region 1
G	Gravitational force
GCs	Glucocorticoids
GH	Growth hormone
GIT	Gastro intestinal tract
Gly	Glycine
GRs	Glucocorticoid receptors
HCl	Hydrochloric acid

H	Hour
His	Histidine
HREs	Hormone responsive elements
Hsp	Heat shock proteins
i.p.	Intraperitonially
Ig	Immunoglobulin
IP_3	Inositol 1,4,5 triphosphate
kDa	Kilo Dalton
L	Liter
M	Molarity
mA	milli Ampere
mAbs	monoclonal antibodies
Met	Methionine
Min	Minute
MW	Molecular weight
N	Normality
NaCl	Sodium chloride
Nts	Nucleotides
PAGE	Polyacrylamide gel electrophoresis
PBMCs	Peripheral blood mononuclear cells
Phe	Phenylalanine
PRPP	Phosphoribosylpyrophosphate
P-site	Purine site
Rm	Relative mobility
SAH	S-adenosyl-L-homocysteine
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
Sec	second
SLE	Systemic lupus erythematosus
T_3	3,5,3'-triiodothyronine
T_4	3,5,3',5'-tetraiodothyronine
TCA	Tri Carboxylic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TRH	Thyroid releasing hormone
Tris	Tris (hydroxymethyl)-aminomethane
TSH	Thyroid stimulating hormone
TTBS	Tween-Tris-phosphate buffer
UTR	untranslated region
V	Volt
V_e	Elution Volume
V_o	Void Volume
v/v	Volume/Volume
W	Watt
w/v	Weight/Volume

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Fig. 74:	Effects of various modulators on activity of purified ADA from the small intestine of immature (1-day) mature (90-day) chicken. DTT, ME, DTNB, Ca^{2+}, Mg^{2+} and Hg^{2+}indicate dithiothreitol, β-mercaptoethanol, 5, 5'-dithiobis-(2-nitrobenzoic acid), calcium, magnesium and mercury respectively. Results are expressed as percentage taking no modulators as 100%.

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1. Introduction

Development is a continuous process by which organisms grow and develop. It is described as the gradual changes in size, shape, and function during an organism's life that translate its genetic potentials called genotype into functional mature systems or phenotype. Beginning with birth at one end and senescence at the other, in between is the reproductive phase, characterized by maximal physiologic competence. Development of animals is divided into prenatal and postnatal periods, both of which are characterized by certain distinguishing features like morphogenic, physiologic, biochemical and psychological (Shaharan *et al.*, 1986). In humans, development progresses through the embryo and fetus stages before birth and continues during childhood. Other mammals follow a similar course. Animal development is a spectacular process and represents a masterpiece of temporal and spatial control of gene expression.

Longevity as a result of prolonged or postponed aging is substantially influenced by genetic determinants. A number of theories have been postulated describing development. Evolutionary theory was first proposed in 1940s based on the observation that, a dominant lethal mutation, remained in the population even though it should be strongly selected against. The evolutionary theories include mutation accumulation theory (Bowles, 2000) and disposable soma theory (Kirkwood, 1977). Mutation accumulation theory states that the late-acting detrimental mutations may accumulate in the population and ultimately lead to pathology and senescence (Chittiprol *et al.*, 2007). Disposable soma theory is based on optical allocation of metabolic resources between somatic maintenance and reproduction. It entails that the longevity has a cost and the balance of resource invested in longevity vs. reproductive fitness determines the lifespan of an organism (Kirkwood, 1996). Whereas, the theory of antagonistic pleiotropy suggests that some genes may be selected for beneficial effects early in life and yet have unselected deleterious effects with age, thereby contributing directly to aging.

As envisaged in molecular theories, mostly the genes regulate the lifespan of an organism by interacting with different environmental factors. These theories include codon restriction, somatic mutation, and error catastrophe and gene regulation. *Codon restriction theory* depicts that fidelity/accuracy of mRNA translation is impaired due to inability to decode codon in mRNA (Streler *et al.*, 1971). The *somatic mutation theory* suggests mutations

occurring randomly in the somatic tissues accumulate with age and lead to structural disabilities and finally to the death of an organism (Szilard, 1959). *Error catastrophe theory* of aging proposed that decline in fidelity of gene expression results in increased fraction of abnormal proteins, which ultimately lead to the senescence (Orgel, 1963). *Gene regulation theory* depicts that aging results from the differential gene expression regulating both development and aging of an organism (Kanungo, 1994).

Cellular theories embody the changes that occur in structural and functional elements of cell with the passage of time. These theories include cellular senescence, telomere shortening, free radical, wear and tear and age pigments. Hayflick and Moorhead (1966) proposed the *cellular senescence theory*, according to which phenotypes of aging are caused by an increase in frequency of senescent cells. Aging can also occur as a result of telomere loss (replicative senescence) (Sitte *et al.*, 1998). *Free radical theory* of aging entails that damages of DNA, proteins and lipids by highly reactive free radicals, produced as a result of oxidative metabolism, are critical in determining the lifespan of an organism (Harman, 1956). The *wear and tear theory* of aging suggests that years of damage to cells, tissues and organs eventually wears them out, killing them and then the body. The damage begins at the level of molecules within cells. The *wear and tear theory* of aging believes that the effects of aging are caused by damage done to cells and body systems over time. If the telomeres are shortened, cells age. Conversely, if telomerase activity is high, telomere length is maintained, and cellular senescence is delayed (Greider and Blackburn, 1989).

The process of development onsets as soon as the fertilized egg begins to grow. This genetic programme determines the maximum lifespan of each species. Lifespan of an individual can be divided into three main phases: (a) development (b) reproduction and (c) senescence. Each of these phases is distinguished by morphological, physiological, biochemical and psychological features. The time and onset, duration and rate of each phase is dependent on the vigor and vitality of the previous phases. In a broad sense, development is divided into two main periods, prenatal and postnatal development (Timiras, 1994). The prenatal development involve the quantitative and qualitative changes that lead to specialization of tissues and organs, termed as differentiation and encompasses three main stages- ovum, embryonic and fetal

stages. The postnatal development begins with birth and continues into the stages of neonatal, infancy, childhood, adolescence and adulthood (Timiras, 1994). Postnatal development includes all the events including development of endocrine control mechanisms from the neonate to the fully grown individual, but does not take place proportionally in all categories of tissues.

1.1. DIFFERENTIATION AND ENZYMES

Differentiation and development are programmed processes which occur due to sequential activation and repression of genes causing alterations in the levels of enzymes (Kanungo, 1975; Davidson and Britten, 1979). The activities of several enzymes decrease and of several others increase as a function of age of an organism. A possible reason for such changes may be because of the decrease or increase, respectively in the template activity of the corresponding genes (Kanungo, 1980). Age induces alterations in the levels of enzymes and their inducibility by certain hormones (Wilson, 1973; Adelman, 1975; Kirkwood, 1977). During aging of an organism, several biochemical and physiological changes occur (Kirkwood, 1977). As enzymes are responsible for specific functions, the initiation, duration and termination of various phases of the lifespan of an organism like differentiation, development and reproductive maturity may depend on the appearance or disappearance or alterations in the levels of specific enzymes or their isoenzymes.

1.2. ENZYMES DURING DEVELOPMENT

All biological reactions in an organism are catalyzed by enzymes. Living cells have evolved complex regulatory mechanisms to control the concentrations of enzymes particularly those catalyzing critical reactions. They are, thus, essential for all functions and structures of the body. They accelerate reactions by factors of 10^6 to 10^{12} as compared to uncatalyzed reactions. The enzymes catalyze a variety of reactions acting like oxidases-reductases, transferases, hydrolases, lyases, isomerases and ligases. The activity of enzymes *in vivo* is regulated, and their rate of synthesis as well as final concentration is under genetic control

(Markert and Møller, 1959). Some enzymes are synthesized as inactive precursor forms and are activated at a suitable physiological milieu. Some other mechanisms are by covalent insertion of small group(s) on the enzyme and feedback inhibition by the end product. The extent to which an enzyme can increase the rate of a reaction depends on the activity of the enzyme vis-à-vis the active enzyme available and the presence of substrate(s), cofactors, inhibitors and activators. Changes in the levels and properties of these enzymes may alter the functional ability of an organism.

The initiation, duration and termination of various phases in the life of an organism like differentiation, development, and maturity may depend on various characteristics of enzymes such as their levels and isoenzyme patterns. Changes in their properties during development may alter the activities of the organism. There is evidence that the levels of certain enzymes decrease and others increase after reproductive maturity, while some enzyme levels remain constant. Each enzyme is coded by a gene, and for some isoenzymes consisting of two types of subunits, two genes are involved. Several reviews on changes in enzyme levels have been published (Wilson, 1973; Florini, 1975; Kirkwood, 1977; Sharma, 1988a). Kanungo (1980) took a different approach by comparing the data on changes in enzymes during the aging of animals. Using the Enzyme Commission's system, he compiled the data on the six classes of enzymes in order to find out if enzymes of a particular class that catalyze a specific reaction show a specific type of change. It was found that within each 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. Some enzymes do not show any difference in activity. In general, it appears that the enzymes responsible for oxidation decrease in activity after adulthood in post mitotic tissues like heart, skeletal muscle and brain. The activities of enzymes located in different compartments of a cell do not show any specific pattern of change. Not only does the reduced rate of transcription and translation decrease the level of an enzyme, but also its posttranslational modifications may reduce the number of active enzyme molecules that control specific metabolic steps.

Proteins undergo several types of posttranslational modifications, like methylation, phosphorylation, acetylation, adenylation and ADP-ribosylation to become functional.

Although it is not known whether any change occurs in the rates of these modifications, it is reasonable to assume that the longer an enzyme stays in a cell, the greater the likelihood of its undergoing one or the other type of modification that may partially or completely decrease its activity. Thus, the fraction of inactive enzyme molecules will increase with the passage of time, which would adversely affect the activity of the cell. Such modifications would alter the K_m, K_i, molecular weight, electrophoretic mobility, antigenicity, and heat lability of enzymes.

Considerable literature is available describing changes in the activities of enzymes during development and aging. Some detail reviews in the subject are available (Kirkwood, 1977; Sharma, 1988a). The rate of synthesis of enzymes usually changes in response to changes in the extracellular environment. In bacterial cells, these changes in enzyme synthesis are very pronounced with several hundred-fold increase in the activity level of specific enzymes being observed. However, in animal cells, the situation is quite different. Although the whole organism may be exposed to marked changes in the environment, mechanism exists which minimizes the number of cells or tissues to exhibit response (Sitte *et al.*, 1998) Vast majority of enzymes in multicellular organisms are expressed constitutively, differing from adaptive enzymes, to describe those enzymes which are induced in a microorganism only when they grow in presence of specific substrate in contrast to constitutive enzymes which are present in a given organism irrespective of the composition of the medium on which the organism grows. The term induction was soon introduced in relation to enzymes, which is defined as an increase in enzyme activity caused by a change in substrate concentration or by hormonal action (Cohn *et al.*, 1953), whereas, enzyme repression was defined as a relative decrease, resulting from exposure of cells to a given substance, in the rate of synthesis of a particular apoenyme (Vogel, 1957). Induction of an enzyme is attributed to several inducers, or effectors which may either be a substrate or a hormone or a metabolite or even an exogenous factor (Sharma, 1988a).

1.3. CHANGES IN GENE REGULATION DURING DEVELOPMENT

The divergent morphology and physiology observed during development with many distinct complex and coordinated processes involve the control of the programmed circuits of gene expression. Certain processes, like release of a hormone or fertilization of an egg triggers

the expression of one set of genes. Various globin chains that are coded by separate genes appear sequentially during the gestation period in humans (Zuckerkandle, 1965). The hemoglobin during gestation is made up of $\alpha_2\xi_2$ followed by $\alpha_2\gamma_2$ during fetal life, so called fetal hemoglobin (HbF). It is shifted to $\alpha_2\beta_2$ just before birth. While the gene for α chain is activated since the early period of fetal life, ξ, γ, β genes are activated one after the other sequentially. The factors responsible for switching on these genes in a programmed and sequential manner are not known, but it appears that varying oxygen tension may contribute to their sequential expression (Kanungo, 1975). The changes in several protein patterns during the development of *Drosophila melanogaster* and developmentally controlled expression of lactate dehydrogenase (LDH) isoenzymes (Markert and Ursprung, 1963) indicate the switching on and off of genes during development. However, the regulatory mechanisms with largest effects on phenotype have been shown to act at the levels of transcription and mRNA processing. It is clear that different organisms have certain genes that control specific events during development. These genes have been conserved during evolution, for example, the homeotic gene controls segmentation in insects to mammals and *myo* D gene controls the differentiation of skeletal muscle in vertebrates. It is probable that more such conserved genes exist that control specific events of development in the animal kingdom. There are abundant data to show that within a species also, early development is the result of sequential activation and repression of specific genes. The product(s) of one (or more) of these genes functions by turning off the transcription of the first set of genes and/or turning on of a second set of genes. In turn the product(s) of the second set of genes may act as regulator for the transcription of a third set of genes and so on. This sequential expression of genes is genetically pre-programmed, and the genes involved in such programmes do not usually escape out of the sequence (Brown, 1981). More genes are expressed during development than in any other phase of the lifespan. During the development of animals, gene expression has been shown to be regulated in different instances at essentially all possible levels-transcription, pre-mRNA processing, mRNA transport, mRNA stability, translation, post translational protein processing and protein stability. However, extensive data indicate that it is regulated primarily at the levels of transcription and pre-mRNA processing (Brown, 1981; West and Proudfoot, 2009).

Differential regulation of gene expression is central to much of modern biology. Animal development can be thought of in terms of an early phase, which begins with an egg and ends with an embryo, and a late phase, which begins with an embryo and ends with the mature organism. Some genes function only in the early phase while others only in the late phase. The inability to express a gene when it should be ON or the excess expression of a gene when it should be OFF is usually dysfunctional and often lethal. For any given gene, expression can be considered a roughly periodic function, which in the simplest case is OFF for a period and ON for another period with the total duration being the lifetime of the organism. The differential regulation of many such genes in time and space determines the pattern of cell-specific expression that underlies development of the organism (Savageau, 1998).

During development, cells start in a pluripotent state, from which they can differentiate into many cell types, and progressively develop a narrower potential. Their gene-expression programmes become more defined, restricted and, potentially, 'locked in'. Pluripotent stem cells express genes that encode a set of core transcription factors, while genes that are required later in development are repressed by histone marks, which confer short-term, and therefore flexible, epigenetic silencing. By contrast, the methylation of DNA confers long-term epigenetic silencing of particular sequences, the transposons, imprinted genes and pluripotency-associated genes in somatic cells. Long-term silencing can be reprogrammed by demethylation of DNA, and this process might involve DNA repair. It is not known whether any of the epigenetic marks has a primary role in determining cell and lineage commitment during development (Reik, 2007). Regulatory fine tuning at translational level is obviously important in the overall control of metabolic processes in living organisms. Regulation of turning gene on and off involves the tightly interrelated coordination of cis-acting elements, trans-acting factors and signaling molecules like hormones.

1.3.1. CIS-ACTING ELEMENTS

Eukaryotic genes are regulated by promoter elements located just upstream (5') from the transcription initiation sites in a manner quite similar to the regulation of prokaryotic genes. In addition to the nearby promoters, many eukaryotic genes are also regulated by more distant cis-

acting elements called enhancers and silencers. Enhancers increase the transcription and are independent of orientation. Enhancers can act over relatively large distances. They are present several thousand bp from the regulated genes and are relatively large elements up to several hundred nucleotide pairs in length.

They sometimes contain repeated sequences that have partial enhancer activity by themselves. Most enhancer elements function in a complete or partially tissue specific manner, that is, they will only enhance the transcription of genes in specific target tissues frequently. Several genes seem to be transcribed coordinately. The different members of a storage protein or photosynthetic protein family are expressed at the same time in development. These genes have sequence modules in common that control the coordinate regulation. These modules are called response elements. These elements are a class of cis-acting elements.

Features of Response Elements

- contain short consensus sequences
- modules are related but not identical
- not fixed in location but usually within 200 bp upstream of the transcription start site
- a single element is usually sufficient to confer a regulatory response
- can be located in a promoter or an enhancer
- assumed that a specific protein binds to the element and the presence of that protein is developmentally regulated

1.3.2. TRANS-ACTING FACTORS

Factors, usually considered to be proteins that bind to the cis-acting elements to control gene expression are known as trans-acting factors. They are subunits of RNA polymerase, bind to RNA polymerase to stabilize the initiation complex, bind to all promoters at specific sequences but not to RNA Polymerase (TFIID factor which binds to the TATA box), bind to a few promoters and are required for transcription initiation; these are positive regulators of gene expression.

Those factors which bind to consensus module sequences can bind to any promoter that contains the sequence. The binding of multiple factors, for example, multiple trans-acting factors each with one of the four properties mentioned above, may be essential for transcription initiation. Enhancers, which normally have a consensus 72 bp repeat sequence, have sites for multiple trans-acting factors to bind. Thus genes with enhancers may require several complexes to be constructed for gene expression to be initiated. Transcription factors recognize target sequences in DNA and interact with other transcription factors.

These trans-acting factors can control gene expression in several ways:

- factor may be expressed in a tissue- specific manner (spatial regulation)
- factor may be expressed in at specific time in development (temporal regulation)
- factor may require modification (phosphorylation)
- factor may be activated by ligand binding
- factor may be sequestered until an appropriate environmental signal allows it to interact with the nuclear DNA

Each transcription factor has a characteristic motif. These motifs are short structures which comprise only a small portion of the protein. These motifs are used to define a specific class of trans-acting factors. The following are specific examples of trans-acting factors, each with a defined and specific motif.

- steroid receptors
- zinc finger proteins – Zn^{2+} binds at a specific site
- leucine zipper proteins - leucine appears every seven amino acids in a region of the protein

The *c-myc* gene is a cellular homologue of the oncogene *v-myc*, the transforming gene of the avian retrovirus MC29 (Colby *et al.*, 1983). Activation of its expression has been implicated in the oncogenesis of a variety of neoplasias in different species: B cell lymphoma in chicken (Hayward *et al.*, 1981; Payne *et al.*, 1982) promyeolocytic leukemia HL60 (Dalla-

Favera, 1982), Burkitt's lymphoma (Taub *et al.*, 1982; Marcu *et. al.*, 1983) and small cell carcinoma (Little *et al.*, 1983) in man. Sequences derived from *c-myc* gene induce or repress expression of a *c-myc*/CAT indicator gene in competition assays. The sequences normally bind negative and positive regulatory factors that influence expression of the c-myc gene. Three regions, one approximately 400-600 bp upstream of the first promoter, a second in the first exon, and a third in the 5' half of the first intron, contain sequences that compete for a putative negative regulatory factor(s) (Chung *et al.*, 1986).

Cell hybrid studies have shown that the translocated *c-myc* gene is activated only in B-cell background (Nishikura *et al.*, 1984). In rare instances of Burkitt's lymphoma (Hayday *et al.*, 1984) and murine plasmacytoma (Corcoran *et al.*, 1985), the *c-myc* gene translocates to the immunoglobulin (Ig) heavy chain enhancer sequence. The fact that the immunoglobin enhancer is usually not able to activate the translocated *c-myc* gene raises the possibility that there are other enhancer-like sequences or specific chromatin configurations in the immunoglobulin loci capable of activating the translocated *c-myc* gene (Rabbitts *et al.*, 1983).

1.3.3. HORMONE ACTION

Hormones are signal molecules synthesized and secreted in small quantities by specialized group of cells. They act on target cells by interacting with cognate receptors located either on the cell surface (protein/peptide hormones) or within the cytosol/nucleus (steroid and thyroid hormones). Hormones of endocrine, paracrine and autocrine systems control wide variety of cellular and metabolic processes. Adaptation to external and/or internal stresses during development depends on control mechanisms of the combined interplay of nervous and endocrine systems (Timiras, 1994). Neurons in the brain of higher animals act as pacemaker, regulating the biological clock which governs development and aging (Sharma, 1994). This pacemaker role of neurons coordinates with the hormones resulting in dramatic changes in metabolic patterns of gene expression. Changes in the levels of hormones and their properties of binding to receptors during development have been extensively studied. Hence, the level of hormones, their receptors and post-receptor events may influence the process of development, growth and reproduction in animals. Hormone action on target cells is controlled not only by

the concentration of hormones and their cognate receptors but also by modulators of these hormonal actions (Katunuma *et al.*, 1988). Given the distinct differences that exist in hormonal regulation of metabolism between species, references, wherever applicable have been cited in relation to the avian system.

In the avian system, growth and development are under an intricate, but judicious control mechanism, involving autocrine, paracrine and endocrine factors. A number of hormones have long been recognized as playing major roles in the control of growth. These include growth hormone, thyroid hormones (T_3 and T_4) and the sex steroids (Scanes and Johnson, 1984). In addition, other factors like epidermal growth factors, insulin like growth factor (IGF), and nerve growth factor also influence growth (O'Keef *et al.*, 1988).

1.3.3.1 GROWTH HORMONES

Considerable strides have been made in the knowledge of avian growth hormones (Harvey and Scanes, 1977; Palmer and Stiles, 1995). In aves, growth hormone (GH) is required for normal rate of growth. It has been observed that hypophysectomy reduces growth while replacement therapy with mammalian GH partially restores growth rates (King and Scanes, 1986). Administration of GH to intact young chicken does not stimulate growth (Libby and Schaible, 1955; Scanes *et al.*, 1984). The absence of major effects of chicken GH in intact young chicks was speculated to be due to the maximal effect exerted by endogenous GH at these ages studied (Palmer and Stiles, 1995). However, repeated administration of biosynthetic chicken GH stimulated growth in the older (12-weeks) chicks but not in the younger (8-weeks) chicks (Palmer and Stiles, 1995). Further, plasma GH decreases considerably before puberty/mid-postnatal period (Scanes and Johnson, 1984). Growth hormone shows similar patterns in development of chicken as that of T_3 and a casual relationship has been described to exist between T_3 and GH during the late embryonic and early post hatch development in chicken (Darras *et al.*, 1992)

1.3.3.2. ADRENAL HORMONES

1.3.3.2.1. ***Glucocorticoids***

Cohen (1973) and Martin *et al.*, (1977) found that glucocorticoids are present even before birth in rats and exhibit significant changes after neonatal period. In avian system, the level of glucocorticoids increases during the early stages of development at about day 15 (Wise and Frye, 1973). The rate of synthesis of corticosteroid hormones decreases with age (Serio *et al.*, 1969). Exogenous glucocorticoid hormones have effects on several enzyme activities.

In avian systems, the synthesis and secretion of adrenal hormones are also initiated during embryogenesis and have been suspected to be involved in development (Freeman and Vince, 1974). The principal glucocorticoid in chicken is corticosterone (Lauber *et al.*, 1987; Bisbis *et al.*, 1994) and is known to increase during the early stages of development with a marked rise at about 15^{th} day (Schrader and West, 1985). Further, it was also reported that exogenous hydrocortisone induces induces several enzymes (Cohen *et al.*, 1972). Subsequent studies have shown that corticosterone treatment in chicken impairs growth and induces fattening and insulin resistance (Taouis *et al.*, 1993; Simon, 1984).

Previous work in our laboratory have demonstrated quantitative changes in GR level in liver of chicken during postnatal development, with maximum GR level at a low age, probably associated with changing dietary and metabolic adjustments at this stage of lifespan. The yolk sac absorbed during hatching can supplement nutritional requirements for a limited period only, thus, by the earlier postnatal age there is total dependency on external diet. An alteration in nuclear chromatin organization might have contributed to the higher binding of H-R complexes in newly hatched chicken (Nongbri and Sharma, 2007). The effect of DR on GR has also been established in the laboratory. It has been seen that there may not be an appreciable change in the organization of chromatin in DR subjected animals as compared to the AL fed ones (Dutta and Sharma, 2004). Findings in the laboratory clearly entail that the long-term dietary restriction leads to a cumulative increase in the level of GR in the liver of mice (Dutta and Sharma, 2003). It was also demonstrated in our laboratory that hydrocortisone increased the

rate of c-MDH in the liver of chicken. Since the magnitude of increase of c-MDH at different postnatal ages is variable, it indicates that glucocorticoids do play a role in regulation of this isoenzyme of the malate aspartate shuttle enzymes (Lyngdoh and Sharma, 2001). Hydrocortisone also lowered PPiase activity in liver of immature chicken, while it did not influence activity in the brain or kidneys (Syiem and Sharma, 1997).

1.3.3.2.2. ***Insulin/glucagon***

The role of insulin in development is well acknowledged in avian systems (Ingolia *et al.*, 1986). These early works have established the fact that insulin is present in chicken as early as the 4^{th} day of incubation and is known to increase glycogen and affect maturation of hepatocytes (Benzo and De la Haba, 1972) and uptake of neutral amino acids (Levi and Lattes, 1969). Insulin's role in embryonic development of chicken was later established in great detail (de Pablo *et al.*, 1985; Wada *et al.*, 1990).

In chicken development, carbohydrate accounts for less than 1% of available nutrients and this is primarily utilized within the first 7 days of the 21 days incubation period. Throughout the rest of the incubation period, proteins and lipids serve as the major fuel source for both energy provisions and for production of intermediates for biosynthetic reactions (Ingolia *et al.*, 1986)

1.3.3.2.3. ***Thyroid hormones***

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

It has been established that growth in poultry as well as other species is regulated by thyroidal and pituitary hormones (Ingolia *et al.*, 1986; Wilson and Quiocho, 1993). An observation suggests that there is a reduction in growth of chicks following chemical ablation of the thyroid and return to normal growth rate following thyroxine replacement therapy (King and King, 1973). Attempts to stimulate growth in chicks with exogenous T_3 or T_4 resulted in reduced growth (May, 1980), whereas growth of sex-linked dwarf chicks was stimulated (Marsh *et al.*, 1984a & b). In chicken, the biological active form of thyroid hormone is 3, 5, 3'-triiodothyronine (T_3) which is however predominantly produced by monodeiodination of 3, 5, 3', 5'-tetraiodothyronine (T_4) (McNichols and McNabb, 1987).

1.4. SECONDARY MESSENGER SYSTEM

Cellular signaling constitutes an important component of information flow in biological systems. It has been evolutionarily conserved from micro-organisms to humans. All such organisms use one or the other form of signal(s) to generate the desired response. 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. The hydrophilic signals (amino acid derivatives and proteins), being water soluble, cannot cross the plasma membrane and hence act by binding to specific membrane-bound receptors. These receptors are mostly coupled to transducer G-proteins which influence the amplifier enzymes to produce a variety of second messengers like cAMP, cGMP, IP_3, DAG, Ca^{2+}. The second messengers thus generated, modify the effector proteins and enzymes to elicit the cellular response (Sharma, 1993; Litwack and Schmidt, 1997). On the other hand, lipophilic signals (steroids and their derivatives), being lipid soluble, can cross the plasma membrane and bind to specific intracellular receptors, located either in the cytosol (for glucocorticoids) or in the nucleus (for sex steroids, thyroid hormones, vitamin D3 and retinoic acid) (King and Greene, 1984).

1.5. HORMONE RECEPTORS

Steroid and protein/peptide hormones have two different types of signal cascade systems used in intracellular communications. All the steroid hormones exert their action by

passing through the plasma membrane and binding to intracellular receptors. The mechanism of action of the thyroid hormones is similar; they interact with intracellular receptors. When these receptors bind ligand they undergo a conformational change that renders them activated to recognize and bind to specific nucleotide sequences. These specific nucleotide sequences in the DNA are referred to as hormone-response elements (HREs). When ligand-receptor complexes interact with DNA they alter the transcription level (responses can be either activating or repressing) of the associated gene. Thus, the steroid-thyroid family of receptors has three distinct domains: a ligand-binding domain, a DNA-binding domain and a transcriptional regulatory domain. Although there is a commonly observed effect of altered transcriptional activity in response to hormone-receptor interaction, there are family member-specific effects with ligand-receptor interaction. Several receptors are induced to interact with other transcriptional mediators in response to ligand binding. Binding of glucocorticoid leads to translocation of the ligand-receptor complex from the cytosol to the nucleus. Different steroid hormone-receptor complexes have different hormone response elements containing different DNA sequences as would be expected since distinct sets of genes respond to each hormone (Evans, 1988; Beato, 1989; Tsai and O'Malley, 1994).

The receptors to which glucocorticoids bind are ligand-activated proteins that regulate transcription of selected genes. Glucocorticoid receptors are found in the cytosol. These receptors bind ligand to undergo a conformational change and get activated to recognize and bind specific nucleotide sequences and modulate cognate gene expression. The effect of glucocorticoids on various processes depends on the level as well as on the physicochemical properties of its receptor. Age-dependent changes in the inducibility of enzymes by this hormone are influenced by the level of receptors and/or by the post-receptor events (Knudsen *et al.*, 1991). The concentration of glucocorticoid receptor increases during prenatal and postnatal development (Singer and Litwack, 1971; Feldman, 1974, Borbhuiya and Sharma, 1995). Glucocorticoid receptor levels and nature of chromatin organization change during development of mice (Borbhuiya and Sharma, 1995). A control is also exerted by the receptor over the responses that are dependent upon the amount of complexes generated between receptors and hormones (Roth, 1988). Glucocorticoids (GCs) are synthesized and secreted from the adrenal cortex and are responsible for the control of many metabolic processes. They are

the key regulators of homeostasis and adaptation in animals, and have several effects on various animal tissues including liver, where they enhance the expression of hepatic enzymes and increase protein and glycogen content (Bawtwe and Forsham, 1972; Kalimi and Gupta, 1982). Glucocorticoid receptors (GRs) mediate the action of glucocorticoids, the key regulators of different physiological activities of target tissues in human and animal tissues (Kinyamu and Archer, 2003).

Steroid and thyroid hormone receptors are members of a large group ("superfamily") of transcription factors. In some cases, multiple forms of a given receptor are expressed in cells, adding to the complexity of the responses. All of these receptors are composed of a single polypeptide chain that has, in the simplist analysis, three distinct domains:

- *The amino-terminus*: In most cases, this region is involved in activating or stimulating transcription by interacting with other components of the transcriptional machinery. The sequence is highly variable among different receptors.
- *DNA binding domain*: Amino acids in this region are responsible for binding of the receptor to specific sequences of DNA.
- *The carboxy-terminus or ligand-binding domain*: This is the region that binds hormone.

In addition to these three core domains, two other important regions of the receptor protein are a nuclear localization sequence, which targets the receptor protein to nucleus, and a dimerization domain, which is responsible for latching two receptors together in a form capable of binding DNA.

1.6. GENE REGULATION DURING DEVELOPMENT

Three strategies by which cells are instructed to express specific sets of genes during development are mRNA localization, cell-to-cell contact and signaling through the diffusion of a secreted signaling molecule.

mRNA localization: mRNA is distributed asymmetrically during cell division to the daughter cells inheriting different amounts of the regulators ensuring different pathways of development. The roles of mRNAs are to encode for RNA-binding proteins or cell signaling molecules and/or transcriptional activators and repressors. The common mechanism for localizing mRNAs is by transporting them from one end to the other by means of an adapter protein, which binds to a specific sequence within the 3' untranslated region (UTR) of an mRNA and cytoskeleton. So the mRNA adapter complex can be moved with the cytoskeleton.

Cell-to-cell contact: a cell produces extracellular signaling proteins, then the given signal is recognized by specific receptor on the surface of recipient cells and triggers changes in gene expression in the recipient.

Signaling through the diffusion of a secreted signaling molecule: This communication from the cell surface to the nucleus often involves signal transduction pathways. There are a few basic features of these pathways:

a. sometimes ligand-receptor interactions induce an enzymatic cascade.
b. activated receptor causes the release of DNA-binding proteins from the cell surface or cytoplasm into the nucleus.
c. Upon cleavage, the intracytoplasmic domain of the receptor is released from the cell surface and enters the nucleus, to associate with DNA-binding proteins and then influence transcript signaling through the diffusion of a secreted signaling molecule.

During development, the formation of the placenta is one of the first and most important developmental events that occur in early mammalian embryogenesis. An in-depth study of gene regulation in the trophoblast layer of the placenta using murine adenosine deaminase (ADA) as a model system was undertaken. It has been determined that ADA is highly expressed in the placenta and is critical for embryo development. A 1.8 kb fragment of the ADA 5' flanking region has been described that is capable of directing trophoblast specific expression in a transgenic model system. Brickner *et al.*, (1995) identified a 236-bp first intron segment of the

mouse adenosine deaminase gene (ADA) that shares 71.1% identity with the human ADA thymic enhancer. Preliminary studies have demonstrated several critical portions of this fragment that are necessary for the correct tissue- specific expression in the placenta. The trans factor binding to one of these sequences, the FP3 was elucidated. The functional importance of this binding was demonstrated by a transgenic approach. A significant difference in expression of the reporter in the placenta was seen when the 5 bp sequence was mutated. This finding is a novel use for the AML-1 transcription factor which is the DNA binding portion of the heterodimer Core Binding Protein (CBP). This region has also been demonstrated to contain functionally significant sequence. The area has been narrowed to two pertinent regions that are predicted to contain GATA binding motifs. Trophoblast cells are specialized extra-embryonic cells present only in eutherian mammals. They play a major role in the implantation and placentation processes. To understand better the molecular mechanisms that control the development and function of trophoblast cells, the transcription factors that regulate murine ADA gene expression in the placenta were identified. It was reported that the RUNX1 transcription factor plays a significant role in regulating ADA gene expression in the trophoblast cell lineage (Schaubach *et al.*, 2006).

Studies in ADA-deficient mice have demonstrated that the absence of ADA in the trophoblast cells is associated with perinatal lethality (Migchielsen *et. al.*, 1995; Wakamiya *et al.*, 1995). ADA expression in the placenta is under stringent control during trophoblast differentiation (Knudsen *et al.*, 1988; Knudsen *et al.*, 1991; Witte, 1991). The level of ADA expression increases as the diploid trophoblast cells grow and differentiate. In the mature placenta, ADA is enriched in all trophoblast cells with highest level of expression found in the spongiotrophoblast cells of the junctional zone. The expression pattern and functional importance of ADA in the placenta make it a good model to identify transcription factors important in trophoblast gene expression. The temporal and spatial information for ADA expression in trophoblast cells resides in a 770-bp sequence located 5.4 kb pairs upstream of the ADA transcription start site (Winston *et al.*, 1992; Shi *et al.*, 1997). Within this region, there are binding sites for transcription factors including bHLH and GATA. Recently, two bHLH factors, Mash-2 and Hand1, have been identified in the mouse placenta (Johnson *et al.*, 1990; Cross *et al.*, 1995). Mash-2 is essential for development of spongiotrophoblast cells (Guillemot

et al., 1994; Tanaka *et al.*, 1997), whereas Hand1 promotes trophoblast differentiation *in vitro* and is important for trophoblast giant cell formation in the mouse placenta (Firulli *et al.*, 1998; Riley *et al.*, 1998). Possible involvement of bHLH and GATA factors in the regulation of ADA expression in trophoblast cells was supported by deletion and mutational analysis (Cohen, 1973).

A detailed characterization of a placenta-specific footprinting region (FP1) in the ADA placental regulatory element was accomplished. The sequence of FP1 was mapped by DNase I footprinting and was found to match a consensus AP-2 transcription factor-binding site. Electrophoretic mobility shift assays demonstrated that FP1 interacted with AP-2-like proteins. Further analysis using AP-2 antibody confirmed that AP-2 protein was indeed present in the placenta and bound to FP1 (Oztürk *et al.*, 2006). Mutation at the AP-2 site in FP1 abolished the ability of the ADA placental regulatory element to bind AP-2 proteins and failed to target chloramphenicol acetyltransferase reporter gene expression to placentas in transgenic mice, indicating that AP-2 is required for ADA expression in the placenta. In addition, RNase protection assays demonstrated that AP-2 gamma was the predominant AP-2 family member expressed in the placenta. In situ hybridization analysis revealed that AP-2 gamma expression was enriched in the trophoblast lineage throughout development, suggesting that AP-2 gamma may be critical for trophoblast development and differentiation (Shi and Kellems, 1998).

The murine ADA gene has a GC-rich promoter that is structurally typical of many mammalian "housekeeping" gene promoters. The ability of the ADA gene promoter to support diverse tissue-specific gene expression was investigated (Chen and Mitchell, 1994). Endogenous ADA gene expression in different mouse tissues was found to vary over a >3000-fold range in a highly complex pattern. Several of the mouse tissues examined also showed stage-specific variation during postnatal development. In order to determine whether tissue-specific *ADA* expression was controlled by cis-acting sequences upstream of the coding region, constructs containing a reporter gene regulated by the ADA gene's 5' flanking sequences were used to generate transgenic mice. All transgene-expressing mice obtained showed diverse reporter gene expression in the tissues analyzed. It was demonstrated that both *in vivo* and in

the context of an integrated transgene, this GC-rich promoter can support highly diverse gene expression in all tissues of the animal (Rauth *et al.*, 1990).

1.7. ADENOSINE

In 1929, the first documentation of the physiological effects of adenosine was reported when extracts of heart, muscle, brain, kidney and spleen had pronounced effects on cardiovascular function (Drury and Szent-Györgyi, 1929). The active substance found in this extract was known to be adenosine. Specific receptors for adenosine were later discovered as P_1 and P_2 (Burnstock, 1978). The P_1 receptor was subdivided based on adenylate cyclase response to adenosine (Londos and Wolff, 1977). Two adenosine sensitive sites were defined: the R-site which requires integrity of the ribose moiety, and P-site which requires integrity of the purine moiety. Based on its ability to activate or inhibit adenylate cyclase, the R-site was later divided into Ra and Ri, respectively. Van Calker *et al.*, (1979) proposed the terminology A_1 and A_2 for inhibition and activation of adenylate cyclase, respectively. Belardinelli *et al.*, (1981) demonstrated that adenosine impaired atrioventricular conduction. Adenosine is used for the treatment of supraventricular tachyarrhythmias (diMarco *et al.*, 1985; Overholt *et al.*, 1988). Cardiac effects of adenosine are believed to be mediated by A_1 adenosine receptor (Belardinelli *et al.*, 1983). Activation of adenylate cyclase in smooth muscle and the subsequent increase in cyclic AMP (cAMP) has been suggested to be the mechanism by which adenosine produces relaxation. A lot of data suggest that adenosine increases cAMP production and subsequently activates a cAMP-dependent protein kinase (Kukovetz *et al.*, 1978; Cassis *et al.*, 1987; Ramkumar *et al.*, 1990). Mechanisms other than adenylate cyclase activation could also be associated with adenosine-mediated relaxation. Kurtz (1987) suggested that cyclic guanosine monophosphate production may be involved in adenosine-mediated relaxation since adenosine increased guanylate cyclase activity.

Adenosine has been postulated by many investigators to be involved in neural transmission. Sattin and Rall (1970) were the first to report the stimulation of adenylate cyclase by adenosine in brain slices and that the alkylxanthines were antagonists of this response. Adenosine and its agonist analogs produce sedation, analgesia, hypothermia, and muscular

weakness, and prevent seizure activity. Adenosine has a respiratory depressant effect which is believed to be the result of a direct effect of adenosine on brain centers controlling respiration (Moss *et al.*, 1986). Adenosine is produced in the brain in states of hypoxia (Rubio *et al.*, 1974), and hypoxia is demonstrated to depress respiration.

Adenosine has also been involved in asthma. It is produced in the lung due to hypoxia (Mentzer *et al.*, 1975) and causes constriction of the airway smooth muscle. Along with being an important physiological regulator of blood flow, respiration, and neural modulation, adenosine has been shown to play an important physiological role in lipolysis. Adenosine is an immunomodulator, with anti-inflammatory properties such as the promotion of endothelial barrier function (Lennon *et al.*, 1998) and the regulation of cytokine production by macrophages (Hasko *et al.*, 1996), superoxide production by neutrophils (Cronstein *et al.*, 1986) and mediator release by mast cells (Ramkumar *et al.*, 1993).

Adenosine has proven to play an important role in modulation of insulin action on glucose metabolism in different tissues (Richelson, 1985; Shaharan *et al.*, 1986; McLane *et al.*, 1990). Insulin may affect metabolism of adenosine by changing activities of principal enzymes involved in nucleoside production and degradation (5'-nucleotidase and adenosine deaminase, respectively) (Ohisalo, 1987). Plasma ADA can be a sensitive marker of an ongoing biological insult to host tissues either because of infection and/or side effects of medication. Measurement of plasma ADA activity, along with serological evidence for HIV infection may provide an alternate laboratory tool to monitor intracellular parasitism including secondary infection vis-à-vis the after effects of therapeutic outcome (Chittiprol *et. al.*, 2007).

The propensity of sleep increases in the course of wakefulness. The inhibitory neuromodulator, adenosine, is a promising candidate for a sleep-inducing factor: its concentration is higher during wakefulness than during sleep, it accumulates in the brain during prolonged wakefulness, and local perfusions as well as systemic administration of adenosine and its agonists induce sleep and decrease wakefulness. During sleep the extracellular adenosine concentrations decrease, and thus the inhibition of the wakefulness-active cells also decreases allowing the initiation of a new wakefulness period. It is believed that the drive to

sleep is determined by the activity of the basal forebrain (BF) cholinergic neurons, which release adenosine, perhaps because of increased metabolic activity associated with the neuronal discharge during waking, and the accumulating AD begins to inhibit these neurons so that sleep-active neurons can become active (Blanco-Centurion *et al.*, 2006). A functional polymorphism in the adenosine metabolizing enzyme, adenosine deaminase, contributes to the high inter-individual variability in deep slow-wave sleep duration and intensity (Landolt, 2008). In the brain sleep/wake regulatory areas of rats, there are significant spatial differences in the distribution of ADA but age has no impact on its enzymatic activity (Mackiewicz *et al.*, 2006). It was observed that inhibitors of ADA significantly inhibited the proliferation of leukemia and lymphoma cells (Honma, 2001). In patients with *H. pylori* infection, ADA activity was significantly high reflecting the regulatory role of the enzyme in acid secretion (Bulbuloglu *et al.*, 2005).

Adenosine and adenosine receptor ligands influence numerous processes in the chicken including angiogenesis in the developing embryo and chorioallantoic membrane (Dusseau *et. al.*, 1986). Adenosine also plays an important role in the development and growth regulation of chicken sympathetic neurons (Wakade *et. al.*, 1995). In aves, as in mammals, growth and development are controlled by interplay of various cellular signaling with the genes and their products. A number of hormones and growth factors exert an intricate but judicious control on the process of development in aves. ADA is a well-characterized enzyme involved in the depletion of adenosine levels. Adenosine, a local hormone, produced by mesangial cells is also a metabolic regulator of renal blood flow, capable of decreasing glomerular filtration rate (GFR), exerting immunosuppressive, antiproliferative and anti-inflammatory properties (Kocic *et al.*, 2002). It has also been shown that in chicken embryonic neurons, deficiency of ADA is associated with an accumulation of adenosine and generation of neurotoxicity (Wakade *et al.*, 1995; Zhao *et al.*, 1999). It was observed that an accumulation of 2'-deoxyadenosine causes cell death in embryonic chicken sympathetic ganglia and brain (Zhao *et al.*, 1999). Signaling through extracellular adenosine receptors is believed to account for the wide-ranging effects of adenosine (Gomez *et al.*, 2001). ADA metabolizes extracellular adenosine, so the deficiency of it results in an exacerbation of inflammation (Kuno *et al.*, 2006). Hence, ADA is a crucial enzyme involved in the down regulation of the substrates, adenosine and 2'- deoxyadenosine.

ADA has been reported to modulate cell growth in colon cancer cell lines (Lelievre *et al.*, 1998a; Lelievre *et al.*, 1998b). It was earlier shown that agents that trigger colon cancer cell line HT29 differentiation also trigger modifications in the activity of ADA (Dexter *et al.*, 1981). A concomitant down regulation of adenosine A1 receptors and up regulation of adenosine A2 receptors occurred in cloned cells of colon cancer cell line with a strong reduction in proliferation (Lelievre *et al.*, 2000). Removal of exogenous adenosine by growth in the presence of adenosine deaminase also inhibited the proliferation of other human tumor cells (Colquhoun and Newsholme, 1997).

1.8. ADENOSINE RECEPTORS

Adenosine exerts its effect by binding to specific cell-surface receptors, called adenosine receptors. Adenosine receptors are integral membrane proteins in virtually all cell types and mediate the important physiological actions of adenosine. The receptors may exert a certain degree of control over the responses which they mediate. In many cases, the degree of biological response is dependent upon the amount of complexes which form between receptors and hormones (Roth, 1979). A lot of biochemical, functional and receptor-cloning studies is beginning to provide convergent data supporting the existence of A_1, A_{2A}, A_{2B} and A_3 receptors (Collis and Hourani, 1993). Thus, adenosine receptors can be divided into major classes designated A_1, A_{2A}, A_{2B} and A_3, all of which have been isolated, cloned and expressed (Palmer and Stiles, 1995). These receptors are widely distributed throughout the body. They are involved in many central and peripheral processes, including immunological and inflammatory responses (Marx *et al.*, 2001). Each is encoded by a separate gene and has different functions, although with some overlapping (Gao and Jacobson 2007). For instance, both A_1 receptors and A_{2A} play roles in the heart, regulating myocardial oxygen consumption and coronary blood flow, while the A_{2A} receptor also has broader anti inflammatory effects throughout the body (Haskó and Pacher, 2008). These two receptors also have important roles in the brain (Kalda *et al.*, 2006), regulating the release of other neurotransmitters such as dopamine and glutamate (Fuxe *et al.*, 2007; Schiffmann *et al.*, 2007; Cunha *et al.*, 2008), while the A_{2B} and A_3 receptors are located mainly peripherally and are involved in processes such as inflammation and immune responses. Of the two subtypes of adenosine receptors, the A_1 receptor inhibits

adenylyl cyclase by interaction with a GTP- binding protein G_i, whereas the A_2 receptor acts via G_s to activate the enzyme. In addition to the cell-surface adenosine receptors, a purine site (P-site) for adenosine is located on the cytoplasmic face of the catalytic subunit of the adenylyl cyclase, which mediates the inhibitory effects of high concentrations of adenosine. This secondary inhibitory site may be a physiological target for 3'-AMP and 2'-deoxy-3'-AMP (Johnson *et al.*, 1990). While the A_1 and A_3 receptors transduce signals via G_i and G_0, the A_2 receptors interact with G_S. When A_1 or A_3 adenosine receptors predominate in a particular cell type, extracellular adenosine may inhibit adenylate cyclase or modulate the activity of cationic channels, whereas A_2 receptors may stimulate adenylate cyclase (Xu *et al.*, 1998).

Table 1: Various adenosine receptors and their modes of action

Receptor	Gene	Mechanism	Effects
A_1	*ADORA1*	Gi/o $\rightarrow$ cAMP$\downarrow$	* Decrease heart rate * In brain, slows metabolic activity
A_{2A}	*ADORA2A*	GS $\rightarrow$ cAMP$\uparrow$	* Coronary artery vasodilation
A_{2B}	*ADORA2B*	GS $\rightarrow$ cAMP$\uparrow$	* Bronchospasm
A_3	*ADORA3*	Gi/o $\rightarrow$ cAMP$\downarrow$	* Cardioprotective in cardiac ischemia * Inhibition of neutrophil degranulation

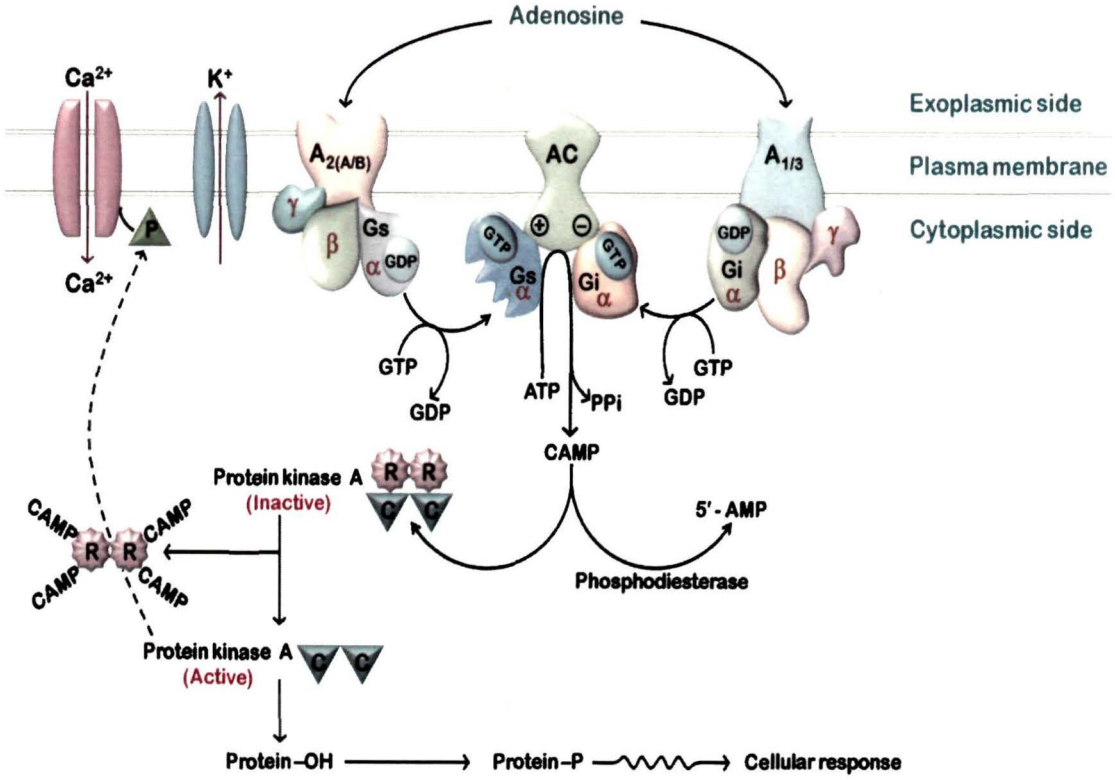


Fig. 1: Action mechanism of adenosine: Adenosine binds either to $A_{2A/B}$ receptor and activates adenylate cyclase (AC) through stimulatory G-protein (Gs) or to $A_{1/3}$ receptor and inhibits AC via inhibitory G-protein (Gi). When adenosine binds to $A_{2A/B}$ receptor, adenylate cyclase is activated, which converts ATP to cAMP that in turn activates protein kinase A (PKA) phosphorylating proteins leading to various cellular responses. Active PKA may also phosphorylate Ca^{2+} channels, opening them up and helping in the influx of Ca^{2+} and efflux of K^{+} ions. Alternatively, when adenosine binds to $A_{1/3}$ subunit the entire process is negatively regulated. R: regulatory subunit; C: catalytic subunit; P: phosphate; PPi: inorganic pyrophosphate.

1.9. METABOLISM OF ADENOSINE

Two principal enzymatic reactions are involved in the formation of adenosine: dephosphorylation of 5'-AMP by action of 5'-nucleotidase (EC 3.1.3.5) as well as hydrolysis of S-adenosyl-L-homocysteine (SAH) by SAH-hydrolase (EC 3.3.1.1) to yield adenosine and L-homocysteine. 5'-nucleotidase serves in a cascade to extracellularly degrade ATP to adenosine (Pearson and Coade, 1987). SAH-hydrolase is an exclusively cytosolic enzyme, ubiquitously distributed (Ueland, 1982). The reaction catalysed by the enzyme is reversible, with the

equilibrium lying far in the direction of SAH hydrolysis. Adenosine can be formed from the extracellular breakdown of ATP due to ecto-nucleotidase activities (Fredholm, 2002).

1.10. ADENOSINE AND IMMUNE SYSTEM FUNCTION

The discovery that certain immunodeficiency diseases are related to deficiency of ADA (Gilbett *et al.*, 1972) or purine nucleoside phosphorylase (Gilbett *et al.*, 1975) brought about an interest in searching for immunomodulatory roles of adenosine. Lymphocytes are shown to be capable of secreting ADA into the extracellular compartment (Strauss, 1986). Intracellularly, adenosine can originate from ATP degradation or S-adenosyl-homocysteine hydrolysis. Intracellular catabolism of ATP was found to be different in T and B lymphoblasts (Barankiewicz *et al.*, 1990). In T lymphoblasts, degradation of ATP occurs mainly via AMP deamination due to relatively low AMP-5'-nucleotidase activity. High activities of ADA and adenosine kinase (AK) in these cells prevent accumulation of adenosine and its possible release into extracellular space. B lymphoblasts catabolize ATP via AMP dephosphorylation, and because of low ADA and AK activities the adenosine is released from these cells. The effects of adenosine in various tissues have been shown to be mediated via membrane-bound receptors which cause either inhibition (A_1 receptors) or stimulation (A_2 receptors) of adenylate cyclase. A third class of adenosine receptors has been found at an intracellular site (P receptors) and mediates adenylate cyclase inhibition.

Both B and T lymphocytes appear to be extremely sensitive to adenosine. In normal individuals, relatively high ADA activity in lymphoid tissues appears to maintain low extracellular levels of adenosine level, permitting lymphocyte survival (Mills *et al.*, 1976). Lack of ADA activity leads to accumulation of S-adenosylhomocysteine and dATP and arrest of lymphocyte blastogenesis. Many fold action of adenosine appears to be mediated via specific cell-surface receptors linked to adenylate cyclase. Corticosteroids are known to exert a multitude of effects on a number of systems (Baxter and Forsham, 1972). Their immuno-suppressive role is well established. They influence the cellular responses by interacting with cognate intracellular receptors and by binding of the hormone-receptor complexes to these specific DNA sequences termed hormone responsive elements (HREs), usually located 100-

300 bp upstream from the RNA polymerase start site, and ultimately causing a change in transcription of specific genes (Yamamoto, 1985; Beato, 1989). The hormone-receptor complexes undergo activation and/or transformation and subsequent translocation into the nucleus in order to bind to chromatin and affect cognate gene expression. Activation is a time dependent (Markovic and Litwack, 1980), multifactorial process, involving dissociation of non-steroid binding proteins (heat shock proteins, Hsp 90) resulting in a conformational change in the receptor that exposes the DNA-binding domain and lead to an increased affinity for nuclear chromatin (Pratt, 1990). They influence cellular growth and differentiation, influence mood, motivation and behaviour (Meyer, 1985). The tissue and age specific changes of corticosterone inhibition on ADA activity may be due to changes in hormone receptor and/or in the post receptor events specific to different tissues studied at various postnatal ages studied (Kalimi, 1984; Sharma, 1988b; Singh and Sharma, 1995). Inhibitory action of corticosterone on ADA may be correlated to greater accumulation of its substrates adenosine and 2'-deoxyadenosine which produce lymphoid toxicity and suppress immune responses (Priebe and Nelson, 1991; Weissman and Cooper, 1995).

1.11. ADENOSINE DEAMINASE

Adenosine deaminase (ADA; EC 3.5.4.4), a key enzyme participating in the purine salvage pathway, catalyzes the irreversible hydrolytic deamination of the substrates adenosine and 2'-deoxyadenosine to yield the products inosine and 2'-deoxyinosine, respectively (Mohamedali *et al.*, 1993; Wilson and Quiocho, 1993). The steady state concentration of these metabolites is regulated with marked pleiotropic effects, especially on immunological, neurological and vascular systems (Payne *et al.*, 1982; Priebe and Nelson, 1991). ADA has an enhancement rate of about $2x10^{12}$. Deficiency of ADA enzyme due to different missense mutations in exon 4 of its gene has been correlated with the failure of both B- and T-lymphocytes mediated functions (Wiginton *et al.*, 1986).

Some of the molecular mechanisms of this hydrolytic process and the biochemical properties of ADA isolated from tissues of several species are well established (Geiger and Nagy, 1990); some unresolved issues relevant to consideration of the contribution of ADA to

purine metabolism remain. ADA is a predominantly cytosolic enzyme, consisting of a single polypeptide and one mole of Zn^{2+} per mole of protein with which catalytic activity is associated (Wilson *et al.*, 1991). However, this low molecular weight catalytic moiety can interact with proteins termed ADA-binding proteins (ADA-BP) to form high molecular weight aggregates upto 280 kDa in size. The ADA-BP has been purified and localized immunohistochemically to cytoplasmic membranes and, in some cases, to external surfaces of cells (Andy and Kornfield, 1982). The ADA-BP may anchor ADA at sites where the enzyme could exert control of adenosine levels near adenosine receptors (Schrader and West, 1985). ADA is a major component of lymphoid cells (Shibagaki *et al.*, 1996). Some human tissues like spleen, liver, stomach display as many as four molecular species of ADA with different molecular weights. The highest molecular form is associated predominantly with subcellular organelles and particulate in nature while the remaining smaller molecular species are cytosolic and soluble. Distribution of different molecular forms of ADA is tissue-specific with the small form predominating in the stomach and spleen and no detectable conversion activity into larger form observed (Van der Weyden and Kelley, 1976).

The gene for ADA in mouse is located on chromosome 2 which is approximately 27 kb in length and encodes 1.5-, 1.7-, and 5.2-kb polyadenylated mRNAs, the 1.7 kb being the most abundant (Ingolia *et al.*, 1986; Yeung *et al.*, 1985). The promoter region of the mouse gene is GC-rich, has multiple transcription initiation sites and lacks the TATA and CAAT boxes. The human ADA gene is located on chromosome 20 (20q12-q13.11). It is approximately 32 kb in length and has a promoter with only one transcription initiation site (Wiginton *et al.*, 1986). The levels of ADA mRNA are posttrancriptionally regulated and closely parallel levels of ADA activity (Berkvens *et al.*, 1988). In chicken, the ADA gene is located on chromosome 20 and is approximately 64 kb in length. Sp1 protein is essential for both enhancer-mediated and basal activation of ADA promoter (Yeung *et al.*, 1985).

The three dimensional structure of recombinant murine ADA produced in *E. coli* was reported by Wilson and co-workers. The enzyme contains a parallel α/β-barrel motif with eight central β strands and eight peripheral α helices, which is a common structure found in 1/10 of known enzymes (Farber and Petsko, 1990); it also contains five additional helices. The oblong-

shaped deep active site is lined by the COOH- terminal segments and connecting loops of the β-barrel strands. The active site also contains a zinc atom, which participates directly in the deamination mechanism. There are several hydrogen bonds between the substrate and the enzyme that stabilize the binding of substrate and the transition state. It was proposed that catalytic functions of the enzyme are carried out by Cys 262, Asp 295, Asp 296 and His 214 of the mammalian adenosine deaminases (Chang *et al.*, 1991).The zinc ion is coordinated by His 15, His 17, His 214 and Asp 295. From further studies (Wilson and Quiocho, 1993; Mohamedali *et al.*, 1993; Sidekari *et al.*, 1996), the mechanism for deamination was proposed, in which the zinc cofactor activates liganded water from which the nearby His 238 abstracts a proton, thus creating the attacking hydroxyl group. The incipient hydroxyl is oriented for attack in the C6 of the substrate through its interaction with Asp 295, His 238 and the zinc. Asp 295 is thought to hydrogen bond to the catalytic water and share a zinc ligand with it. The protonated Glu 217 facilitates the reaction by donating a hydrogen bond to N1 of the purine, thus enabling the formation of tetrahedral C6. His 238 is possible candidate as a source of the proton added to the amino leaving group. Residues Asp 296 and Gly 184 participate in hydrogen bonds with N7 and N9 of adenosine. In vitro mutagenesis of three residues coordinating the zinc atom (His 17, His 214 and Asp 295) eliminated ADA activity (Bhaumik *et al.*, 1993). Many of the point mutations thus far examined affect residues lodged in the β strands. Since the active site pocket, with bound zinc, is lined by parts of the β strands, it is likely that any mutation that causes a misalignment of the β strands would have a deleterious effect on activity (Wilson *et al.*, 1991). The role of Zn^{2+} in the functioning of ADA helps to explain the observations of the effects on the immune system produced by Zn^{2+} deficiency, where lymphoid tissues become abnormal as well as T- and B-cells become dysfunctional (Cossack and Prasad, 1991; Wilson *et al.*, 1991). Data also suggest that tryptophan residues are also essential for the functioning of the enzyme (Mardanyan *et al.*, 2001).

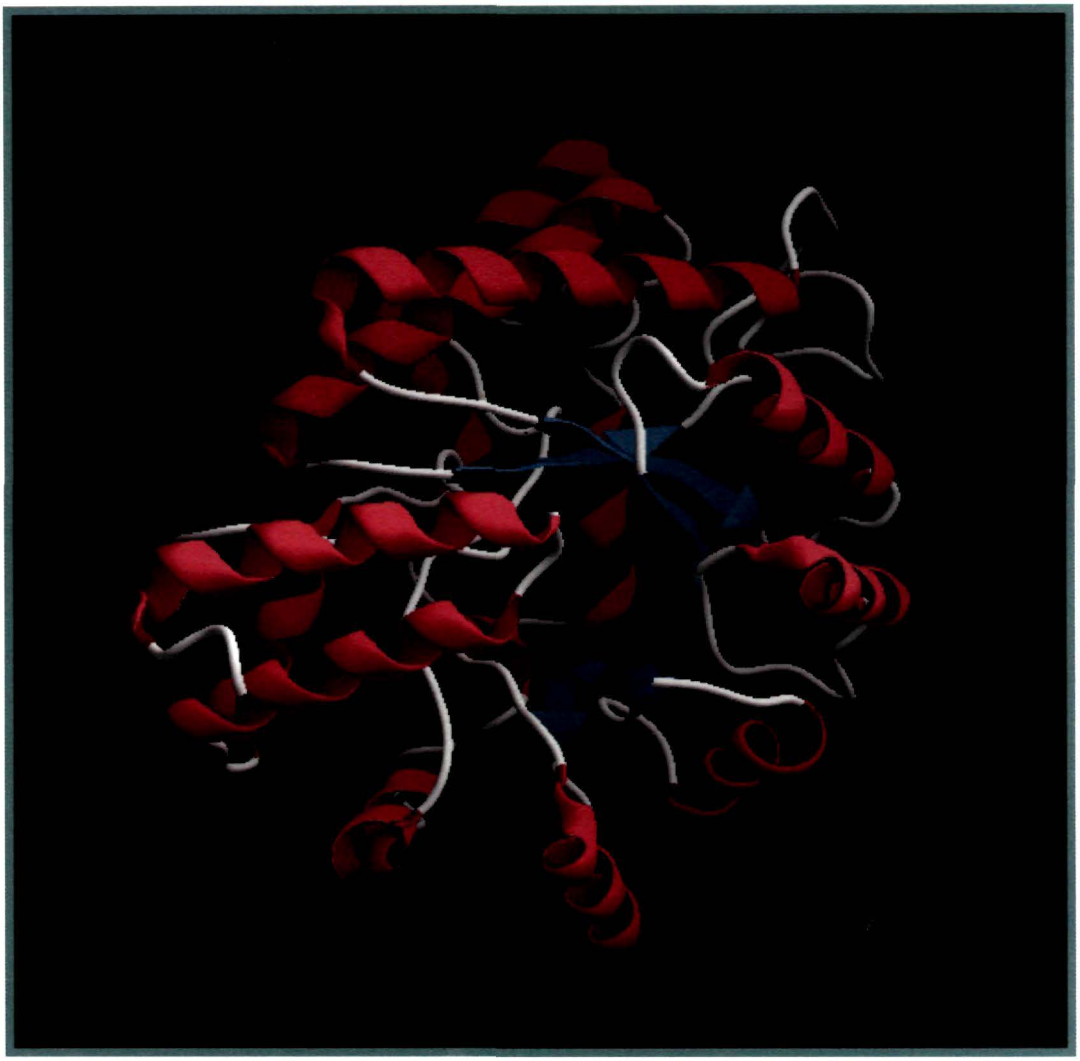


Fig. 2: Predicted three dimensional structure of chicken ADA using Modeller software. The structure was modeled using a human ADA template 31AR. The chicken protein sequence (QZK56) was retrieved from NCBI server. The sequence identity was found to be 66%. The structure consists of a parallel α/β-barrel motif and the active site contains a zinc atom (not shown). The probable active site residues are found to be His 16, His 18, Asp 20, Phe 66, Gly 107, Met 156, His 236, Cys 262, Asp 294 and Asp 295.

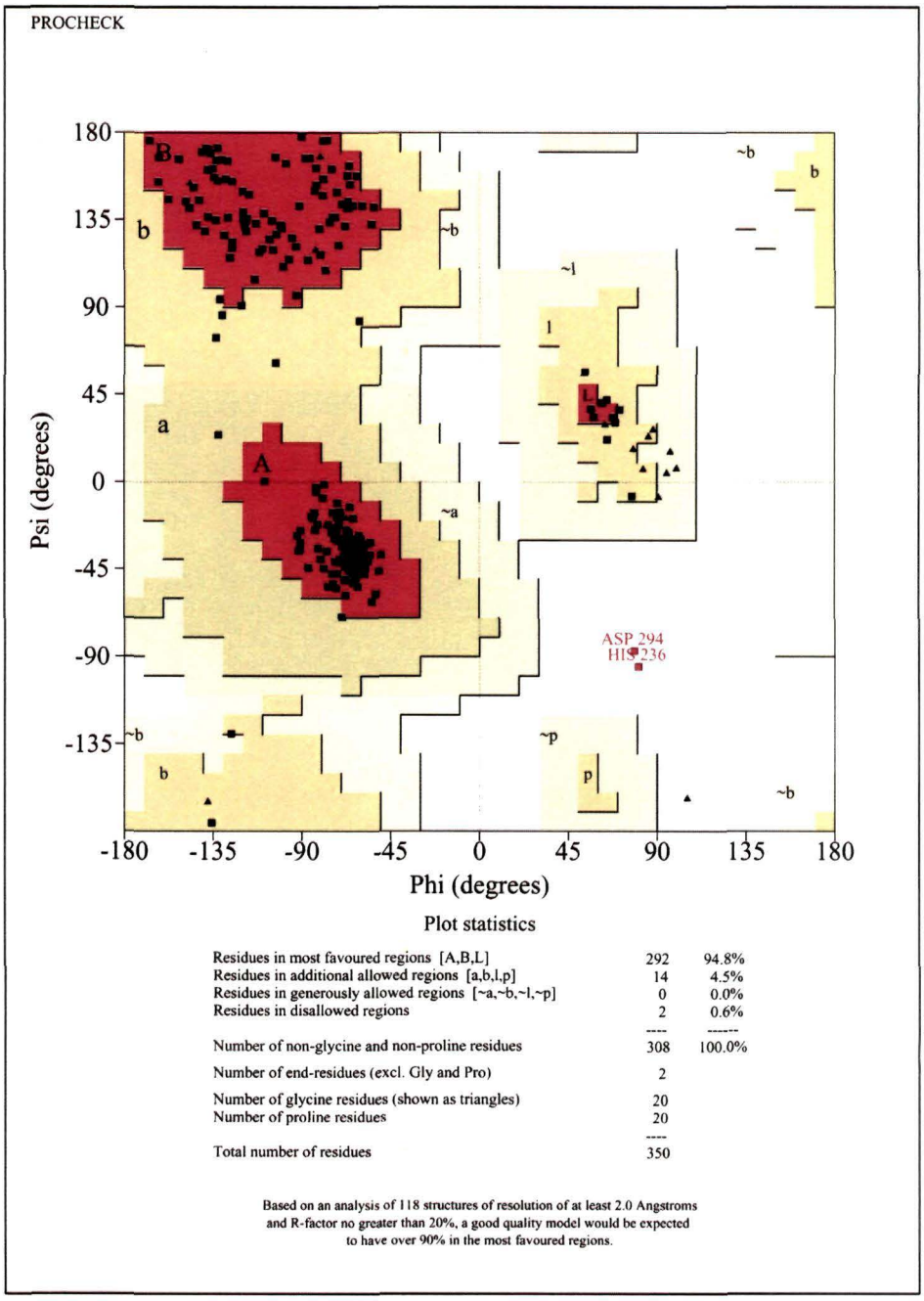


Fig. 3: Ramachandran plot for the predicted model of chicken ADA.

Adenosine deaminase is an enzyme with principal biological activity in T lymphocytes. It is required for lymphocyte proliferation and differentiation. The enzyme activity is known to be elevated in certain infections where immunity is cell mediated like in cerebrospinal fluid of tuberculosis patients (Piras and Gakis, 1973; Ribera *et al.*, 1987; Prasad *et al.*, 1991). Regarding

its main biological activity (Piras *et al.*, 1978), ADA is related to lymphocytic differentiation and proliferation, showing a significant increase in its values during the mitogenic and antigenic response of lymphocytes. In addition, Carson and Seegmiller (1976) found a restriction of lymphocytic blastogenesis following the activation of ADA inhibitors through biological and nonclarified mechanisms, possibly connected with conversion of deoxyadenosine into deoxyATP, which, gathering selectively in lymphocytic cells, would cause its destruction by the inhibition of DNA synthesis (Carson and Seegmiller, 1976). On the other hand, the congenital and genetically determined deficit of this enzyme with its autosomal recessive trait, is usually associated with severe forms of combined immunodeficiency and is responsible for an increase in toxic nucleotides that prevent the differentiation or proliferation (or both) of T lymphocytes.

In human, rat, and mouse, the highest level of enzyme activity were found in thymus, spleen, placenta, and in organs comprising the gastrointestinal tract, whereas low activity was found in muscle, lung and kidney. In humans, rat and mice, the highest level of ADA is found in thymus and alimentary canal where the enzyme accounts for as much as 20% of the total protein (Chinsky *et al.*, 1990; Mohamedali *et al.*, 1993). In the rat CNS, immunohistochemical and biochemical studies have shown that ADA has a high heterogenous distribution (Geiger and Nagy, 1986). ADA positive neurons have been found in dorsal root ganglia, spinal cord, retina, brainstem and spinal parasympathetic nuclei and posterior hypothalamus (Nagy *et al.*, 1990).

Adenosine deaminase activity is widely distributed in human tissues and is highest in lymphoid tissues. In adult mice, the highest level of ADA is present in the keratinized squamous epithelium that lines the alimentary canal, where the enzyme accounts for as much as 20% of the total soluble proteins. Two ADA isozymes are known as ADA1 and ADA2. Human tissue extracts contained ADA1 predominantly. Meanwhile, ADA2 was the main component of serum ADA. ADA activity was significantly elevated in the sera from patients with hepatic diseases, hematological malignancies and infectious diseases. Serum concentration of ADA1 was high in patients with acute leukemias, chronic myeloid blast crisis leukemia and acute liver injury. Serum ADA2 level was raised in patients with adult T-cell leukemia, multiple myeloma (B–J type), infectious mononucleosis, rubella, acquired immunodeficiency syndrome, chronic

hepatic diseases and tuberculosis. It is observed that ADA1 is derived mainly from injured tissues or cells while ADA2 comes from stimulated T cells (Kurata, 1995). The absence or low levels of ADA in humans result in severe combined immunodeficiency (SCID), which is characterized by hypoplastic thymus, T lymphocyte depletion and autoimmunity. Deficiency of ADA causes increased levels of intracellular and extracellular adenosine, of which the intracellular lymphotoxicity is considered in the pathogenesis of ADA SCID (Apasov and Sitkovsky, 1999). In T- lymphocytes of SCID patients, there is a 50-1000 fold increase in the concentration of dATP which inhibits ribonucleotide reductase and caused reduction in the other deoxyribonucleotides required for DNA synthesis, eventually inhibiting proliferation of lymphocytes as well as other cells. This presumption is affirmed by the findings that these metabolites arrest lymphocytes proliferation, particularly of T-cells but donot impair mature T- and B-lymphocytes (Ballet *et al.*, 1976). There could be another possible way of cytoxicity in the absence of ADA, and it is when high level of adenosine and deoxyadenosine cause the accumulation of S-adenosylhomocysteine which acts as an inhibitor of S-adenosylmethionine mediated methylation reactions required for normal functioning of cells (Johnston and Kredich, 1979). It has also been ascribed to the inhibition of pyrimidine nucleotide synthesis by adenosine, known as pyrimidine starvation. This probably arises from the inhibition of conversion of orotic acid to orotidine 5'-monophosphate or inhibition of PRPP synthesis by excessive synthesis of adenine nucleotide. ADA may also be directly involved in T-cell activation, since it is associated with human CD26, an essential accessory molecule for T-cell activation (Kameoka *et al.*, 1995). CD26 is a binding site for ADA on T cells and is costimulated by some anti-CD26 monoclonal antibodies and anti-CD3 induces CD4+ T cell proliferation (De Meester *et al.*, 1995). The specific binding of adenosine deaminase to the multifunctional membrane glycoprotein CD26 is thought to be immunologically relevant for certain regulatory and co-stimulatory processes (Ludwig *et al.*, 2004). Generally, ADA deficiencies cause death from murine infection before the patient reaches 2 years of age.

ADAs have been purified, characterized and reviewed from a wide variety of biological sources including amphibians (Ma and Fisher, 1968 a, b), aves (Ma and Fisher, 1968c; Iwaki-Egawa and Watanabe 2002; Iwaki-Egawa *et al.*, 2004), micro-organisms (Jun *et al.*, 1994) and mammals (Jaroszewicz and Kowalczyk, 1995; Singh and Sharma, 2000). ADA1 was purified

from human and chicken liver. The purified enzyme had a molecular weight of approximately 42 kDa on SDS-PAGE (Iwaki-Egawa and Watanabe 2002). ADA2 was purified to homogeneity from chicken liver. The purified enzyme had a molecular mass of approximately 110 kDa on gel filtration. The enzyme was also shown to be a homodimer with an estimated molecular mass of 61 kDa on SDS-PAGE (Iwaki-Egawa *et al.*, 2004). ADA activity was assayed in thymocytes and bursal lymphocytes at different times during chicken development. It was observed that the enzyme activity was significantly higher in the bursa than in the thymus. During development of chicken, studies on the activity of ADA in B- and T- cells revealed a higher expression in the B- lymphocytes. Thus, it plays a pivotal role in the maturation of the immune system in chicken, and is related more to the differentiation of the B- rather than the T-cell lineage (Senesi *et al.*, 1990). Chicken egg yolk contains an adenosine deaminase that was investigated after purification. It was compared with the adenosine deaminases of chicken liver and blood plasma. From this comparison, it is evident that this protein had undergone certain changes during the successive events leading to its final structure (Boeck *et al.*, 1975).

It was observed that fasting for 24 h decreased ADA activity in the stomach and small intestine of mice. Refeeding of the fasted mice for 24 h brought back the level of ADA in those tissues. These findings indicate that the GIT ADA is under the control of caloric-intake and corroborate with the biochemical adaptation of GIT enzyme during DR intervention (Holt and Kotler, 1987). Lowered activity of ADA in the stomach and small intestine of DR mice probably reflects a biochemical adaptation so that there is no futile synthesis of excess ADA when there is no need for it to metabolise dietary adenosine or to compensate the changes required for hydration of GIT during such intervention (Strohmeier *et al.*, 1995). The studies in our laboratory have indicated that lowered ADA activity during DR corroborate the fact that ADA level could be related to body fat and obesity in mammals. ADA activity was selectively reduced in GIT by DR indicating tissue-specific dietary regulation of ADA in mice (Ray and Sharma, 2002).

The physicochemical properties of ADA have been studied and well characterized. K_m of ADA towards adenosine ranges from 45-150 μM in human tissues, while in other sources; it

is between 35-400 μM. Previous work in our laboratory has shown that ADA has K_m value of 23 μM for adenosine and 16 μM for 2'-deoxyadenosine (Singh and Sharma, 2000). Studies on chicken ADA1 showed a K_m of 23 μM (Iwaki-Egawa and Watanabe, 2002) for adenosine whereas ADA2 showed a K_m of 144 μM (Iwaki-Egawa *et al.*, 2004) for adenosine. On the other hand, chicken ADA2 showed a K_m of 333 μM (Iwaki-Egawa *et al.*, 2004) for 2'-deoxyadenosine. The optimum pH for ADA1 was 7.5 to 8.0 whereas for ADA2 it was observed to be 6.5. Both forms were seen to be inhibited by 2'-deoxycoformycin (DCF), but EHNA could inhibit only ADA1.

1.12. ADA INHIBITION

Inhibition of ADA can serve separate purposes. Inactivation of the enzyme allows evaluation of the degree to which ADA contributes to adenosine metabolism. The compounds most commonly used to inactivate ADA are dooxycoformycin (DCF) and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA). These compounds are potent inhibitors of ADA, appear to be selective for the enzyme when used at appropriate concentrations, and are easily absorbed and distributed within tissues. There are a number of known ADA inhibitors both of natural and synthetic origin. DCF (also known as Pentostatin) is the most potent naturally occurring inhibitor. It is a 2'-deoxynucleoside with a K_i value of 2.5×10^{-12} M. This potent inhibitory activity is described as tight-binding because regeneration of the enzyme is extremely slow and the inhibition is sometimes described as irreversible. Pentostatin is in clinical use for the treatment of hairy cell leukemia. There are also a number of synthetic ADA inhibitors. Among the most important is EHNA (Schaeffer and Schwender, 1974). The difference between EHNA and DCF is the potency of inhibition of the enzyme. EHNA has a K_i value of 10^{-9} M which makes it one thousand times less active than DCF. Another major difference between the two drugs is their duration of inhibition of ADA. Unlike DCF, inhibition with EHNA is reversible with a half life of half an hour. This difference is based on the fact that EHNA is apparently metabolized by liver enzymes to oxidized (hydroxylated) metabolites which are excreted in the urine (McConnell *et al.*, 1980; Lambe *et al.*, 1983). Hydroxylated derivatives of (+)-EHNA at positions 8- and 9- in the alkyl chain have been shown to have, in addition to ADA inhibitory activity (Varghese *et al.*, 1994), a protective effect on the heart muscle against ischemic

damage. Adechlorin exhibiting a potent inhibitory activity against calf intestinal ADA was isolated from the cultured broth of *Actinomadura sp.* OMR-37. The aglycone of adechlorin was identical with that of the known adenosine deaminase inhibitors coformycin and 2'-deoxycoformycin (Omura *et al.*, 1985). Upon screening of four nucleoside analogues containing a common heterocyclic base, 4(7)-amino-6(5) H-imidazo[4,5- d]pyridazin-7(4)one, against calf-intestine adenosine deaminase, it was observed that two compounds with K_i values of 10-12 μM are more than four times as potent inhibitors of ADA compared with the other two, with K_i values of 51-52 μM. All four are only minimally or nontoxic to the normal cells (Ujjinamatada *et al.*, 2008).

1.13. DISEASES DUE TO ADA

A number of diseases are encountered due to an abnormal production of ADA and its distribution. Familial Mediterranean Fever (FMF) patients with acute attacks had higher serum ADA levels than attack-free periods and healthy controls. ADA may have a role in the cytokine network of the inflammatory cascade of FMF (Kisacik *et al.*, 2009). Several diseases are characterized by T-cell activation and this indicates the importance of ADA in the aetiopathogenesis of such diseases. A relationship between ADA levels and disease activities in patients with systemic lupus erythematosus (SLE) suggests its concentration as a useful parameter (Canpolat *et al.*, 2006). ADA and its isoenzymes play a role during pathophysiology of progressive systemic sclerosis, rheumatoid arthritis and SLE (Meunier *et al.*, 1995). The presence of ADA in various body fluids reflects the activity of cell-mediated immunity. The level of ADA is significantly higher in patients who have undergone successful pleurodensis (Yildirim *et al.*, 2008).

Adenosine deaminase deficiency is a rare inherited disorder of purine metabolism that leads to a form of severe combined immunodeficiency (SCID). Without treatment, the condition is fatal in the first year of life and therefore requires early intervention (Donofrio *et al.*, 1978; Gaspar *et al.*, 2009). Suggestions are made that high serum ADA activity may be involved in the pathogenesis of Parkinson's disease through peripheral T lymphocyte activation (Chiba *et al.*, 1995). New data provide support for a key role for adenosine in asthma, which

has become increasingly persuasive in recent years. The evidence is now convincing, and the time has matured to the design and evaluation of molecules that mimic or block the biological effects of adenosine as potential novel therapeutic agents for this condition (Fozard, 2003).

The immune system is under the modulation of adenosine which inhibits inflammation via reduction of cytokine biosynthesis and neutrophil functions. Inhibition of ADA and thus accumulation of adenosine has been found to be associated with a significant attenuation of intestinal inflammation (Antonioli *et al.*, 2007). Lack of ADA leads to an accumulation of adenosine in solid tumors and stimulates tumor growth and tumor angiogenesis while imparting tumor resistance to the immune system (Spychala, 2000). Adenosine has been found to be mitogenic for breast carcinoma cells (Mujoomdar *et al.*, 2004). Adenosine is being used in hospitals as treatment for severe tachycardia (Peart and Headrick, 2007). Abnormalities of this enzyme have also been reported in other diseases of immune system including AIDS, lymphomas, leukemias, anemia and several other unrelated disorders like short-limbed dwarfism, hepatitis and jaundice.

Studies have been conducted to observe that adenosine modulates the immune system and inhibits inflammation via reduction of cytokine biosynthesis and neutrophil functions. Adenosine removal which is facilitated by the enzyme is seen to play an inhibitory role in human tumor cell proliferation (Colquhoun and Newsholme, 1997). It was observed that adenosine deaminase inhibitors produce anti-inflammatory activity by increasing extracellular adenosine concentration. Thus, they have a good potential as anti-rheumatic and anti-inflammatory (Kuno *et al.*, 2006). Adenosine, the substrate of ADA, is known to stimulate the cAMP cascade in the epidermis (Iizuka *et al.*, 1976), leading to inhibition of cell proliferation through cAMP levels (Koizumi *et al.*, 1983). Elevated ADA activity in psoriatic epidermis has been reported (Koizumi *et al.*, 1983; Tikhonov *et al.*, 1998), and probably the increase in ADA activity is compatible with hyperproliferative states of the keratinocytes with pronounced DNA synthesis (Koizumi *et al.*, 1983). Hence, it could be concluded that the ADA activity was higher in psoriatic patients (Bukulmez *et al.*, 2000; Köse *et al.*, 2001).The enzyme activity is higher in hypothyroid patients and lower in hyperthyroid patients (Litvinenko, 1988). A significant elevated serum or pleural fluid ADA level was observed in tuberculous patients (al-

Shammary, 1997). The determination of ADA activity in pathologic pericardial fluids is of great value in the diagnosis of tuberculous pericardial effusions (Dogan *et al.*, 1999). Severe B- and T-cell lymphopenia was accompanied by a pronounced accumulation of 2'-deoxyadenosine and dATP in the thymus and spleen, and a marked inhibition of S-adenosylhomocysteine hydrolase in these organs. ADA-deficient mice exhibited severe pulmonary insufficiency, bone abnormalities and kidney pathogenesis (Blackburn *et al.*, 1998). In patients with liver diseases like chronic hepatitis, liver cirrhosis, increased serum ADA activities have been reported (Kobayashi *et al.*, 1993). Adenosine deaminase (ADA) activity, as a marker of cell-mediated immunity, was evaluated in the serum (S-ADA) and lymphocytes (L-ADA) of children with idiopathic nephrotic syndrome. The mean S-ADA and L-ADA levels were significantly raised in active nephrotic syndrome (ANS) and in its sub-groups in comparison with controls (Mishra *et al.*, 2005). A study was conducted to identify the role of ADA activity in patients with visceral leishmaniasis for management. There was a significant increase in mean ADA activity in sera of patients with visceral leishmaniasis (Baral *et al.*, 2005). In pregnant women, the activity of plasma 5'-nucleotidase and plasma adenosine were significantly elevated and plasma adenosine deaminase activity was significantly reduced. Enzymatic activities of both plasma enzymes appear to be changed in a way that would favor increased adenosine concentrations (Yoneyama, 2002).

1.14. GENE THERAPY

The first ever gene therapy trials were initiated by Anderson (Zwiebel *et al.*, 1990). The patient was a four-year-old girl who was suffering from a very rare disease known as severe combined immunodeficiency (SCID). In the patient's case, the disease was caused by the absence of the enzyme ADA. This deficiency prevented her body from producing the lymphocytes (both B- and T-cells) that are required to fight off infections. Before the advent of gene therapy, there were two ways to treat ADA related SCID. The first was regular injections with the ADA enzyme, administered as a mixture with polyethylene glycol (PEG) to increase its stability. The second was a bone marrow transplant from a compatible donor. When neither of these treatments was possible, the only way affected children could survive was by total isolation of them in an artificial, germ-free environment. For this reason, children suffering

from SCID are often known as 'bubble babies'. ADA deficiency was an ideal target for the first gene therapy trials for a number of reasons:

- the pathological effects of the disease are reversible
- the disease results from the loss of function of a single gene
- ADA levels vary widely in the normal population so tight control of the introduced gene is not important
- the ADA gene is very small and easy to manipulate in the laboratory
- the target cells for the therapy are lymphocytes, which are accessible, easy to culture and easy to put back into the body of the patient
- alternative treatments are expensive and/or hazardous.

Further trials were initiated in which bone marrow cells or umbilical cord blood cells were used as targets. These contain the stem cells that produce lymphocytes throughout our lives. The modification of these stem cells did result in the long-term production of a small number of ADA-positive lymphocytes. However, the levels of ADA produced by the cells were low and it is not clear whether the patients would survive without concurrent ADA-PEG treatment.

In 2002, there was a major breakthrough in ADA gene therapy. This resulted from the use of a technique called non-myeloblative conditioning, in which bone marrow in the SCID patient is partially killed in order to give the modified stem cells the chance to proliferate (Aiuti, 2002). Another important factor was that none of the children in this trial had been treated with ADA-PEG. It appears that enzyme treatment may have contributed to the lack of success in previous gene therapy trials. The first patient was a two-year-old Palestinian child called Salsabil who had never received ADA-PEG therapy. The new treatment seems to have cured her condition and Salsabil is now enjoying a normal life. Her body is producing antibodies and she has even managed to fight off chicken pox, which would almost certainly have been fatal to her (Aiuti, 2002).

Clinical gene therapy trials for adenosine deaminase deficiency have shown limited success of corrective gene transfer into autologous T lymphocytes and $CD34^+$ cells (Cassani *et*

al., 2009). In these trials, the levels of gene transduction and expression in hematopoietic cells have been assessed by DNA- or RNA-based assays and measurement of ADA activity. A flow cytometry (fluorescence-activated cell sorting, FACS) assay capable of estimating the levels of intracellular ADA on a single-cell basis has been formulated. This technique has been validated with T cell lines and peripheral blood mononuclear cells (PBMCs) from ADA-deficient patients that showed severely reduced levels of ADA expression (ADA-dull) by FACS and Western blot analyses. After retrovirus-mediated ADA gene transfer, these cells showed clearly distinguishable populations exhibiting ADA expression (ADA-bright), thus allowing estimation of transduction efficiency. By mixing ADA-deficient and normal cells and using enzymatic amplification, the staining procedure could detect as little as 5% ADA-bright cells. This technique, therefore, is useful to quickly assess the expression of ADA in hematopoietic cells of SCID patients and represents an important tool for the follow-up of patients treated in clinical gene transfer protocols (Otsu *et al.*, 2002). Gene therapy for the most common forms of SCID can lead to immune reconstitution in most patients, although a minority of patients has derived minimal clinical benefit (Sokolic *et al.*, 2008). Gene therapy was able to provide full correction of disease phenotype and, hence, clinical benefit of SCID-X1 (Cavazzana-Calvo *et al.*, 2000).

1.15. CHICKEN GENOME

Various avian species provide models of evolution, development and differentiation, behaviour and ecology. Avian genomes appear to show relatively high levels of conservation, at least of the karyotype, and the genome sequence and related information and infrastructure that are available for chicken provide added benefits for the genetic analysis of all wild and domestic birds. The chicken is a premier non-mammalian vertebrate model organism. It is one of the primary models of embryology and development because its embryonic development occurs *in ovo* rather than *in utero*. The immune system of chicken have the thymus, a paired lobulated gland along the neck of the chicken which is considered to be the source of T-cells or small lymphocytes which mediate the rejection of hemografts, graft-virus-host reaction and delayed hypersensitivity. They also have the bursa of fabricus, an organ situated dorsally to the

cloaca which is considered to be the source of B-cells which forms plasma cells and produce antibody.

It is a major model organism for the study of viruses and cancer. The chicken immune system provided the first indications of the distinctions between B- and T- cells, with the B-cell nomenclature based on the avian Bursa of Fabricus (Cooper *et al.*, 1966). The chicken is also an important model system for studies of gene regulation. Many of the pioneering studies of steroid hormone regulation involved the chicken oviduct like the ovalbumin gene regulation. As the chicken red blood cells retain their nucleus, they have been the classical model system in which the chromatin structure has been studied. As one of many possible examples, the CCCTC-binding factor (CTCF) that now appears to play critical role in imprinting and X inactivation was first discovered via its activity at the chicken *c-myc* gene. Evidence continues to grow that gene order is conserved between the human and chicken genomes to a remarkable extent. The chicken genome has a haploid content of 1.2×10^9 base pairs of DNA organized on 38 autosomes plus the Z and W sex chromosomes. Most autosomes are called microsomes which cannot be distinguished by size alone.

Both the thymus and bursa regress as the bird approaches maturity and may also regress with diseases. Early damage to the bursa has been shown to increase susceptibility to certain diseases. Grossly recognizable lymphoid tissues equivalent to Peyer's patches are found along the intestinal tract of the chicken, particularly in the ileum, at Meckel's diverticulum and at the base of the ceca (cecal tonsils). Other important accumulations of lymphoid tissue are found under respiratory epithelium, in Harderian glands, and around lacrimal and nasal gland ducts. Bursal-dependent lymphoid tissue appears in histological section as well circumscribed immune bodies (follicles). The avian spleen has white and red pulp. Its functions are similar to those of the mammalian spleen except that there is some argument as to whether or not it functions as a blood reservoir.

The draft genome of the chicken has been sequenced in 2004. It has been revealed that the bird has 20,000 to 23,000 genes and has only 1 billion DNA base pairs. The evolutionary distance between chicken and human provides high specificity in detecting functional elements,

both non-coding and coding. Many of the conserved non-coding sequences are located far from genes and cannot be assigned to defined functional classes. In coding regions, the evolutionary dynamics of protein domains and orthologous groups demonstrate processes which distinguish the lineages leading to birds and mammals (Schmutz and Grimwood, 2004).

Not much is known about the role of chicken ADA as a function of postnatal development. Studies on the level of ADA in different tissues at various postnatal ages of chicken have not been done earlier. The endeavor has, thus, been to ensure a proper understanding of the distribution of ADA in various tissues. Consequent upon all the above information and keeping in mind the role of ADA in the regulation of a wide array of physiological processes, the present work has been directed towards the following:

1. Determination of the normal endogenous activity level of ADA in different tissues of GIT (esophagus, crop, proventriculus, small intestine) and spleen at different postnatal ages during development, i.e. at day 1, 10, 30, 60 and 90 of male chicken. This will be done in order to find out the changes, if any, in the activity level of ADA in different tissues at various postnatal ages which might be able to comprehend the tissue- and age-specific implication of ADA in early postnatal development of chicken.

2. The role of hormones and their analogues like corticosterone (adrenalcortical hormone), testosterone (sex hormone), triiodothyronine (thyroid hormone) and a membrane permeable analog of c-AMP, dibutyryl cAMP, on the activity of adenosine deaminase in different tissues of chicken at two specific postnatal ages (day 10 and 60) ascertained, in order to postulate the effect of these hormones, if any, in modulation of ADA in a tissue- and age- specific pattern.

3. The activity and protein levels of ADA monitored by enzyme assay and Slot as well as Western blot analyses. This was conceived to contemplate if the alterations, if any, took place at the level of transcription or translation.

4. Purification of ADA from small intestine of two age groups (day 1 and 90) of chicken and characterization of its physicochemical and kinetic properties, with a view to analyze the alterations, if any, in these parameters of the enzyme during the course of development.

2. Experimental Procedures

2.1. ANIMALS AND DIET

Male chicken (Babcock Venkateswara 380, BV 380 breed) were purchased locally from a veterinary farm. They were maintained at 25 ± 2°C under normal laboratory conditions and fed with a chick mash diet (Premier Hatchery Ltd.) and tap water *ad libitum*. Chicken of five postnatal ages (1-, 10-, 30-, 60- and 90- days) were used to determine the normal endogenous level of ADA activity. For the hormonal studies, two postnatal ages (10- and 60- day) were selected for the experiments. Institutional guidelines on use of animals were followed during experimentation.

2.2. BIOCHEMICALS AND REAGENTS

Sigma Chemical Company, St. Louis, USA: Adenosine (9-β-D-Ribofuranosyladenine), corticosterone, Bt_2cAMP (N^6-2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate), T_3 (3,5,3'- triiodothyronine), testosterone propionate, imidazole, 2'-deoxyadenosine, inosine, DEAE-cellulose, phenylmethylsulfonyl fluoride (PMSF), Tris, acrylamide, ammonium persulphate, bromophenol blue sodium salt, TEMED (N,N,N',N'-tetramethylethylenediamine), EDTA (Ethylene diaminetetraacetic acid), purine riboside, 2-mercaptoethanol, DL-Dithiothreitol, sephadex G-100, BSA (bovine serum albumin), sodium azide, DTNB [5,5'-Dithiobis (2 nitrobenzoic acid)], urea, nitrocellulose membrane (0.45 μm pore size), dialysis tubing.

Merck, India: Calcium chloride, magnesium chloride, sodium chloride, citric acid-1-hydrate.

Suree Interfoods, Thailand: Sesame oil.

Glaxo, India: Mercury chloride.

Qualigens, India: Sucrose, glycerol, potassium chloride purified, tri-sodium citrate, sodium hydroxide, hydrochloric acid, acetic acid, orthophosphoric acid, sodium chloride.

Pharmacia, Sweden: Blue dextran

Boehringer Manheim, Indianapolis, USA: Bis-acrylamide, sodium dodecyl sulphate.

Bangalore Genei, India: BCIP/NBT, Tween-20, Goat-anti-rabbit-IgG-ALP conjugate

Bengal Chemicals and Pharmaceuticals, India: Ethanol, Methanol

Santa Cruz Biotechnology, Inc USA: A rabbit polyclonal antibody raised against amino acids 64-363 of human adenosine deaminase.

SRL, India: Glycine, Coomassie Brilliant Blue G250, Coomassie Brilliant Blue R250.

Whatman, England: Qualitative filter paper, Whatman No. 1 filter paper

Miscellaneous: Sterile disposable syringes and injection needles were purchased locally.

2.3. BUFFERS

The buffers were made as per requisition and stored at 4°C. The buffers used were as follows:

A. Homogenising Buffer - 0.25 M sucrose/100 mM sodium citrate, pH 5.5

B. Assay Buffer - 100 mM sodium citrate, pH 5.5

C. Tris buffer saline (TBS) - 20 mM Tris-HCl, pH 7.5/50 mM NaCl

D. Tween Tris buffer saline (TTBS) - 20 mM Tris-HCl, pH 7.5/50 mM NaCl/0.5% Tween 20

E. Phosphate buffer saline (PBS) - 0.14 M NaCl/3 mM KCl/10 mM Na_2 HPO_4/2 mM KH_2PO_4,
pH 7.3

F. Towbin buffer-25 mM Tris/192 mM glycine/20% w/v methanol, pH 8.3

G. Imidazole Buffer-20 mM imidazole/ 0.25 M sucrose, pH 6.8

H. Imidazole Buffer-20 mM imidazole buffer/20 µM sodium azide, pH 6.8

I. Imidazole Buffer-10 mM imidazole buffer/10 µM dithiothreitol/20 µM sodium azide, pH 6.8

2.4. INSTRUMENTATION

2.4.1. pH measurements: A Cyberscan 510 digital pH meter was used at room temperature after calibrating with standard buffer solutions of pH 4, 7 and 10.

***2.4.2. Absorbance measurements*:** A Hitachi U-2000 Double Beam Spectrophotometer was used for all absorbance measurements in the visible as well as ultraviolet region using glass or quartz cuvettes of path length 1 cm.

***2.4.3. Homogenisation*:** A Remi motor type RQ-127 A, HP 8, rpm 8000 homogenizer fitted with Teflon pestle was used. Glass homogenizing tubes of 10 ml to 50 ml were used for tissue homogenization.

***2.4.4. Centrifugation*:** Centrifugations were carried out in an Allegra TM-64R and a Hitachi Himac CR22GII high speed refrigerated centrifuge at 4°C.

***2.4.5. Electrophoresis*:** For carrying out PAGE and SDS-PAGE, a Bio-Rad Mini Protean Tetra cell was used.

***2.4.6. Weighing balance*:** For weighing of chemicals, a Sartorius CP225D and a Mettler electronic balance were used.

***2.4.7. Rocker*:** A rocker from Bangalore Genei TM Rocker-100 was used for shaking the gels and nitrocellulose membranes.

***2.4.8. Fraction collector*:** A mini collector SJ-1410 from Atto, Japan, was used for collecting the fractions after chromatography.

***2.4.9. Millipore water*:** Millipore Prograd ® 2 (Elix R) was used for distilled water.

***2.4.10. Slot blot*:** A Bio-Rad®-SF Microfiltration apparatus was used for slot blot.

***2.4.11. Western blot*:** A Bio-Rad Mini Trans-Blot®-Electrophoretic transfer cell was used for Western blot.

2.5. PREPARATION OF TISSUES

Chicks were sacrificed by decapitation at a fixed time of the day (15:00 h). The regions of GIT (esophagus, crop, proventriculus and small intestine) and spleen were dissected out, washed in normal saline (0.9% NaCl) and blotted dry. A 20% (w/v) homogenate was prepared in chilled buffer A. The homogenates were centrifuged at 20,000 x g for 45 min at 4°C to obtain a clear fat free cytosol. The supernatant thus obtained was used for the assay of the enzyme.

2.6. ASSAY PROTOCOL FOR ADA

The activity of ADA was measured spectrophotometrically in a Hitachi Model U-2000 spectrophotometer using the method of Kalckar (1947) and Yoshida and Aikawa (1993) with certain modifications of our own (Bhattacharjee and Sharma, 2009). The rate of adenosine degradation is measured spectrophotometrically, catalysed by adenosine deaminase. The reaction occurs as follows:

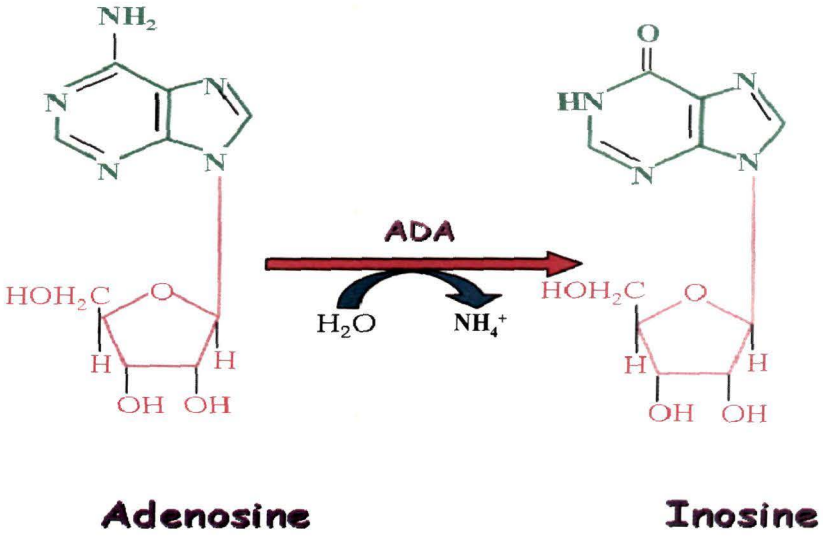


Fig. 4: Adenosine deaminase catalytic deamination reaction of adenosine.

Adenosine has characteristic absorption maxima at 265 nm with an extinction coefficient of 8.5 x 10^6 / mole. The rate of disappearance of adenosine was measured at 265 nm using a cuvette of 1.0 cm path length. The standard assay was carried out at 25°C. The final volume of the reaction mixture was 3.0 ml which contained:

Sodium Citrate buffer, pH 5.5	- 100 mM
Adenosine	- 100 µM
Enzyme (suitably diluted)	- 50 µl

The reaction was initiated by addition of the enzyme. The decrease in absorbance was recorded at 30 sec intervals for 3 min and the enzyme activity was calculated from the initial rate of hydrolysis of adenosine. The linear decrease in absorbance per min was used for the calculation of the enzyme activity.

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

2.7. PROTEIN ESTIMATION

Protein content of the enzyme preparation was measured according to the dye-binding method of Bradford (1976) using bovine serum albumin (BSA) as standard. The reagents and procedure were as follows:

Reagent A: Coomassie Brilliant Blue G-250 (0.1 g in 50 ml of 95% ethanol)
Reagent B: Phosphoric acid (85%)

***Stock solution*:** This was prepared by mixing reagent A (50 ml) with B (100 ml) and stored in a brown bottle at 4°C for future use.

***Bradford's working solution*:** It was prepared by diluting 15 ml of the stock solution to 100 ml with distilled water and filtered through Whatman's filter paper no. 1 just before use. The final concentration of the reagents was 0.01% (w/v) Coomassie Brilliant blue G-250, 4.7% (v/v) ethanol, 8.5% (v/v) phosphoric acid. Appropriately diluted protein sample (0.1 ml) was added to 5 ml of Bradford's working solution and mixed thoroughly with a cyclomixer. The intensity of the color was measured at 595 nm immediately. Protein concentrations were determined using a computed standard curve prepared by different concentrations of BSA (0.01 to 0.1 mg/ml).

2.8. HORMONAL TREATMENTS

Effects of corticosterone, Bt_2cAMP, T_3 and testosterone were studied on the activity of ADA in different tissues of chicken at two postnatal ages. For each hormone and for each age group, the dose and time responses were obtained from pilot experiments. The most optimal dose and incubation time vis-à-vis enzyme activity was selected and reported. Three sets in duplicate, comprising 3-4 animals per set were used. The first set served as normal, where no injection was administered, the second set was administered only the suspension medium (saline, saline/ethanol or sesame oil), whereas the third set was administered the test hormone. To avoid fluctuations which might arise due to circadian rhythms, all the hormone administrations were done at a fixed time of the day (09:00 h) in all cases.

For corticosterone, pilot experiments were undertaken to standardize the time- and dose- response of corticosterone in influencing ADA activity (Singh and Sharma, 1995). The dose and time response of Bt_2cAMP, a membrane permeable analog of cAMP, was ascertained for regulating ADA activity. Trial experiments were carried out to determine the time and dose response of ADA towards T_3. Similarly, for testosterone, the dose and time responses of the hormone towards ADA activity were standardized.

2.8.1. ***Corticosterone injection***

Corticosterone was administered to two different age groups (10- and 60-day) of chicken as a single dose of 1.0 mg/ 100 g body weight in 0.3 ml normal saline (0.9% NaCl) having 6% ethanol intraperitoneally (i.p). A set of animals for each group were used as control and received an equal amount of saline and ethanol solution. All the chicks were sacrificed after 6 h of treatment and tissues were removed, washed in normal saline and blotted dry. These tissues were stored at -80 °C till further use.

2.8.2. ***Bt_2cAMP injection***

Bt_2cAMP, a membrane permeable analog of cAMP, was administered at a single dose of 1.0 mg/ 100 g body weight in 0.3 ml normal saline, i.p. Control chicks received an equal amount of saline only. The controls and treated chicken were sacrificed after 4 h of injection. Tissues were processed as described above.

2.8.3. ***T_3 injection***

Maximum response of the enzyme was obtained at a dose of 200 µg/ 100 g body weight of 3,5,3'-triiodothyronine (T_3). T_3 was administered in 0.3 ml normal saline, i.p for 3 consecutive days. Control chicks received an equal amount of saline only. The controls and treated chicken were sacrificed after 3 h of final injection. Tissues were removed and processed similarly as described above.

2.8.4. ***Testosterone injection***

Testosterone was administered at a dose of 1.0 mg/ 100 g body weight in 0.3 ml sesame oil, i.p, for 3 days. Each set of control chicks received an equal amount of sesame oil only. The controls and treated chicken were sacrificed after 3 h of final injection. The tissues were removed and processed in a similar fashion as described above.

2.9. IMMUNOPRECIPITATION

The antibody used for Slot and Western blotting of chicken ADA was of human origin, and hence, it was necessary to find the cross-reactivity of human anti-ADA antibody with chicken ADA. It was done by following the method described by Rosenberg (1996) with some modifications.

Materials used:

a. Specific primary antibody: Anti-ADA antibody
b. Suspension of protein A-CL agarose beads: This was used directly from the bottle as supplied.
c. 100 mM PMSF.
d. Immunoprecipitation buffer: This buffer consisted of the following components:

Tris-HCl, pH 8.0	50 mM
NaCl	500 mM
EDTA	5 mM
NaN_3	0.02%
Triton X-100	0.5%
SDS	0.1%

To prepare 100 ml of Tris-HCl buffer, 0.6 g of Tris base was dissolved in Millipore water, the pH was adjusted to 8.0 with concentrated HCl, and the final volume was raised to 100 ml with Millipore water. The immunoprecipitation buffer was prepared by dissolving the remaining chemicals in Tris-HCl, and stored refrigerated at 4°C.

100 µl of the centrifuged homogenate was taken in different test tubes which served as the antigen. To each, different volumes of anti-ADA antibody was added ranging from 0-, 1-, 3-, 5-, 10-, 15-, 20-, 25-, 35- µl. PMSF was added to a concentration of 10 mM to each. This was incubated at 4°C overnight with constant stirring. 30 µl of Protein A-CL agarose bead suspension was added with cut tips to prevent lysis of beads. This was mixed thoroughly and incubated at 4°C for 2 h. Protein A-CL agarose beads were recovered by centrifugation at

18000 x g for 3 min at 4°C. The supernatant was used as the source of the enzyme and the assay performed in the way described in section 2.6.

Using BLASTP 2.2.19+, the homology between human (acc. no. NP000013) and chicken (acc. no. Q5ZKP6) ADA was found to be 66% (Altschul *et al.*, 1997). The cross-reactivity of the antibody used to chicken ADA was found to be ~40%.

2.10. SLOT BLOT ANALYSES

The blotting was performed on Bio-Rad Bio-Dot® SF Micro filtration apparatus following the instructions given in the user's manual. A clear fat free cytosol was used for the slot blotting experiment. Nitrocellulose (NC) membrane was soaked for 30 min in buffer C for proper binding. To each slot, an equal amount (100 µl) of cytosol containing 60 µg protein was allowed to filter through activated nitrocellulose membrane by gentle vacuum. The membrane was transferred in blocking solution (5% non-fat milk in buffer C). It was washed twice in buffer D with gentle agitation and incubated overnight with anti- ADA antibody solution (1: 1000). The membrane was then washed twice in buffer D to remove unbound antibodies. After this, the membrane was transferred to a solution containing goat anti- rabbit- IgG- ALP conjugate (1: 10000). This was allowed to incubate for 3 h. The membrane was washed twice in buffer D and finally washed in buffer C to remove the detergent. For the development of color, the substrate (BCIP/ NBT) was added and the membrane kept at 37°C till the development of color. The reaction was stopped by washing the membrane in ddH_2O and photographed using an hp scanjet 7400 c scanner.

2.11. WESTERN BLOT ANALYSES

Thirty µl of cytosol containing 50 µg protein was loaded in each lane. After the protein samples were subjected to SDS-PAGE (8%), the gels were taken out and incubated in buffer F for 10-15 min for equilibration. The transfer was carried out in chilled buffer F using BioRad Mini trans-blot® electrophoretic transfer cell at 100 V (constant) for 60 min. Bio ice cooling unit was also used to maintain appropriate buffer temperature. The electroblotted nitrocellulose

membranes were then processed for immunodetection. The membrane was transferred in blocking solution (5% non-fat milk in buffer C). It was washed twice in buffer D with gentle agitation and incubated overnight with anti- ADA antibody solution (1: 500). The membrane was then washed twice in buffer D to remove unbound antibodies. After this, the membrane was transferred to a solution containing goat anti- rabbit- IgG- ALP conjugate (1: 10000). This was allowed to incubate for 3 h. The membrane was washed twice in buffer D and finally washed in buffer C to remove the detergent. For the development of color, the substrate (BCIP/ NBT) was added and the membrane kept at 37°C till the development of color. The reaction was stopped by washing the membrane in ddH_2O and photographed using an hp scanjet 7400 c scanner.

2.12. INDIA INK STAIN

In order to ascertain equal amount of loads in each slot, for slot blots, a parallel set was run and then the nitrocellulose membrane was stained with 0.2% India ink in Tween solution (0.3% Tween 20 in buffer E). Destaining was performed with Tween solution till the background was clear and the protein bands could be visualized. For Western blots, a parallel set was subjected to SDS-PAGE (8%) and incubated in buffer F. The proteins were then transferred to a nitrocellulose membrane using BioRad Mini trans-blot® electrophoretic transfer cell at 100 V (constant) for 60 min. The nitrocellulose membrane was stained overnight with 0.2% India ink in Tween solution (0.3% Tween 20 in buffer E). Destaining was performed with Tween solution till the background was clear and the protein bands could be visualized. The blots were photographed using an hp scanjet 7400 c scanner.

2.13. PURIFICATION OF ADA

Adenosine deaminase was purified from the small intestine of immature (1-day) and mature (90-day) chicken by the protocol of Jaroszewicz and Kowalczyk (1995) and Mohamedali *et al.*, (1996) with certain modifications of our own (Singh and Sharma, 2000). All the steps were carried out at 4°C.

2.13.1. Crude extract: The small intestinal tubes were collected from 10-12 male immature and mature chicken. The intestinal tubes were washed twice in chilled saline (0.9% NaCl) and blotted dry. 10% (w/v) homogenate was prepared in ice-cold 20 mM imidazole buffer, pH 6.8 containing 0.25 M sucrose. The crude homogenate was centrifuged at 27,000 x g for 60 min. The supernatant thus obtained was used for ammonium sulfate precipitation.

2.13.2. Ammonium sulfate fractionation: The supernatant was brought to 40% ammonium sulfate saturation through gradual addition of solid $(NH_4)_2SO_4$. The solution was stirred gently till all $(NH_4)_2SO_4$ dissolved and was left overnight. This was centrifuged at 15,000 x g for 15 min. The supernatant was brought to 70% ammonium sulfate saturation, pH adjusted to 6.5. This was centrifuged at 20,000 x g for 30 min. The precipitate obtained was dissolved in 3-4 ml of 20 mM imidazole buffer, pH 6.8 and dialysed for 36 h against similar buffer. Dialysate was centrifuged at 20,000 x g for 30 min and pellet discarded.

2.13.3. Sephadex G-100 gel permeation: The supernatant was loaded onto a sephadex G-100 [(40 x 3) cm] column, which was pre-equilibrated with 20 mM imidazole buffer, pH 6.8 containing 20 μM sodium azide. Fractions of 3 ml were collected at a flow rate of 14 ml/h, using automated mini collector SJ-1410 from Atto, Japan. Each fraction was monitored spectrophotometrically at 280 nm for protein and assayed for ADA activity. Enzymatically active fractions were pooled, dialysed against 10 mM imidazole buffer, pH 6.8, containing 10 μM dithiothreitol and 20 μM sodium azide.

2.13.4. DEAE-cellulose ion exchange chromatography: DEAE-cellulose (10 g) was taken in a beaker to which 500 ml of 0.1 N HCl was added with constant stirring. This was washed and to it 500 ml of 0.1 N NaOH was added with constant stirring. Using a Buchner funnel, this was washed till neutral pH. It was then loaded on a column [(14 x 2.5) cm] and equilibrated with 10 mM imidazole buffer, pH 6.8. The dialysate obtained was applied onto a column of DEAE-cellulose, pre-equilibrated with 10 mM imidazole buffer, pH 6.8 containing 10 μM dithiothreitol and 20 μM sodium azide, at a flow rate of 30 ml/h. The column was washed extensively with the equilibrating buffer till absorbance of eluting fractions was nearly zero. A linear gradient of 0-0.4 M NaCl in the same buffer was applied to elute bound fractions. The

active peak fractions were collected, pooled and dialysed against 100 mM sodium citrate buffer, pH 5.5 before further use.

2.14. GEL ELECTROPHORESIS

Native and SDS-polyacrylamide gel electrophoresis (PAGE) were carried out according to the procedures described in Mini-Protean® Tetra Cell instruction manual. The protocols used were the same except some modifications.

2.14.1. ***Native PAGE:*** The following media and reagents were used for native PAGE:

a. Stock acrylamide solution: 30 g acrylamide and 0.8 g bis acrylamide was dissolved in 100 ml H_2O and filtered (Store at 4°C)
b. Separating gel buffer: 1.5 M Tris-HCl, pH 8.8
c. Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8
d. 10% ammonium persulphate in H_2O
e. N, N,N',N' tetramethylethylene diamine (TEMED)
f. Sample buffer (5X): 15.5 ml of 1 M Tris-HCl, pH 6.8, 2.5 ml of 1% solution of bromophenol blue, 7.0 ml H_2O and 25 ml glycerol was taken. Samples were diluted to give a solution that is 1 X sample buffer.
g. Electrophoresis buffer: 3.0 g Tris base and 14.4 g glycine was taken in 1 L H_2O. Final pH was 8.3
h. Protein stain: 0.25 g CBB R 250 was dissolved in 125 ml methanol. To this 25 ml glacial acetic acid and 100 ml H_2O was added.
i. Destaining solution: 100 ml methanol, 100 ml glacial acetic acid and 800 ml H_2O

Native polyacrylamide gel electrophoresis (PAGE) of adenosine deaminase (purified from immature and mature chicken small intestine) was performed according to the method of Davis (1964) with slight modification. A 8% resolving slab gel was prepared by taking 3.3 ml of acrylamide/bisacrylamide solution / 2.5 ml of 1.0 M Tris buffer (pH 8.8) / 4.2 ml of distilled water / 50 µl of 10% ammonium per sulphate (freshly prepared) / 5 µl TEMED. A 4% stacking gel was prepared by taking 1.3 ml of acrylamide/bisacrylamide solution / 2.5 ml of 1.0 M Tris

buffer (pH 8.8) / 6.2 ml of distilled water / 50 µl of 10% ammonium per sulphate (freshly prepared) / 10 µl TEMED.

Prior to addition of ammonium per sulphate, both the gel solutions were degassed for 15 min under vacuum. The resolving gel was poured in between the slab plates fitted with spacers, sealed at both sides and lower end, to the mark. This was allowed to polymerise. Following this, the stacking gel was layered on top of the resolving gel and a comb inserted. The gel was allowed to polymerise and then the comb was removed. Purified enzyme preparations (10 µg) were loaded in the wells and electrophoresed for 45-50 min at constant current of 60 mA at room temperature. Subsequently, the gel plates were prised apart and the gel was stained with Coomassie Brilliant Blue R-250 solution for 3 h. Background stain was removed by immersing the gel in destaining solution for 4-5 h till the background was clear and the bands were clearly visible.

2.14.2. ***SDS-PAGE:*** The reagents, media and procedure for SDS-PAGE have been described below:

a. Acrylamide/ bis: 29.2 g/ 100 ml acrylamide, 0.8 g/ 100 ml N', N'- bis-methylene-acrylamide was dissolved in 100 ml ddH_2O. This was filtered and stored at 4°C in dark.
b. 1.5 M tris-HCl, pH 8.8: 18.15 g Tris base was taken in 80 ml ddH_2O. The pH was adjusted to 8.8 with 1 N HCl. The final volume was made to 100 ml with ddH_2O and stored at 4°C.
c. 0.5 M Tris-HCl, pH 6.8: 6.0 g Tris base was taken in 60 ml ddH_2O. The pH was adjusted to 6.8 with 1N HCl. The total volume was made to 100 ml with ddH_2O and stored at 4°C.
d. 10% sodium dodecyl sulfate (SDS)
e. Sample buffer (SDS reducing buffer) (Stored at room temperature)

Distilled H_2O	4.0 ml
0.5 M Tris-HCl, pH 6.8	1.0 ml
Glycerol	0.8 ml
10% SDS	1.6 ml
2-β mercaptoethanol	0.4 ml (just before adding sample)

0.05% bromophenol blue 0.2 ml

The sample was diluted at least 1:4 with sample buffer and heated at 95°C for 4 min

f. 5 X electrode (running) buffer, pH 8.3

Tris base	15 g/l
Glycine	72 g/l
SDS	5 g/l

This was stored at 4°C. This was used at 1X dilution.

SDS-PAGE of adenosine deaminase (purified from immature and mature chicken small intestine) was performed according to the method of Davis (1964) with slight modification. A 8% resolving slab gel was prepared by taking 2.7 ml of acrylamide/bisacrylamide solution / 2.5 ml of 1.0 M Tris buffer (pH 8.8) / 4.7 ml of distilled water / 100 µl SDS / 50 µl of 10% ammonium per sulphate (freshly prepared) / 5 µl TEMED. A 4% stacking gel was prepared by taking 1.3 ml of acrylamide/bisacrylamide solution / 2.5 ml of 1.0 M Tris buffer (pH 8.8) / 6.1 ml of distilled water / 50 µl of 10% ammonium per sulphate (freshly prepared) / 10 µl TEMED.

The details of gel preparation and separation were the same as described for native PAGE. Purified enzyme preparations (50 µg) were loaded in the wells and electrophoresed for 45-50 min at a constant current of 60 mA at room temperature. The gel plates were then prised apart and the gel was stained with Coomassie Brilliant Blue R-250 for 3 h. The background stain was removed by immersing the gel in destaining solution as described above.

The relative mobility (R_m) of the electrophoresed samples were determined according to the method of Laemmli (1970) using the expression:

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

2.15. MOLECULAR WEIGHT DETERMINATION

Molecular weights of both immature and mature chicken ADA were determined by Sephadex G-100 gel permeation chromatography and SDS-PAGE using Sigma gel filtration low molecular weight markers and Biorad Precision Plus Protein™ All Blue Standards (Catalog No.161-0373) respectively. The standard markers for gel permeation chromatography were cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa).

A linear calibration curve of R_m versus log molecular weight and elution volume/void volume (Ve/Vo) versus log molecular weight was plotted. The molecular weights of ADA from the two ages were determined by comparing R_m and/or V_e/V_o values, employing the standard calibration curves.

2.16. KINETIC STUDIES:

The kinetic studies for both immature and mature intestinal ADA of chicken were carried out using the purified enzyme. All the studies were carried out after dialysis of the purified enzyme against 100 mM sodium citrate buffer, pH 5.5.

2.16.1. ***K_m determination***: K_m values for intestinal ADA of immature and mature chicken were determined by Michaelis- Menten and Lineweaver-Burk plots using various concentrations (0-110 μM) of adenosine and 2'-deoxyadenosine as substrates.

2.16.2. ***K_i determination***: K_i values were determined by Dixon's plot at two fixed concentrations (40 and 100 μM) of adenosine using varying concentrations of purine riboside as an inhibitor.

2.16.3. ***pH stability***: ADA activities were found out using 100 mM sodium citrate buffer, pH 5.5, after pre-incubating the enzyme in buffers of different pH (4- 9) for 1 h.

2.16.4. ***Effect of temperature***: The activity of ADA at different temperatures (25°C to 70°C) was determined to find the optimum temperature for both immature and mature intestinal ADA of chicken. The results were expressed as percentage activity taking the activity at 25°C as 100%.

2.16.6. ***Effect of urea***: Using varying concentrations (0-3 M) of urea as denaturant in sodium citrate buffer, pH 5.5, the activity of purified ADA from immature and mature chicken was studied. At these concentrations of urea, the enzyme samples were preincubated for 30 min. The results were expressed as percentage activity retained in presence of specific urea concentration, taking no urea as 100%.

2.16.7. ***Effect of modulators on ADA:*** ADA activity for adenosine in presence of DL-dithithreitol, β-mercaptoethanol, 5, 5'-dithiobis-(2-nitrobenzoic acid), Ca^{2+}, Mg^{2+}, Hg^{2+} and caffeine were studied.

3. Results

All the results are presented in the form of tables and figures for the sake of clarity. They include the mean values, standard deviation and the level of significance. The activity of adenosine deaminase (ADA) is expressed as unit/mg protein.

3.1. NORMAL ENDOGENOUS LEVEL OF ADENOSINE DEAMINASE

The normal endogenous level of adenosine deaminase (unit/mg protein) was measured in the GIT (esophagus, crop, proventriculus, small intestine) and spleen of 1-, 10-, 30-, 60- and 90-day old chicken. Amongst the regions of GIT, the highest level of ADA activity was found in the small intestine, followed by the esophagus, proventriculus and crop [Fig.1-10]. The specific activity of ADA in all the regions of the GIT exhibited a high level at day 1. At day 10, a sharp decline (-34% in esophagus, -35% in crop and -48% in small intestine) in the activity of ADA was seen in all regions of GIT, except proventiculus where the activity of ADA decreased (-30%) at day 30 and showed further decline (-52%) at day 90 as compared to day 1. While in other regions, the activity of ADA remained fairly constant after day 10. In the spleen, the activity is low in day 1, but increases till day 30 (+78%) and then drops at day 60 and remains almost same till day 90. Slot and Western blots were performed at the two ages where the differences were significant, in order to find out if there was a change in the protein level of the enzyme. Representative data showed that the level of ADA was indeed seen to decrease or remain constant, for which the intensity was determined using KDS-1 software and graphs plotted. Slot blot analyses of ADA protein in the selected postnatal ages, days 1 and 10 indicated that the decline was at the ADA protein level [Fig. 6 a (i), 8 a (i), 10 a (i), 12 a (i), 14 a (i)]. Western blots also confirmed the results of slot blot analyses. [Fig. 6 b (i), 8 b(i), 10 b (i), 12 b (i), 14 b (i)]. India ink stain indicated that an equal amount of protein was loaded in each slot [Fig. 6 (ii), 8 (ii), 10 (ii), 12 (ii), 14 (ii)] during experimentation.

3.1.1. ESOPHAGUS- In the esophagus, the activity of ADA was highest at day 1. There was a significant decrease (-34%) in the activity of ADA at day 10 and showed no significant change thereafter [Table: 2; Fig. 5]. Representative data of the slots and Western blots indicated that the level of the ADA protein decreased at day 10 [Fig. 6].

Age (Days)	*Esophagus* ADA activity (U/mg Protein) x 10^4			
	Mean	SD	*p*	%
1	36.0	5.4		
10	23.6	3.5	<0. 001	-34
30	22.7	5.5	NS	NC
60	15.4	2.01	NS	-32
90	14.1	4.2	NS	NC

Table 2: Normal endogenous level of ADA activity in esophagus. SD, Standard deviation; *p*, Level of significance; %, Per cent decrease (-); NS, Not significant; NC, No change.

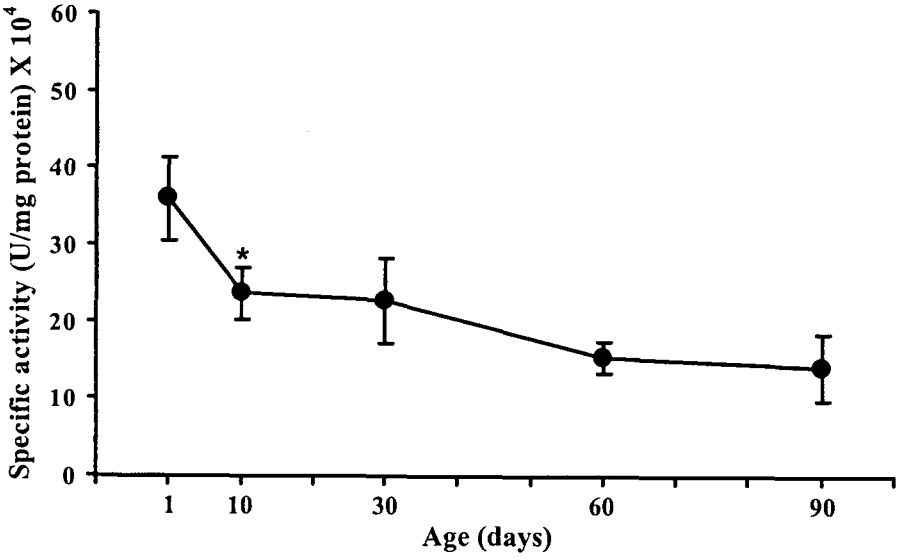


Fig. 5: Normal endogenous activity of ADA in esophagus of chicken. Values are expressed as mean from five chicken at each point. Bars represent standard deviation. Asterisk (*) indicates statistically significant ($p< 0.001$) value as compared to day 1.

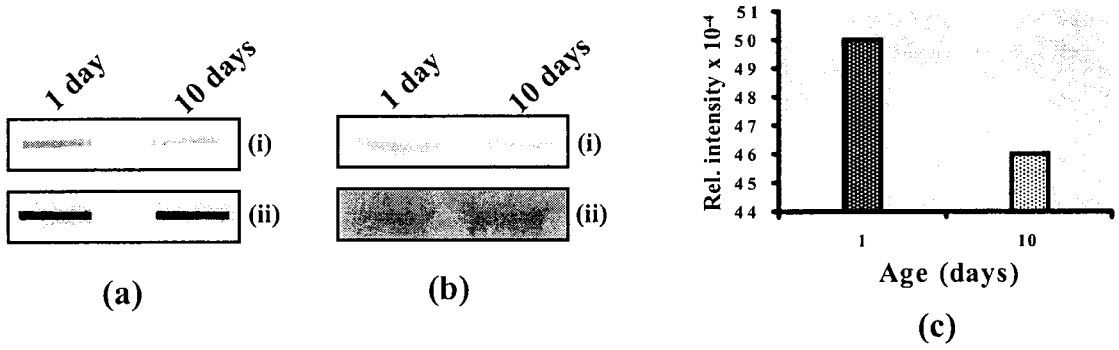


Fig. 6: (a) i. Slot blot analysis of ADA from esophagus of chicken from day 1 and 10. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from esophagus of chicken from day 1 and 10. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by using densitometric analysis (KDS-1 software).

3.1.2. CROP- Here, it can be observed that the activity was highest at day 1, decreasing considerably at day 10 (-35%) and then remained constant. [Table: 3; Fig. 7]. The slot and Western blots indicated a similar pattern at the ADA protein levels [Fig. 8].

Age (Days)	*Crop*			
	ADA activity (U/mg Protein) x 10^4			
	Mean	SD	*p*	%
1	19.18	1.82		
10	12.46	1.87	< 0.001	-35
30	12.90	2.28	NS	NC
60	13.23	0.75	NS	NC
90	12.96	2.75	NS	NC

Table 3: Normal endogenous level of ADA activity in crop. SD, Standard deviation; *p*, Level of significance; %, Per cent decrease (-); NS, Not significant; NC, No change.

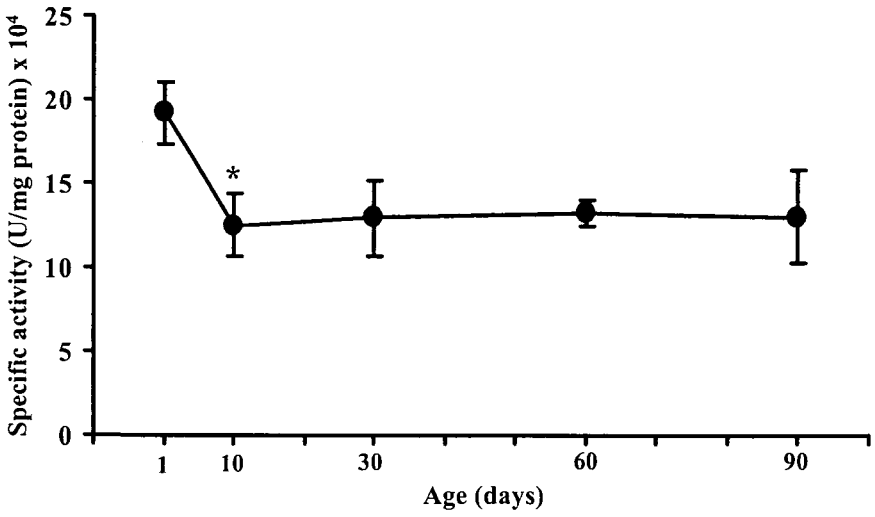


Fig. 7: Normal endogenous activity of ADA in crop of chicken. Values are expressed as mean from five chicken at each point. Bars represent standard deviation. Asterisk (*) indicates statistically significant ($p < 0.001$) value as compared to day 1.

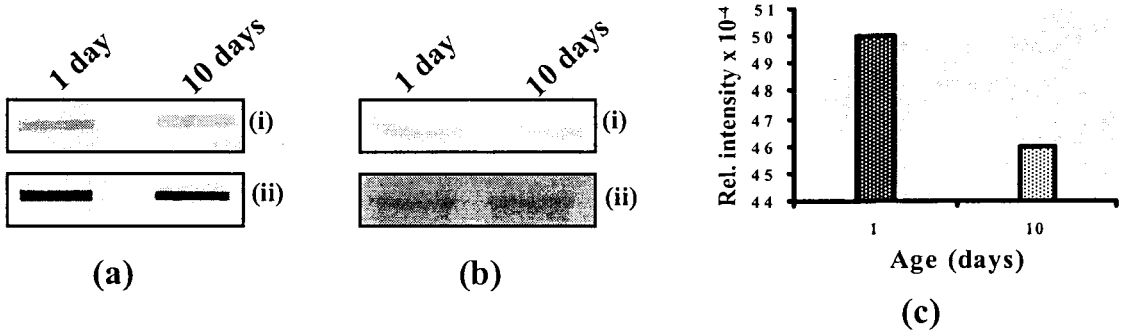


Fig. 8: (a) i. Slot blot analysis of ADA from crop of chicken from day 1 and 10. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from crop of chicken from day 1 and 10. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by using densitometric analysis (KDS-1 software).

3.1.3. PROVENTRICULUS- In the proventriculus, the activity was highest at day 1, went down at day 30 (-20 %) and dropped again at day 90 (-30%) [Table: 4; Fig. 9]. The slot and Western blot done at the ages 1 and 10 further confirmed the spectrophotometric results wherein no change was observed at the protein level of the enzyme [Fig. 10].

Age (Days)	*Proventriculus* ADA activity (U/mg Protein) x 10^4			
	Mean	SD	*p*	%
1	72.85	6.18		
10	63.42	4.2	NS	NC
30	51.26	1.76	< 0.001	-20
60	49.64	3.09	NS	NC
90	34.68	6.48	< 0.001	-30

Table 4: Normal endogenous level of ADA activity in proventriculus. SD, Standard deviation; *p*, Level of significance; %, Per cent decrease (-); NS, Not significant; NC, No change.

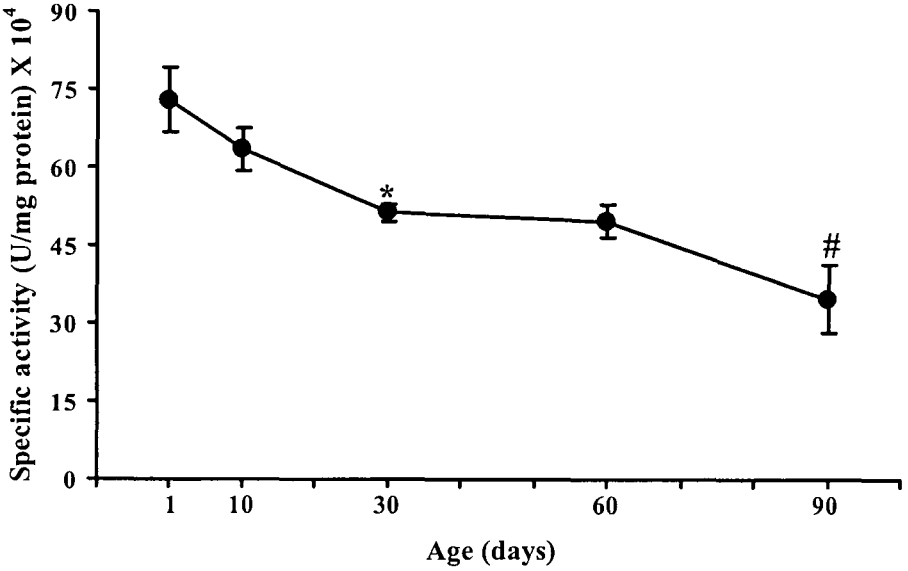


Fig. 9: Normal endogenous activity of ADA in proventriculus of chicken. Values are expressed as mean from five chicken at each point. Bars represent standard deviation. Asterisks (*, #) indicate statistically significant ($p < 0.001$) value as compared to day 1 and 60, respectively.

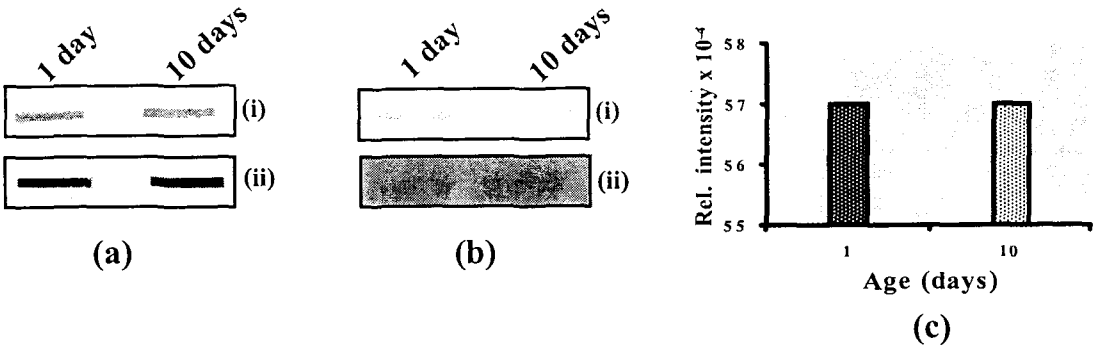


Fig. 10: (a) i. Slot blot analysis of ADA from proventriculus of chicken from day 1 and 10. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 µg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from proventriculus of chicken from day 1 and 10. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 µg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by using densitometric analysis (KDS-1 software).

3.1.4. SMALL INTESTINE- The level of activity of ADA was seen to be highest at day 1 which dropped at day 10 (-48%) and then remained fairly constant at the other postnatal ages. [Table: 5; Fig. 11]. Representative data of the slot and Western blots indicated a decrease in the level of ADA protein [Fig. 12].

Age (Days)	*Small intestine* ADA activity (U/mg Protein) x 10^3			
	Mean	SD	*p*	%
0	35.9	2.76		
10	18.64	2.33	<0. 001	-48
30	20.5	3.41	NS	NC
60	21.5	2.21	NS	NC
90	17.08	3.56	NS	NC

Table 5: Normal endogenous level of ADA activity in small intestine. SD, Standard deviation; *p*, Level of significance; %, Per cent decrease (-); NS, Not significant; NC, No change.

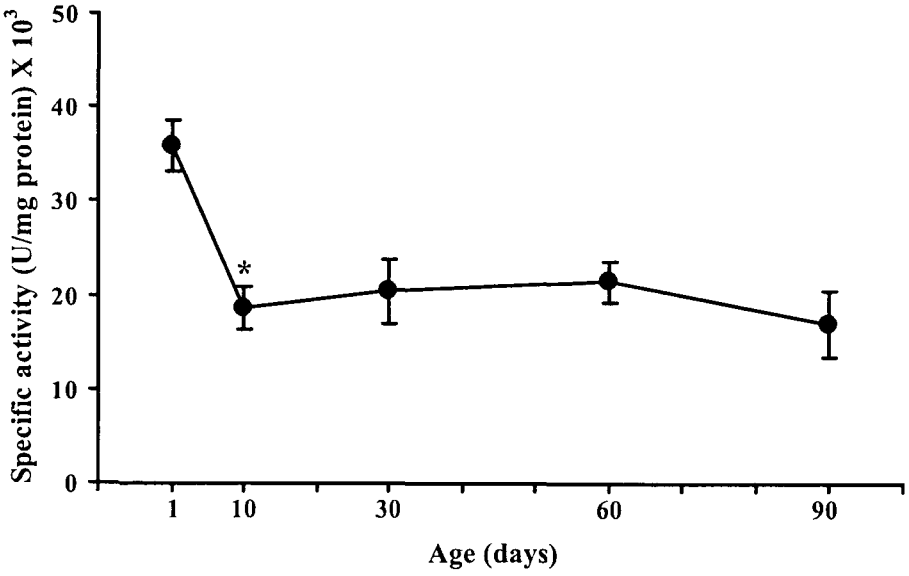


Fig. 11: Normal endogenous activity of ADA in small intestine of chicken. Values are expressed as mean from five chicken at each point. Bars represent standard deviation. Asterisk (*) indicates statistically significant ($p< 0.001$) value as compared to day 1.

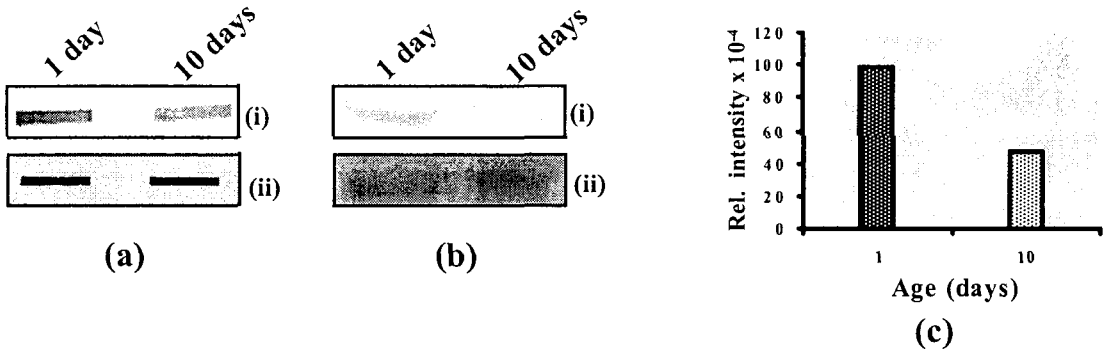


Fig. 12: (a) i. Slot blot analysis of ADA from small intestine of chicken from day 1 and 10. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from small intestine of chicken from day 1 and 10. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by using densitometric analysis (KDS-1 software).

3.1.5. SPLEEN- Unlike all the tissues of GIT, the activity of ADA was seen to increase showing a peak at day 30 (+78%), after which there is a drop in the activity level. [Table: 6; Fig. 13]. Here, the Slot and Western blot at the two ages, day 1 and 10, showed no change in the level of ADA protein as is indicated by spectrophotometric studies [Fig.14].

Age (Days)	*Spleen* ADA activity (U/mg Protein) x 10^4			
	Mean	SD	*p*	%
1	17.52	3.3		
10	23.25	4.39	NS	+33
30	34.26	1.6	< 0.001	+47
60	25.13	3.37	< 0.001	-27
90	23.5	3.83	NS	NC

Table 6: Normal endogenous level of ADA activity in spleen. SD, Standard deviation; *p*, Level of significance; %, Per cent increase (+); NS, Not significant; NC, No change.

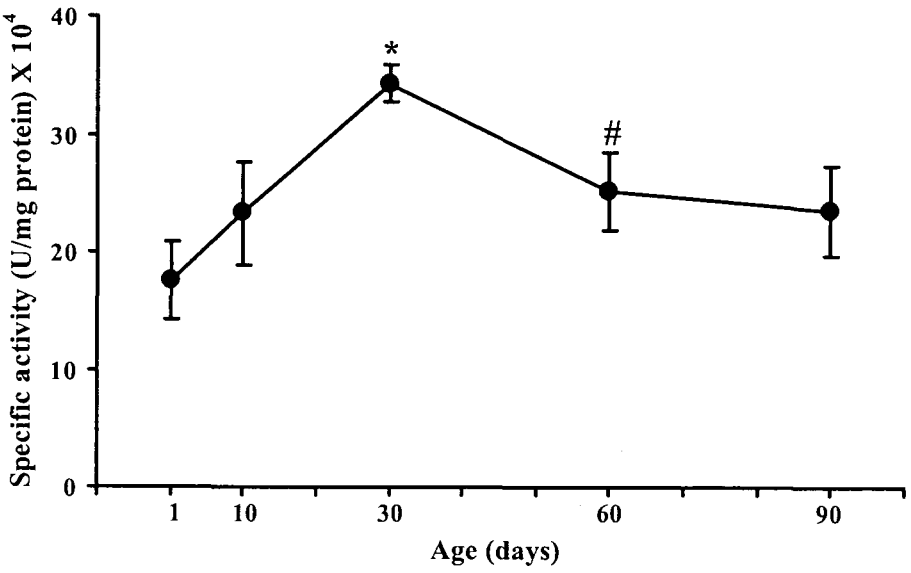


Fig. 13: Normal endogenous activity of ADA in spleen of chicken. Values are expressed as mean from five chicken at each point. Bars represent standard deviation. Asterisks (*, #) indicate statistically significant ($p< 0.001$) values as compared to day 1 and 30, respectively.

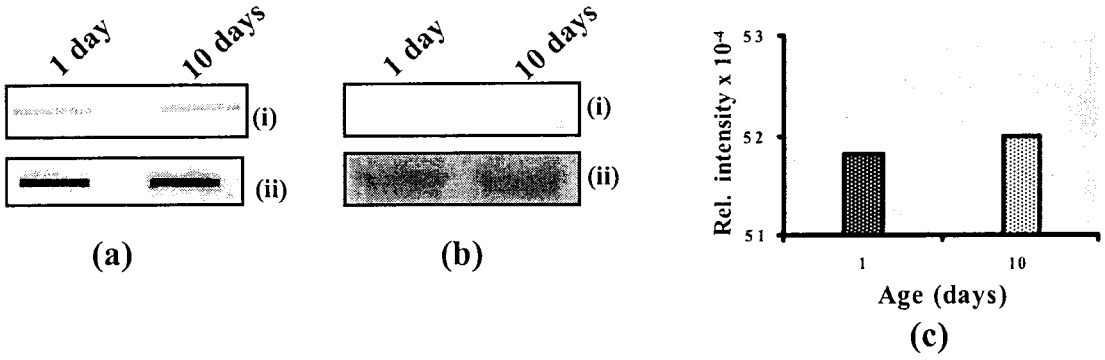


Fig. 14: (a) i. Slot blot analysis of ADA from spleen of chicken from day 1 and 10. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from spleen of chicken from day 1 and 10. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by using densitometric analysis (KDS-1 software).

3.2. HORMONAL REGULATION

Effects of corticosterone (an immunosuppressive hormone), Bt_2cAMP (an immunoinducer, also a membrane permeable analog of cAMP), T_3 and testosterone were studied on the activity of ADA in different tissues of chicken at two postnatal ages, to find out their tissue- as well as age-specific influence on the activity of ADA. For all such studies, slot and Western blots were performed in order to determine if the changes, if any, were at the level of the protein expression for ADA.

3.2.1. EFFECT OF CORTICOSTERONE: Corticosterone was administered in two different age groups (10- and 60-day old) of chicken as a single dose of 1.0 mg/ 100 g body weight in 0.3 ml normal saline (0.9%) having 6% ethanol intraperitoneally, and its effect on ADA activity (U/mg protein) was studied in the GIT (esophagus, crop, proventriculus, crop, small intestine) and spleen. Corticosterone significantly decreased the activity of ADA in all the tissues studied except proventriculus in an age- and tissue-specific manner. In all these studies, it was found that the inhibition was more pronounced at the later stage of development.

3.2.1.1. Esophagus- In the esophagus, corticosterone significantly decreased the activity of ADA at day 10 (-23%), but more at day 60 (-41%). [Table: 7; Fig. 15] The representative Slot and Western blots indicated a change in the level of the protein of the enzyme [Fig. 16].

Esophagus				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	10		60	
	Control	Treated	Control	Treated
Mean	25.36	19.48	17.62	10.4
SD	0.73	0.58	1.31	1.13
p		<0.001		<0.001
%		-23		-41

Table 7: Effect of corticosterone on the level of ADA activity in esophagus. SD, Standard deviation; *p*, Level of significance; %, Per cent decrease (-).

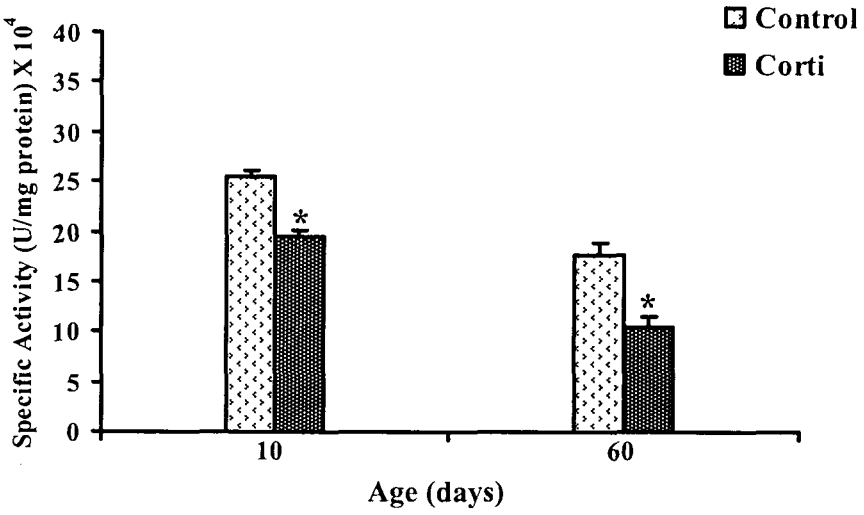


Fig. 15: Effect of corticosterone (Corti) on the activity of esophagus ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation. Asterisks (*) indicate statistically significant ($p< 0.001$) value as compared to control.

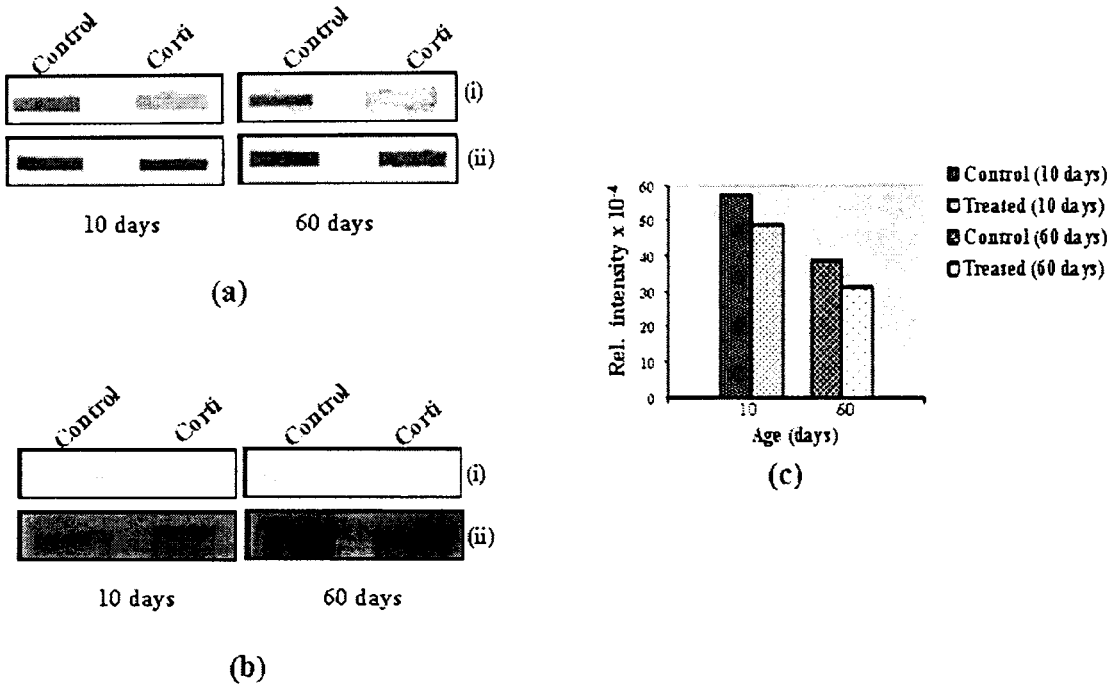


Fig. 16: (a) i. Slot blot analysis of ADA from esophagus of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 µg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from esophagus of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 µg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.1.2. Crop- In the crop, corticosterone decreased the activity of ADA more at day 60 (-79%) than day 10 (-56%). [Table: 8; Fig. 17]. Slot and Western blots confirmed the results of the studies done spectrophotometrically [Fig. 18], indicating that the decreased activity of ADA upon corticosterone administration may be due to reduced expression of ADA protein.

Crop				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	15.34	6.77	17.2	3.54
SD	1.03	0.57	0.69	0.48
p		<0.001		<0.001
%		-56		-79

Table 8: Effect of corticosterone on the level of ADA activity in crop. SD, Standard deviation; *p*, Level of significance; %, Per cent decrease (-).

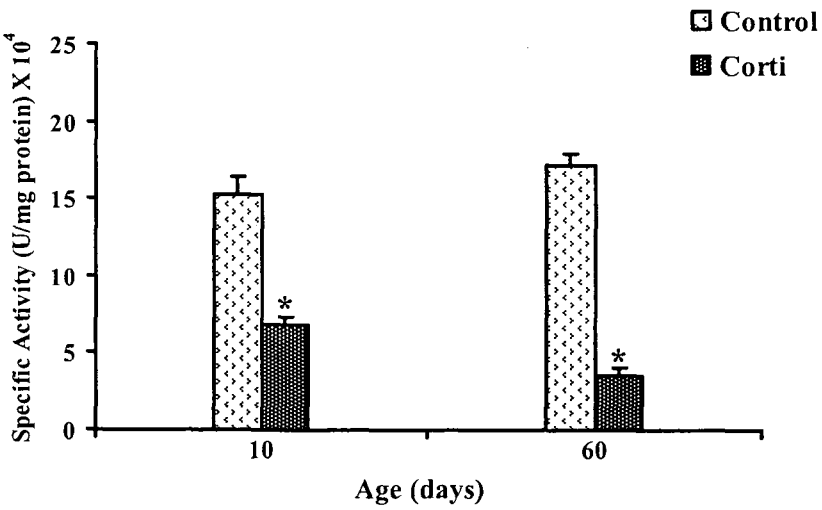


Fig. 17: Effect of corticosterone (Corti) on the activity of crop ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation. Asterisks (*) indicate statistically significant ($p< 0.001$) value as compared to control.

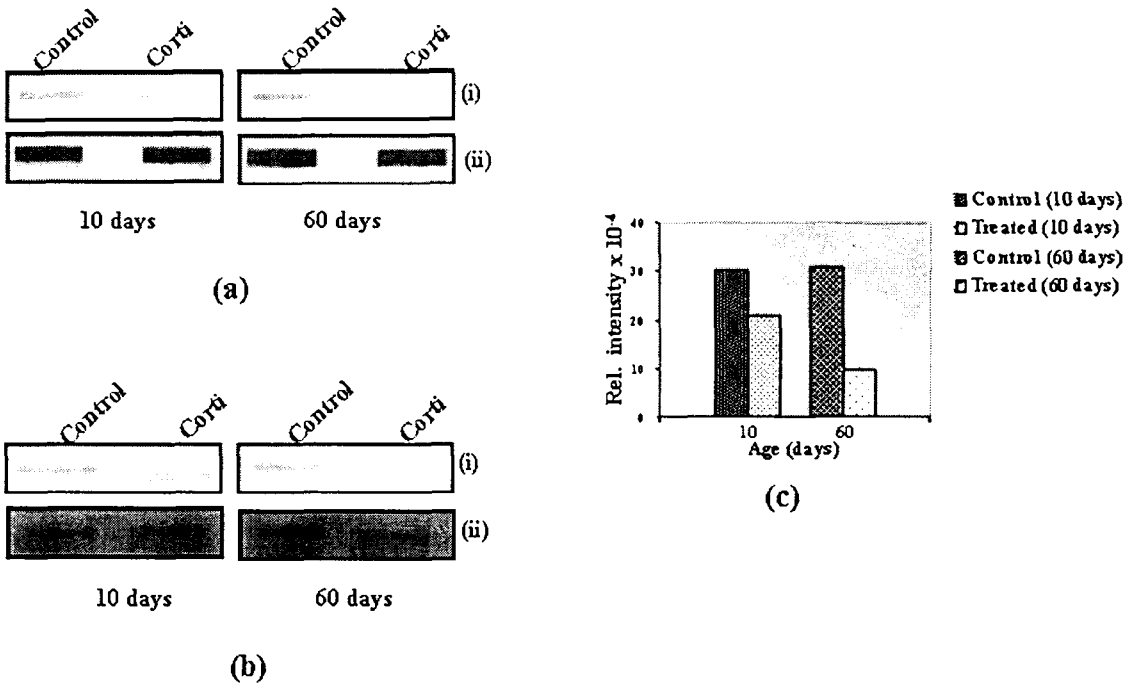


Fig. 18: (a) i. Slot blot analysis of ADA from crop of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from crop of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

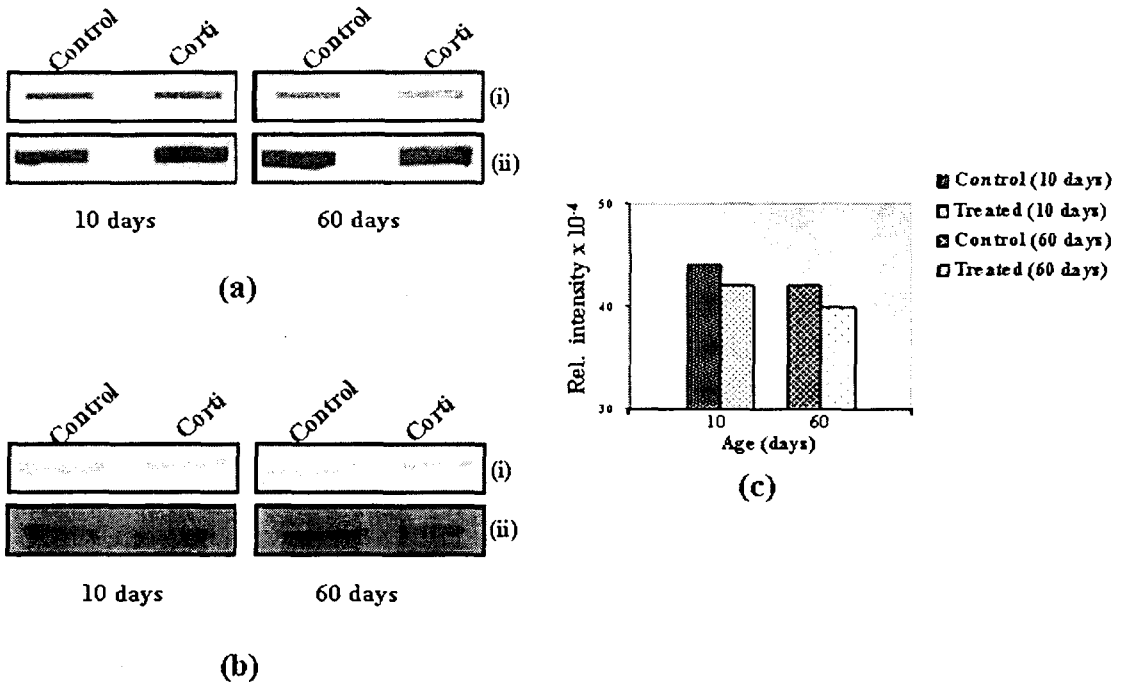


Fig. 20: (a) i. Slot blot analysis of ADA from proventriculus of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from proventriculus of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.1.4. Small intestine- In the small intestine, corticosterone decreases the activity of ADA at both day 10 (-36%) and in day 60 (-41%) [Table: 10; Fig. 21]. Slot and Western blots confirmed that the decrease in ADA activity upon corticosterone administration is due to the decrease in the expression of ADA protein [Fig. 22].

Small intestine				
ADA activity (U/mg Protein) x 10^3				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	19.85	12.71	22.68	13.36
SD	2.02	1.28	2.22	1
p		<0.001		<0.001
%		-36		-41

Table 10: Effect of corticosterone on the level of ADA activity in small intestine. SD, Standard deviation; *p*, Level of significance; %, Per cent decrease (-).

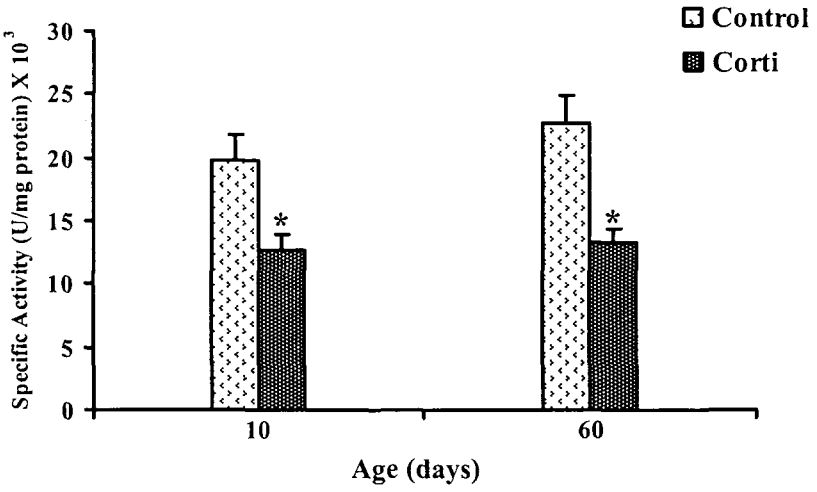


Fig. 21: Effect of corticosterone (Corti) on the activity of small intestine ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation. Asterisks (*) indicates statistically significant ($p< 0.001$) value as compared to control.

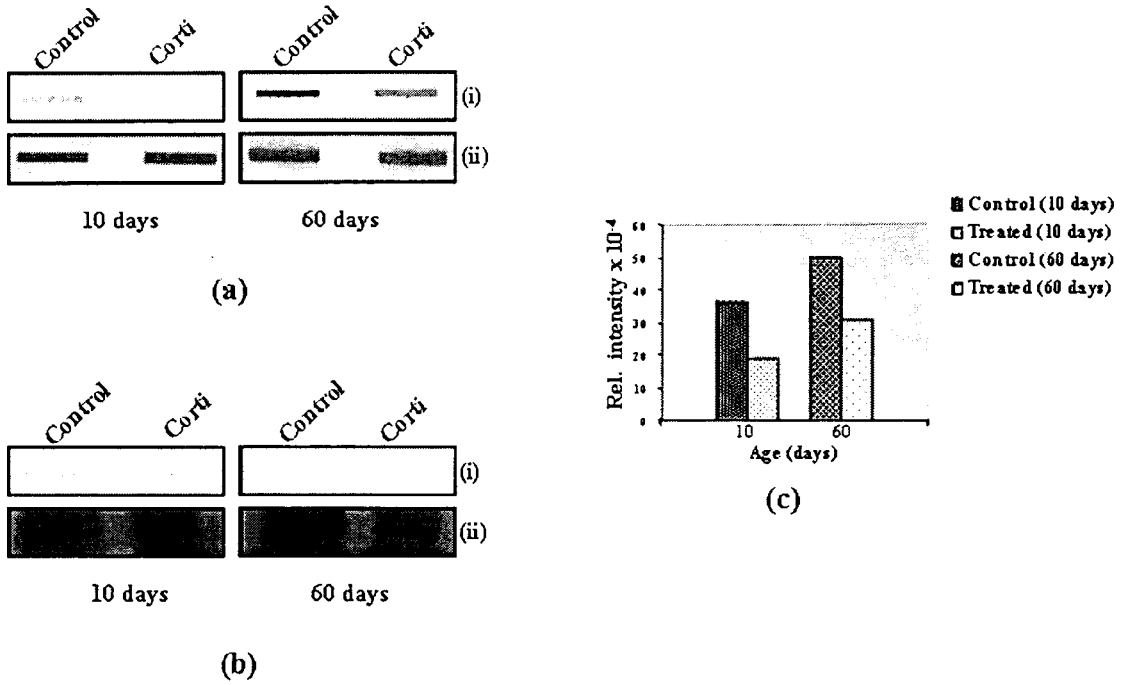


Fig. 22: (a) i. Slot blot analysis of ADA from small intestine of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 µg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from small intestine of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 µg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.1.5. Spleen- In the spleen, corticosterone decreases the activity of ADA at both day 10 (-32%) and day 60 (-40%). [Table: 11; Fig. 23]. Slot and Western blots also indicated that the level of the protein of the enzyme was decreased at the two ages [Fig. 24].

Spleen				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	23.73	16.19	25.47	15.24
SD	0.45	0.62	0.58	1.01
p		<0.001		<0.001
%		-32		-40

Table 11: Effect of corticosterone on the level of ADA activity in spleen. SD, Standard deviation; *p,* Level of significance; %, Per cent decrease (-).

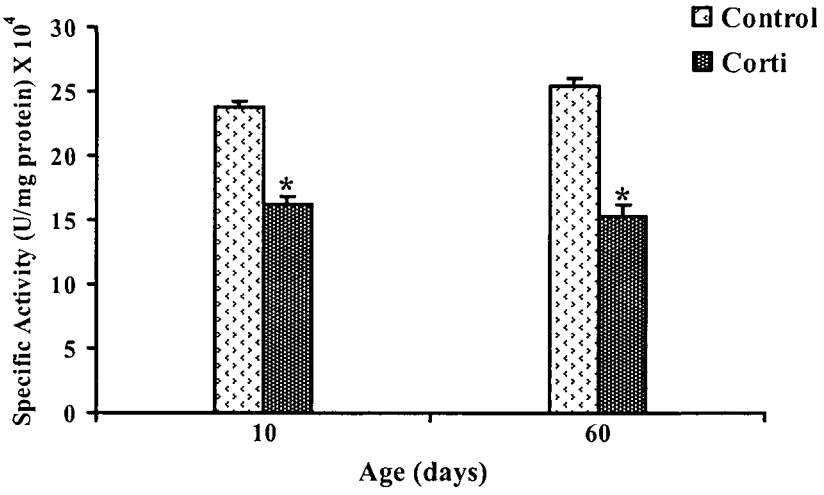


Fig. 23: Effect of corticosterone (Corti) on the activity of spleen ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation. Asterisks (*) indicate statistically significant ($p< 0.001$) value as compared to control.

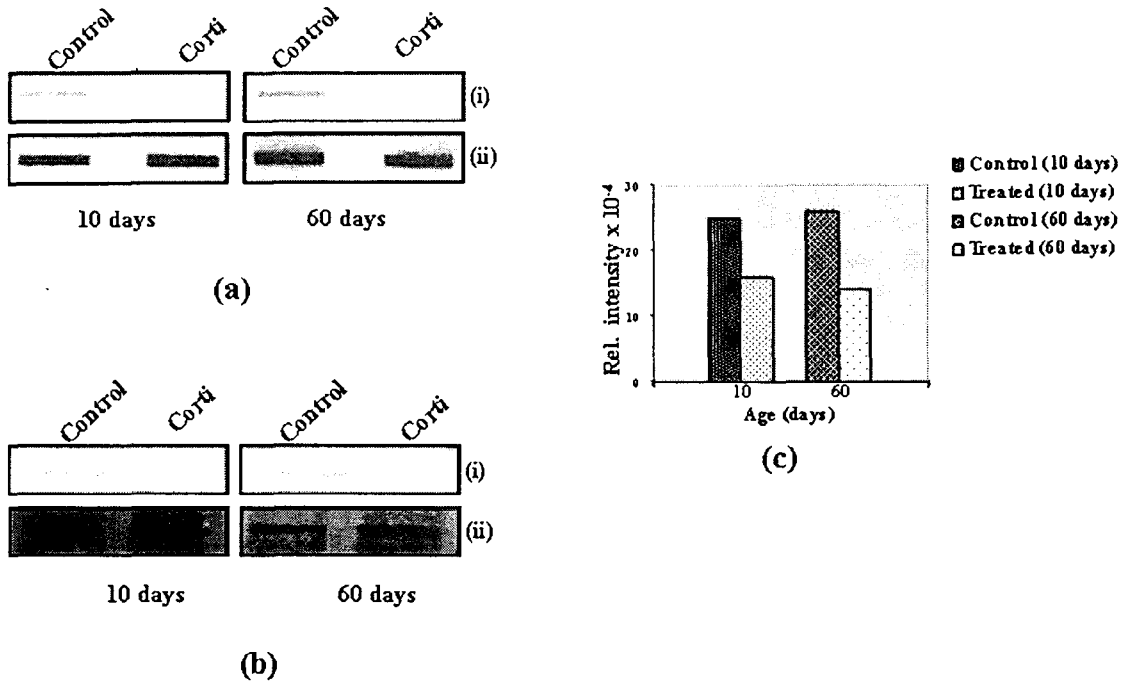


Fig. 24: (a) i. Slot blot analysis of ADA from spleen of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 µg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from spleen of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 µg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.2. EFFECT OF DIBUTYRYL cAMP (Bt_2cAMP): Bt_2cAMP, a membrane permeable analog of cAMP, was administered at a single dose of 1.0 mg/ 100 g body weight in 0.3 ml normal saline (0.9%), intraperitoneally, and its effect on ADA activity (U/mg protein) was studied in the esophagus, crop, proventriculus, crop, small intestine and spleen of chicken at two selected postnatal ages. Bt_2cAMP significantly increased the activity of ADA in all the tissues studied except crop. The magnitude of induction was more pronounced at a later stage of development.

3.2.2.1. Esophagus- In the esophagus, dibutyryl cAMP significantly increased the activity of ADA at both ages, but more at day 60 (+67%) in comparison to day 10 (+42%) [Table: 12; Fig. 25]. Representative data of the slot and Western blots indicated an increase in the level of the protein for ADA [Fig. 26].

Esophagus				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	23.54	33.31	16.61	27.79
SD	0.84	0.83	0.72	0.63
p		<0.001		<0.001
%		+42		+67

Table 12: Effect of Bt_2cAMP on the level of ADA activity in esophagus. SD, Standard deviation; *p,* Level of significance; %, Per cent increase (+).

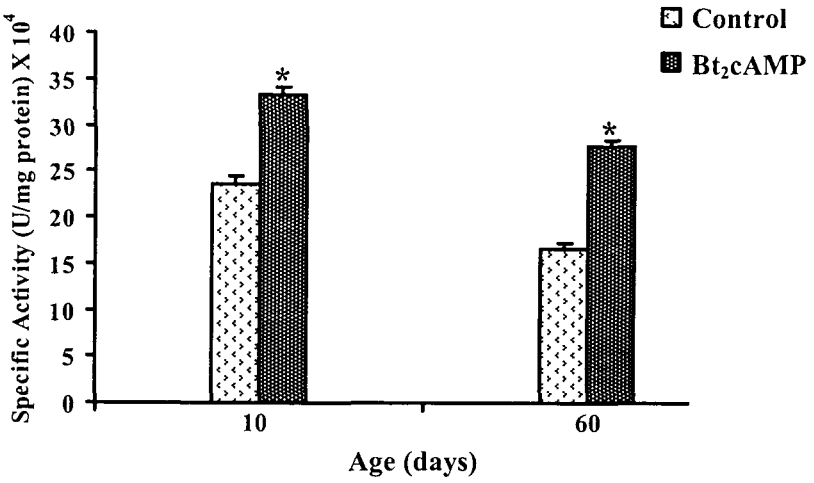


Fig. 25: Effect of Bt_2cAMP on the activity of esophagus ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation. Asterisks (*) indicate statistically significant ($p< 0.001$) value as compared to control.

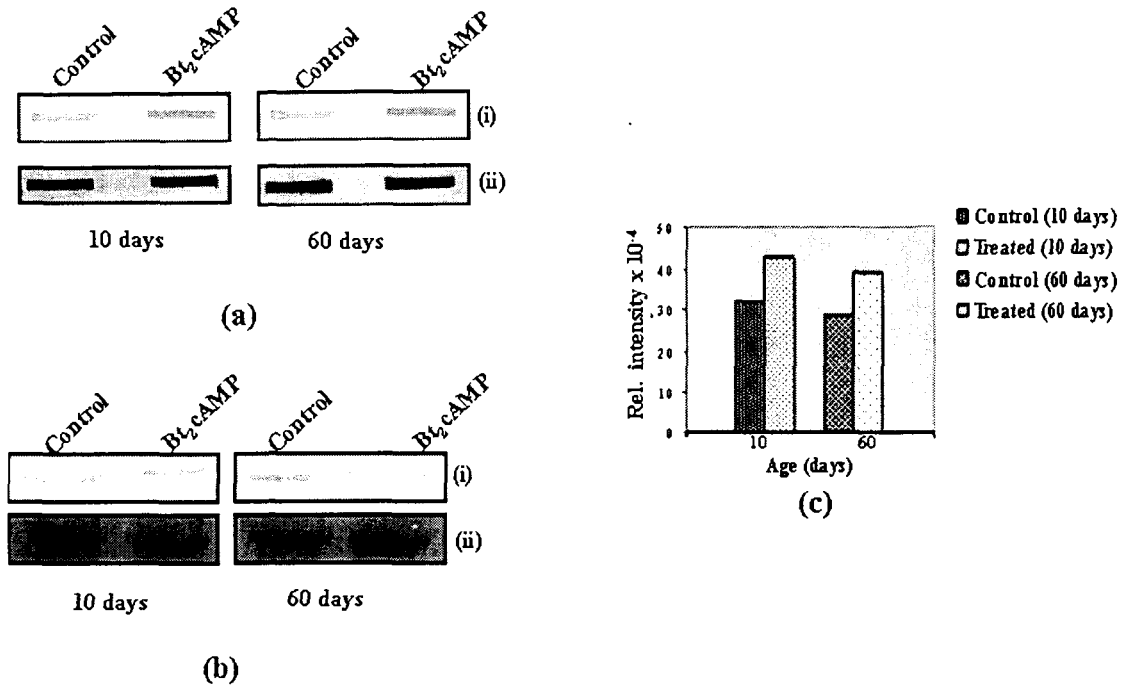


Fig. 26: (a) i. Slot blot analysis of ADA from esophagus of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from esophagus of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.2.2. Crop- In the crop, Bt_2cAMP was seen to have no significant effect at both day 10 and day 60. [Table: 13; Fig. 27]. Slot and Western blots also showed no change in the protein level of the enzyme at the two ages studied [Fig. 28].

Crop				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	16.23	18.52	17.61	19.16
SD	1.07	0.78	1.11	0.98
p		NS		NS
%		NC		NC

Table 13: Effect of Bt_2cAMP on the level of ADA activity in crop. SD, Standard deviation; *p*, Level of significance; %, Per cent change; NS, Not significant; NC, No change.

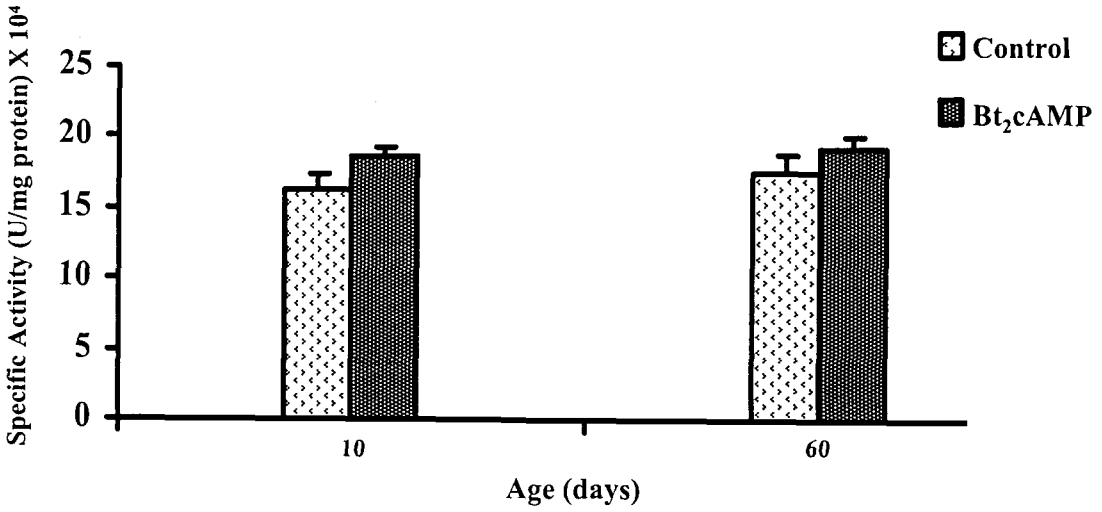


Fig. 27: Effect of Bt_2cAMP on the activity of crop ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation.

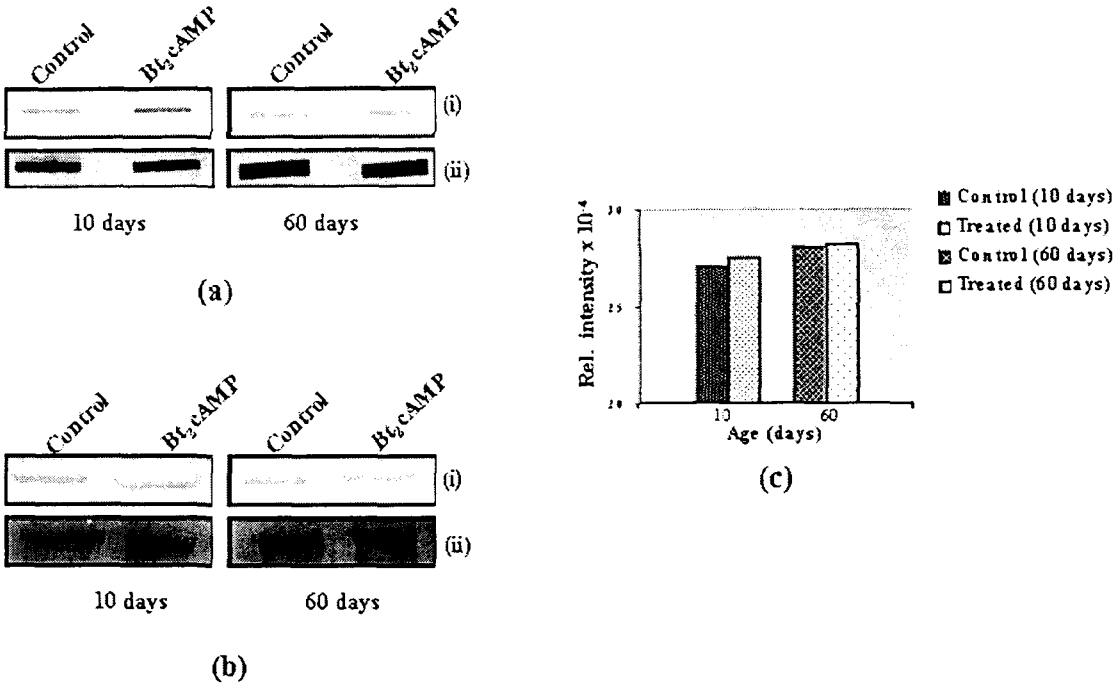


Fig. 28: (a) i. Slot blot analysis of ADA from crop of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 µg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from crop of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 µg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.2.3. Proventriculus- In the proventriculus, Bt_2cAMP increased the activity of ADA at both day 10 and day 60, but the magnitude of increase was more in day 60 (+29%) than day 10 (+21%) [Table: 14; Fig. 29]. Here too, the increase was at the level of the protein of the enzyme as indicated by the slots and Western blots [Fig. 30].

Proventriculus				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	62.05	75.15	52.24	67.2
SD	1.38	2.85	0.95	0.75
p		<0.001		<0.001
%		+21		+29

Table 14: Effect of Bt_2cAMP on the level of ADA activity in proventriculus. SD, Standard deviation; *p*, Level of significance; %, Per cent increase (+).

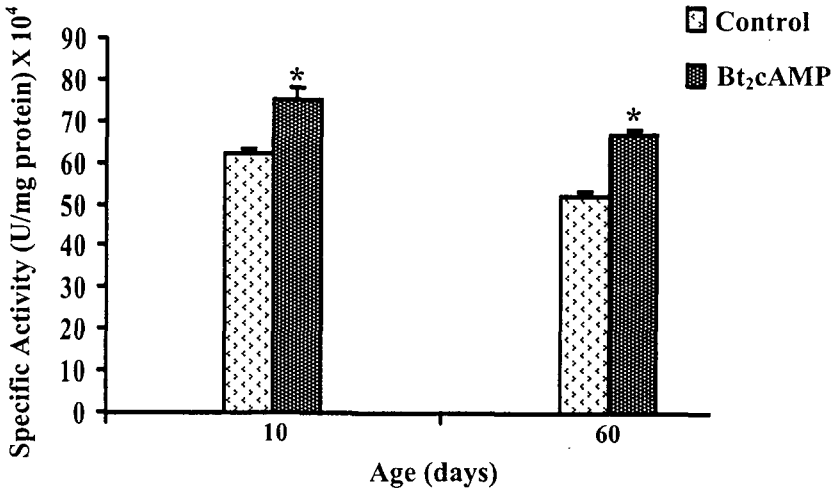


Fig. 29: Effect of Bt_2cAMP on the activity of proventriculus ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation. Asterisks (*) indicate statistically significant ($p< 0.001$) value as compared to control.

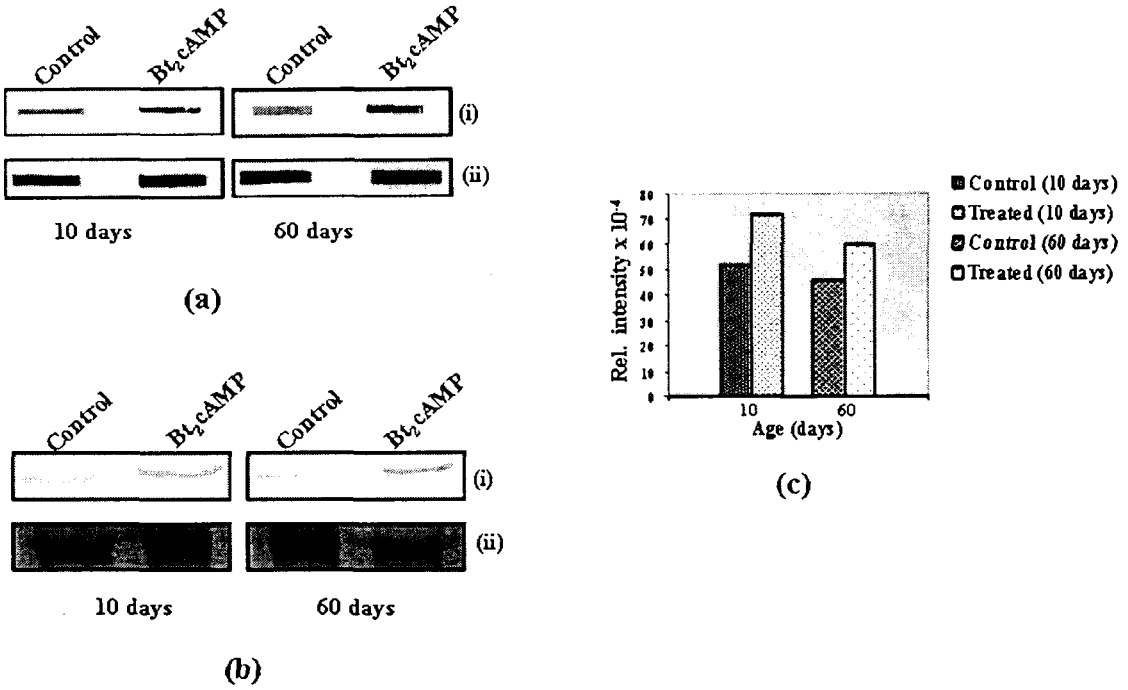


Fig. 30: (a) i. Slot blot analysis of ADA from proventriculus of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from proventriculus of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.2.4. Small intestine- Similarly, Bt_2cAMP induced the activity of ADA in the small intestine at both the ages studied, but increased the activity more at a later stage of development, day 60 (+58%) than at the early age, day 10 (+52%) [Table: 15; Fig. 31]. The level of the ADA protein was indeed enhanced as was seen by slot and Western blot analyses [Fig. 32].

Small intestine				
ADA activity (U/mg Protein) x 10^3				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	24.58	37.26	22.48	35.57
SD	0.81	1.14	1.37	0.92
p		<0.001		<0.001
%		+52		+58

Table 15: Effect of Bt_2cAMP on the level of ADA activity in small intestine. SD, Standard deviation; p, Level of significance; %, Per cent increase (+).

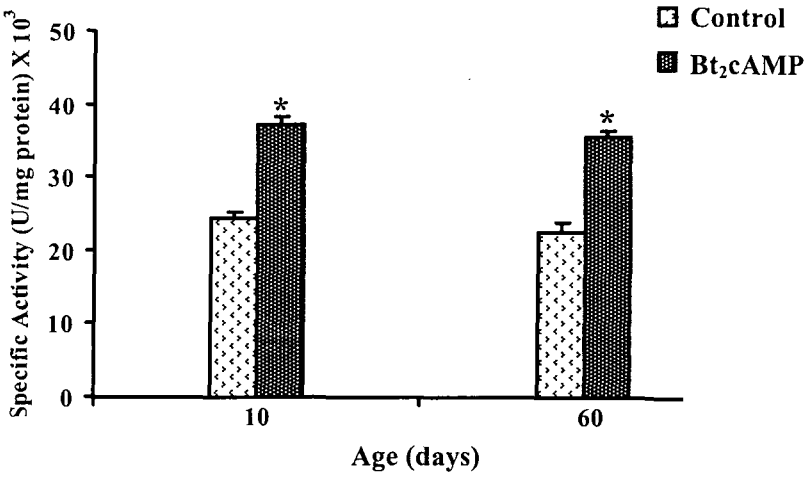
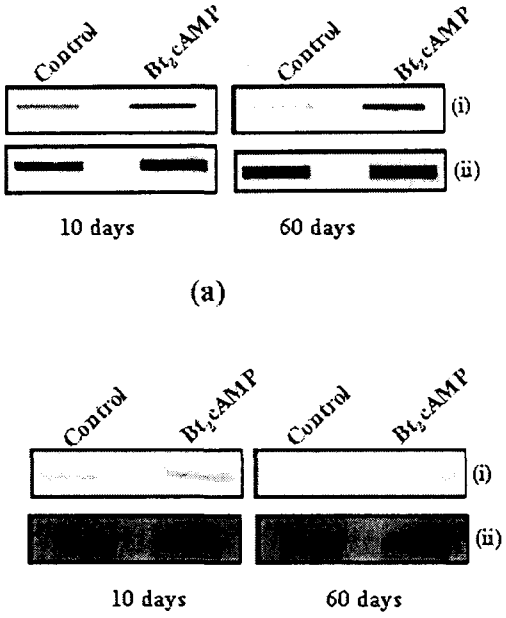


Fig. 31: Effect of Bt_2cAMP on the activity of small intestine ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation. Asterisks (*) indicate statistically significant ($p< 0.001$) value as compared to control.



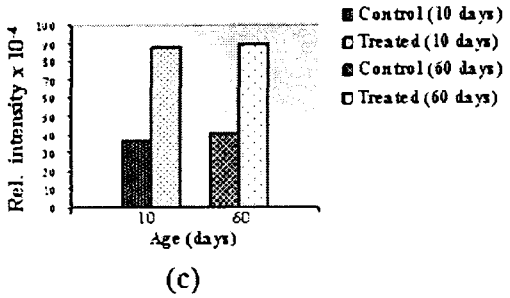


Fig. 32: (a) i. Slot blot analysis of ADA from small intestine of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from small intestune of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.2.5. Spleen- Like the tissues of GIT, in the spleen too, Bt_2cAMP increased the activity of ADA at day 60 (+46%) but unlike the tissues of GIT, there was no effect at day 10. [Table: 16; Fig. 33]. Slot and Western blots confirmed that the increase was at the later stage of development [Fig. 34].

Spleen				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	27.31	29.89	25.9	43.77
SD	1.26	0.99	1.37	1.07
p		NS		<0.001
%		NC		+ 46

Table 16: Effect of Bt_2cAMP on the level of ADA activity in spleen. SD, Standard deviation; *p,* Level of significance; %, Per cent increase (+); NS, Not significant; NC, No change.

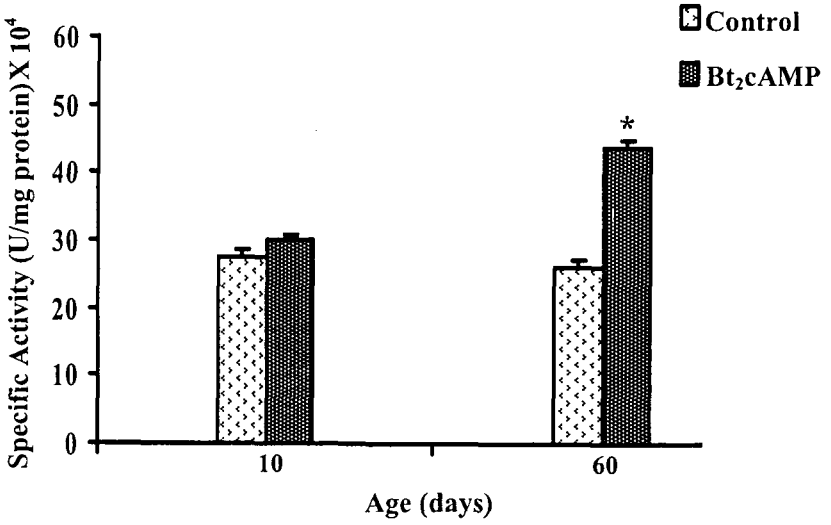


Fig. 33: Effect of Bt_2cAMP on the activity of spleen ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation. Asterisk (*) indicates statistically significant ($p< 0.001$) value as compared to control.

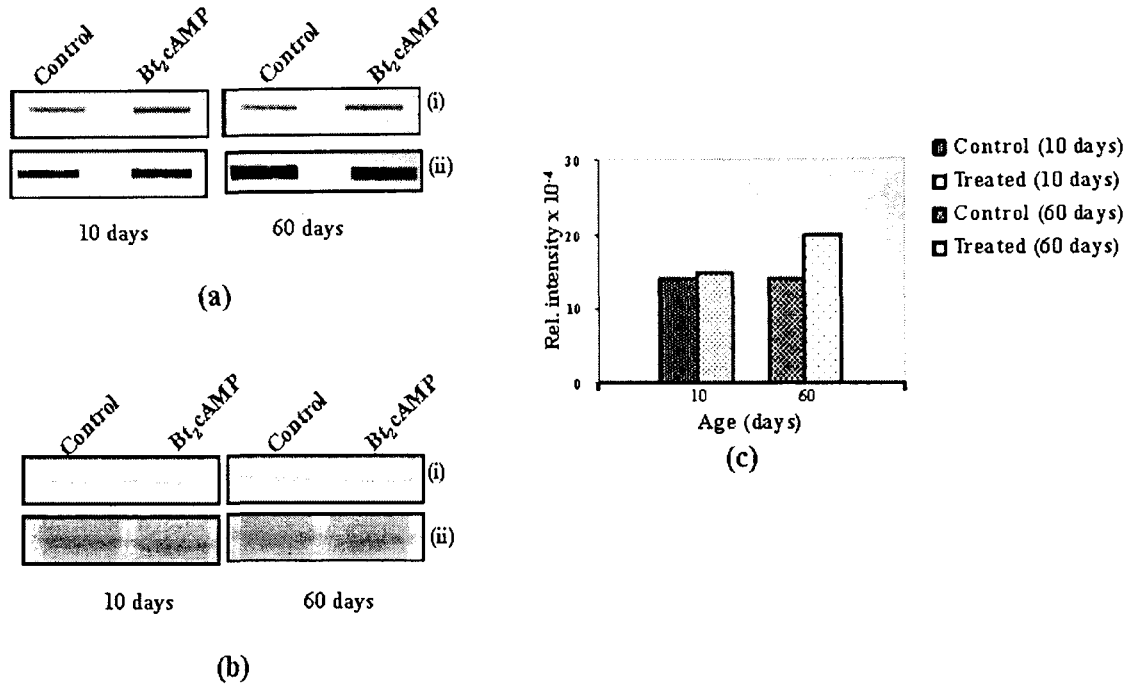


Fig. 34: (a) i. Slot blot analysis of ADA from spleen of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from spleen of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.3. EFFECT OF 3, 5, 3'-TRIIODOTHYRONINE (T_3): T_3 was administered at a dose of 200 μg/ 100 g body weight in 0.3 ml saline (0.9%), intraperitoneally for 3 consecutive days and chicken were sacrificed after 3 h of final injection. Its effect on ADA activity (U/mg protein) was studied in the GIT (esophagus, crop, proventriculus, crop, small intestine) and spleen. However, no significant change on ADA activity was observed in all the tissues of GIT and spleen studied. Slots and Western blots analyses confirmed the results of spectrophotometric studies.

3.2.3.1. Esophagus- In the esophagus, T_3 was seen to have no significant effect in the two ages studied. [Table: 17; Fig. 35]. No change in the level of ADA protein was seen by slot and Western blots as well [Fig. 36].

Esophagus				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	25.18	23.27	18.45	16.19
SD	1.02	1.54	1.01	0.76
p		NS		NS
% (+/-)		NC		NC

Table 17: Effect of T_3 on the level of ADA activity in esophagus. SD, Standard deviation; *p*, Level of significance; % (+/-): Per cent increase or decrease; NS, Not significant; NC, No change.

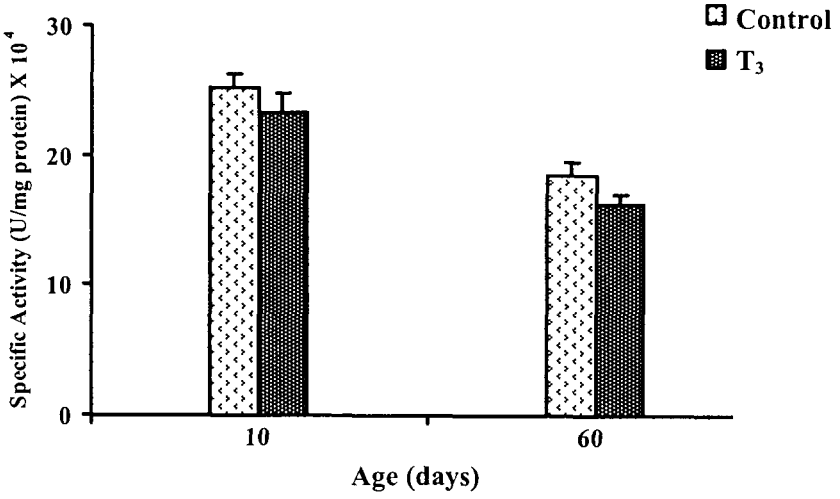


Fig. 35: Effect of T_3 on the activity of esophagus ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation.

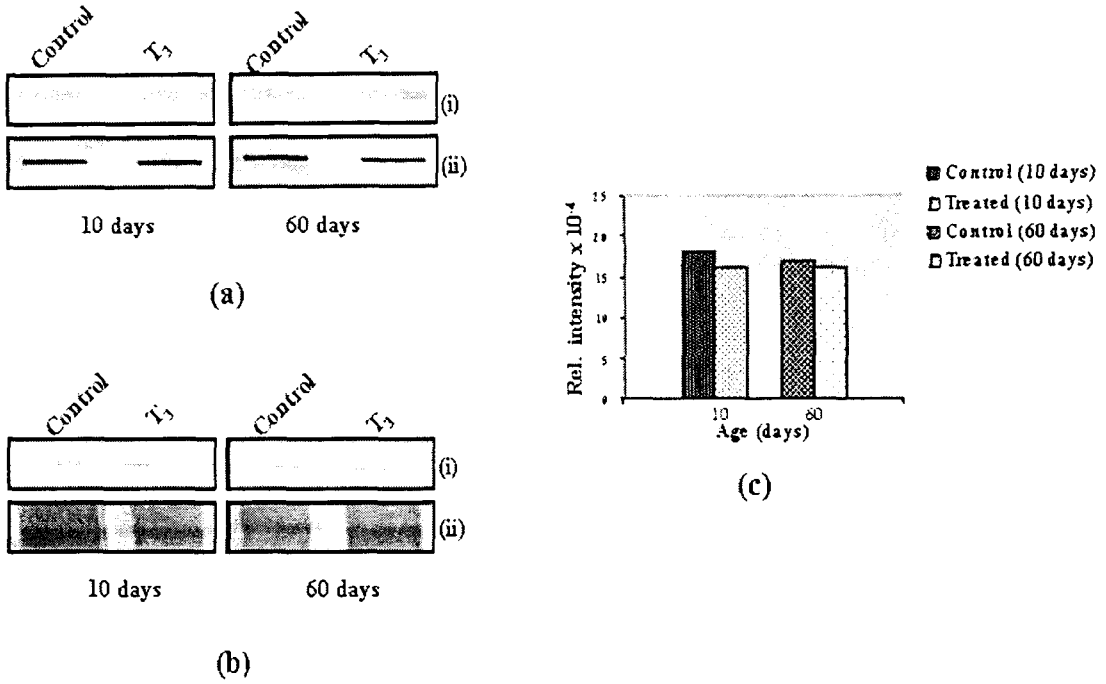


Fig. 36: (a) i. Slot blot analysis of ADA from esophagus of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 µg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from esophagus of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 µg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.3.2. Crop- In the crop, T_3 was seen to have no significant effect in the two ages studied [Table: 18; Fig. 37]. Slot and Western blots confirmed the spectrophotometric results [Fig. 38].

Crop				
ADA activity (U/mg Protein) x 104				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	15.51	13.4	17.37	16.91
SD	0.99	0.84	0.98	0.72
p		NS		NS
% (+/-)		NC		NC

Table 18: Effect of T_3 on the level of ADA activity in crop. SD, Standard deviation; *p*, Level of significance; % (+/-), Per cent increase or decrease; NS, Not significant; NC, No change.

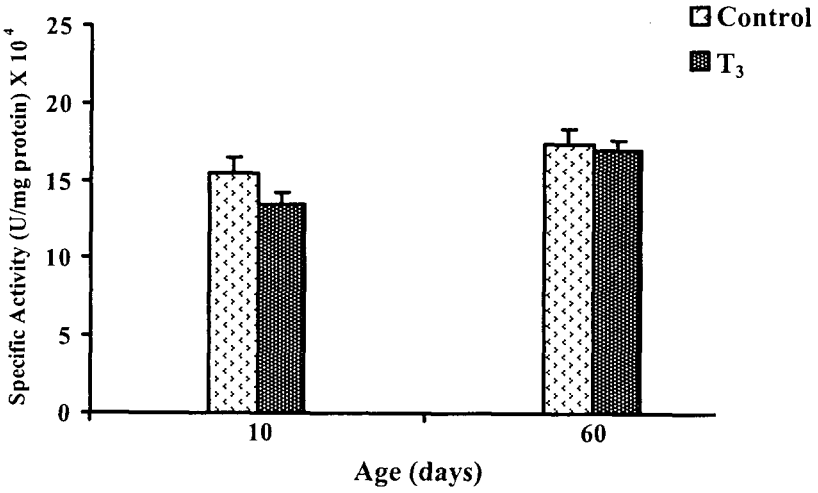


Fig. 37: Effect of T_3 on the activity of crop ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation.

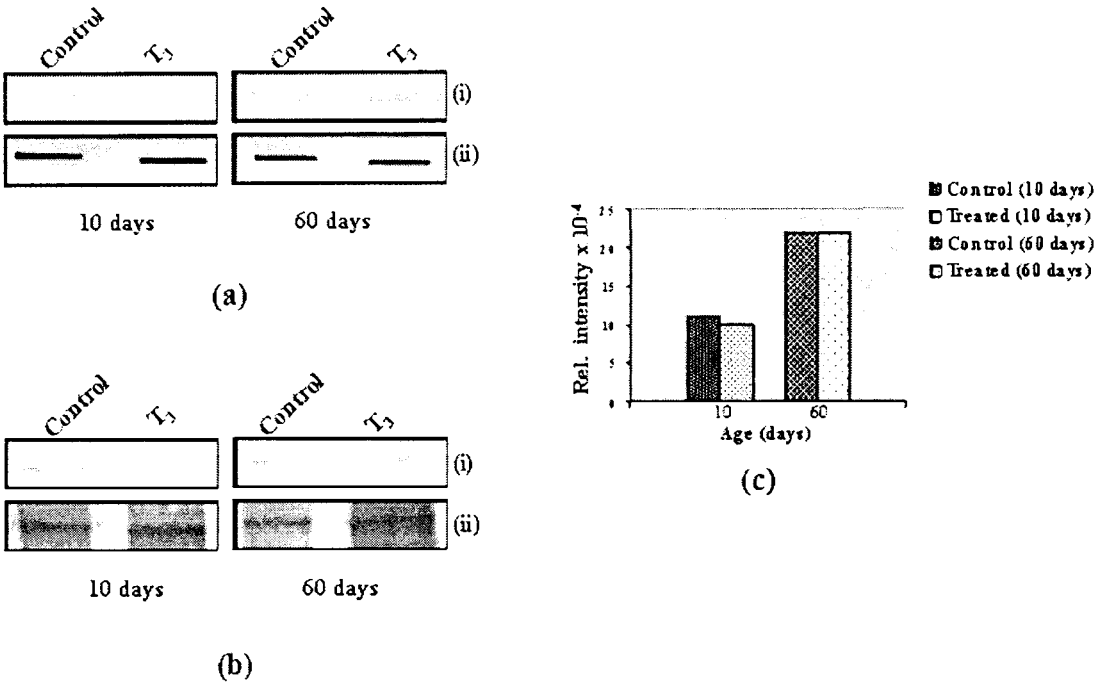


Fig. 38: (a) i. Slot blot analysis of ADA from crop of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from crop of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.3.3. Proventriculus- In the proventriculus, T_3 was seen to have no significant effect on the activity of ADA in the two ages studied. [Table: 19; Fig. 39]. Slot and Western blots also showed no change in the level of ADA protein [Fig. 40].

Proventriculus				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	64.21	62.79	53.2	52.43
SD	1.95	1.71	1.73	2.16
p		NS		NS
% (+/-)		NC		NC

Table 19: Effect of T_3 on the level of ADA activity in proventriculus. SD, Standard deviation; *p*, Level of significance; % (+/-): Per cent increase or decrease; NS, Not significant; NC, No change.

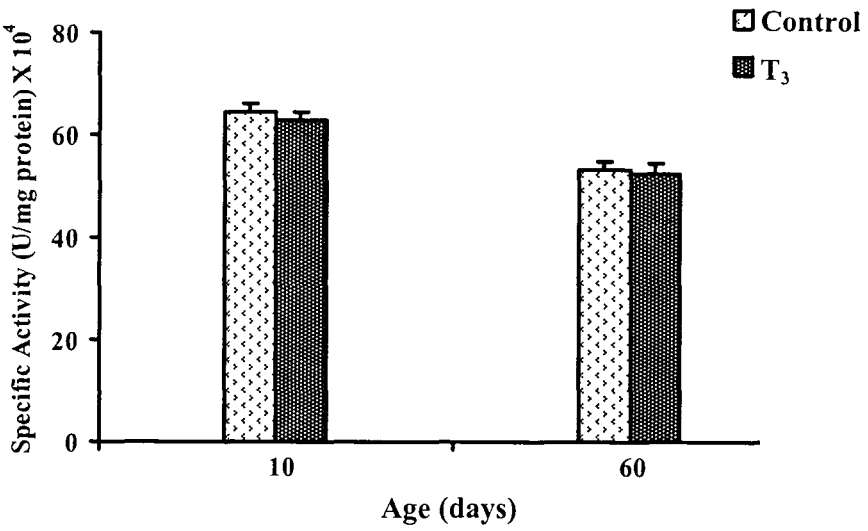


Fig. 39: Effect of T_3 on the activity of proventriculus ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation.

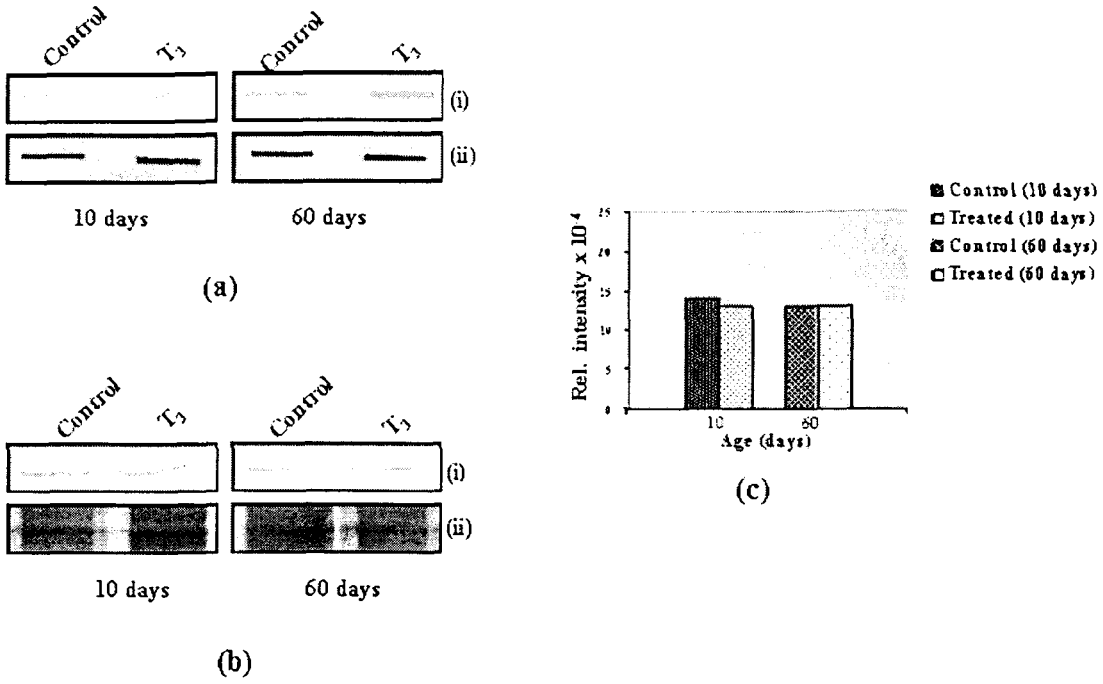


Fig. 40: (a) i. Slot blot analysis of ADA from proventriculus of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 µg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from proventriculus of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 µg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.3.4. Small intestine- In the small intestine, T_3 was seen to have no significant effect on the activity of ADA in the two ages studied. [Table: 20; Fig. 41]. Slot and Western blots showed no change in the level of ADA protein [Fig. 42].

Small intestine				
ADA activity (U/mg) x 10^3				
Age (Days) →	10		60	
	Control	Treated	Control	Treated
Mean	21.52	20.95	23.72	21.49
SD	1.81	1.47	2.4	0.63
p		NS		NS
% (+/-)		NC		NC

Table 20: Effect of T_3 on the level of ADA activity in small intestine. SD: Standard deviation; *p*, Level of significance; % (+/-), Per cent increase or decrease; NS, Not significant; NC, No change.

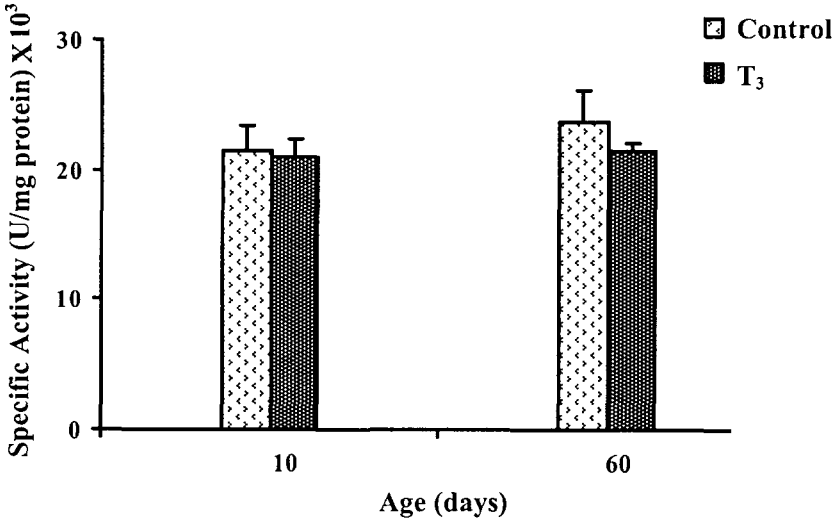


Fig. 41: Effect of T_3 on the activity of small intestine ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation.

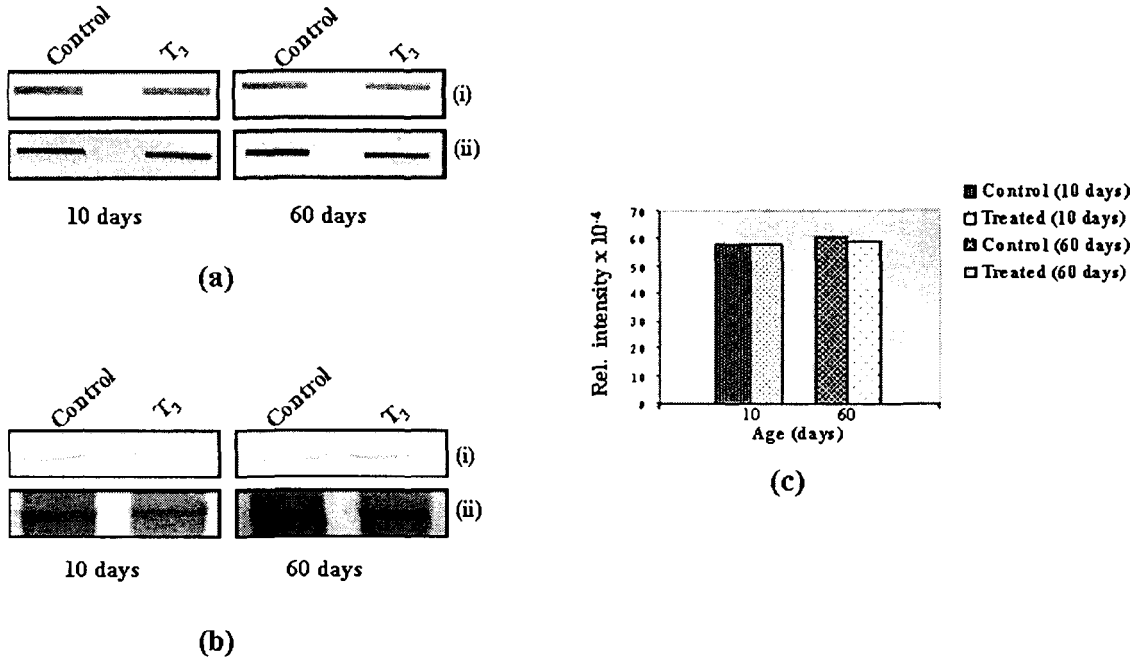


Fig. 42: (a) i. Slot blot analysis of ADA from small intestine of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from small intestine of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.3.5. Spleen- In the spleen, T_3 was seen to have no significant effect on the activity of ADA in the two ages studied. [Table: 21; Fig. 43]. Representative data from slot and Western blots also showed no change in the ADA protein [Fig. 44].

Spleen				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	23.94	21.38	26.31	25.31
SD	0.75	0.86	0.97	1.13
p		NS		NS
% (+/-)		NC		NC

Table 21: Effect of T_3 on the level of ADA activity in spleen. SD, Standard deviation; *p*, Level of significance; % (+/-), Per cent increase or decrease; NS, Not significant; NC, No change.

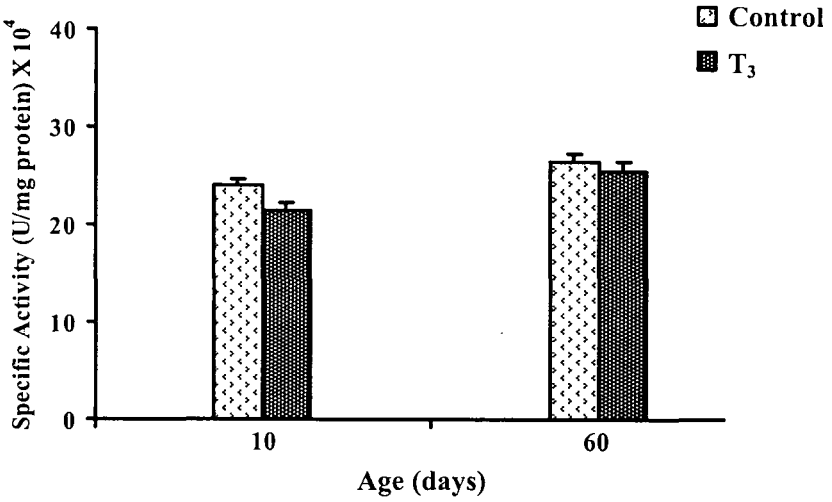


Fig. 43: Effect of T_3 on the activity of spleen ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation.

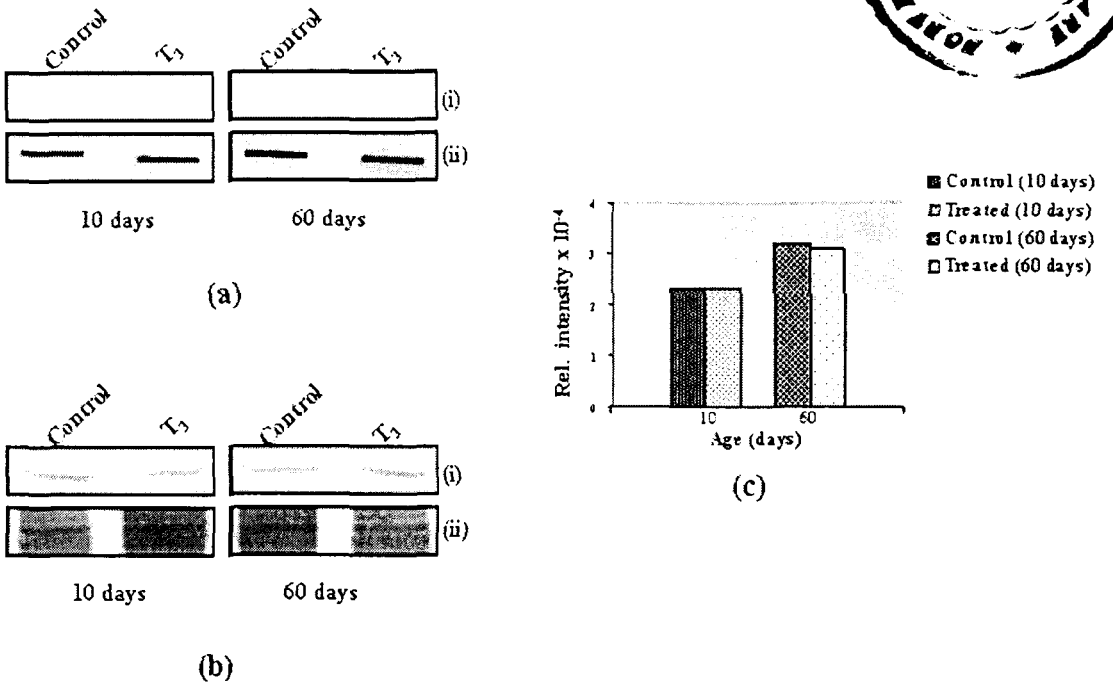


Fig. 44: (a) i. Slot blot analysis of ADA from spleen of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from spleen of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.4. EFFECT OF TESTOSTERONE: Testosterone was administered at a dose of 1.0 mg/ 100 g body weight in 0.3 ml sesame oil, intraperitoneally, for 3 consecutive days and chicken were sacrificed after 3 h of final injection. Its effect on ADA activity (U/mg protein) was studied in the GIT (esophagus, crop, proventriculus, crop, small intestine) and spleen. However, no significant change on ADA activity was observed in all the tissues of GIT and spleen studied. Representative data of the slot and Western blots confirmed the spectrophotometric results.

3.2.4.1. Esophagus- In the esophagus, testosterone was seen to have no significant effect on the activity of ADA in the two ages studied. [Table: 22; Fig. 45]. Slot and Western blots also showed no change at the two ages studied [Fig. 46].

Esophagus				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	25.55	23.92	17.55	15.45
SD	1.71	1.57	1.35	0.4
p		NS		NS
% (+/-)		NC		NC

Table 22: Effect of testosterone on the level of ADA activity in esophagus. SD, Standard deviation; *p*, Level of significance; % (+/-), Percent increase or decrease; NS, Not significant; NC, No change.

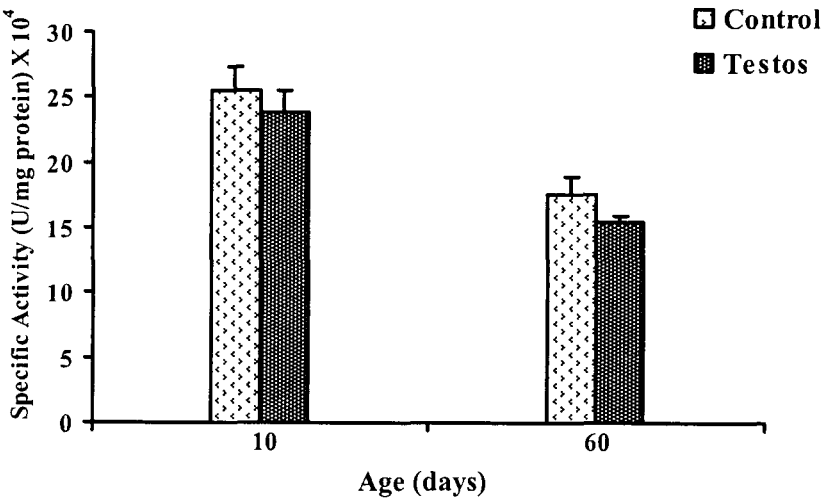


Fig. 45: Effect of testosterone (Testos) on the activity of esophagus ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation.

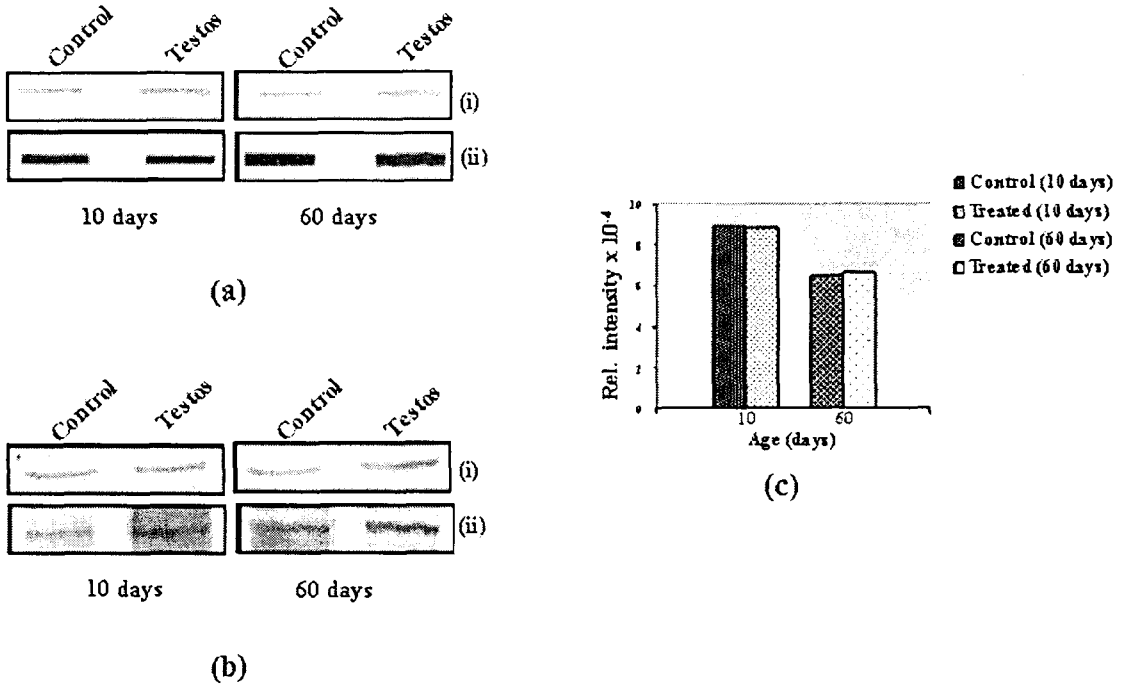


Fig. 46: (a) i. Slot blot analysis of ADA from esophagus of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 µg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from esophagus of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 µg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.4.2. Crop- In the crop, testosterone was seen to have no significant effect on the activity of ADA in the two ages studied. [Table: 23; Fig. 47] Slot and Western blots also showed no change in the level of ADA protein [Fig. 48].

Crop				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	15.02	13.43	17.32	16.1
SD	1.58	1.12	0.81	0.88
p		NS		NS
% (+/-)		NC		NC

Table 23: Effect of testosterone on the level of ADA activity in crop. SD: Standard deviation; *p*, Level of significance; % (+/-), Per cent increase or decrease; NS, Not significant; NC, No change.

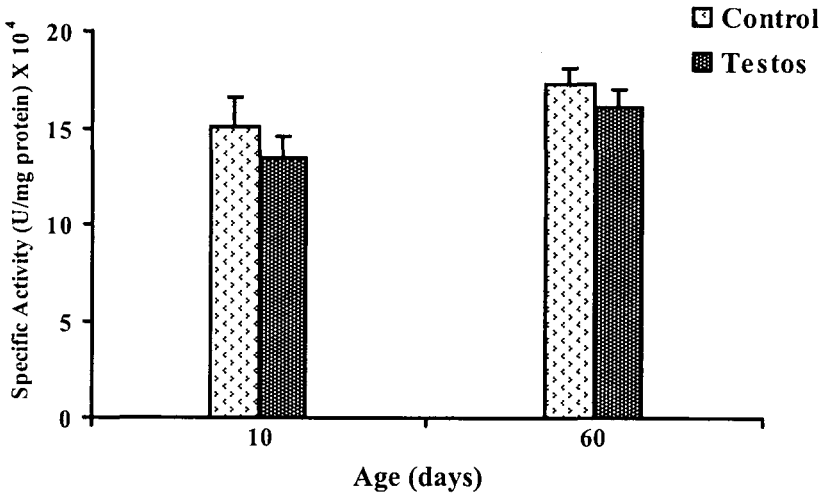


Fig. 47: Effect of testosterone (Testos) on the activity of crop ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation.

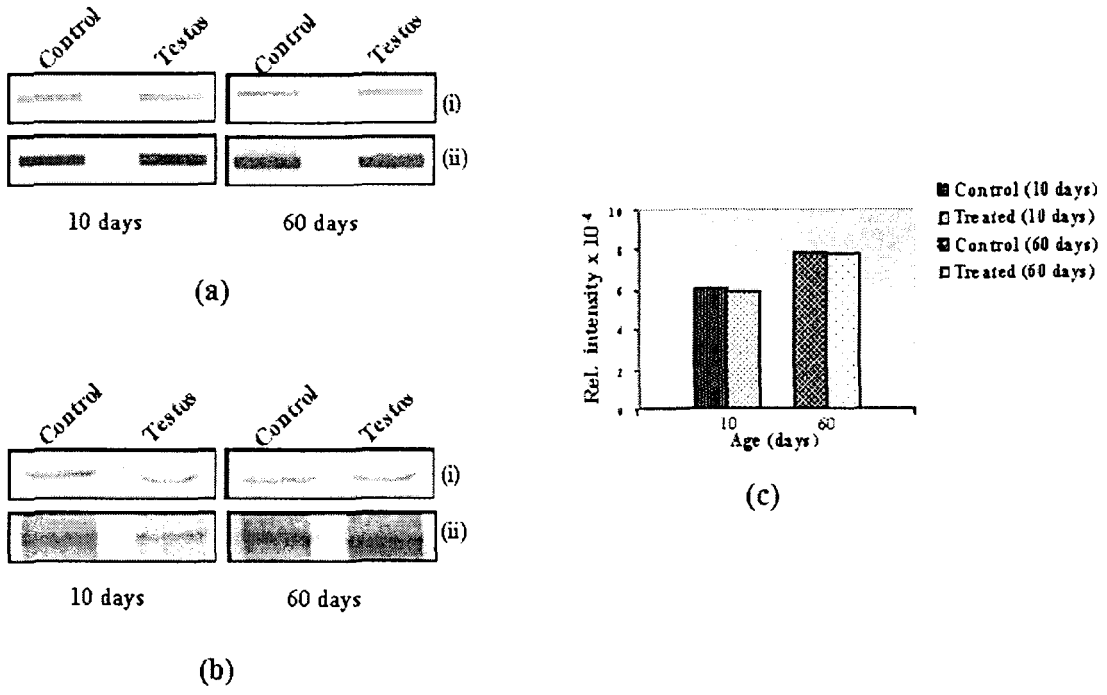


Fig. 48: (a) i. Slot blot analysis of ADA from crop of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 µg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from crop of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 µg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.4.3. Proventriculus- In the proventriculus, testosterone was seen to have no significant effect on the activity of ADA in the two ages studied. [Table: 24; Fig. 49]. Representative data of slot and Western confirmed the spectrophotometric results [Fig. 50].

Proventriculus				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	64.93	63.95	52.75	50.66
SD	1.32	1.43	0.43	1.37
p		NS		NS
% (+/-)		NC		NC

Table 24: Effect of testosterone on the level of ADA activity in crop. SD, Standard deviation; *p*, Level of significance; % (+/-): Per cent increase or decrease; NS, Not significant; NC, No change.

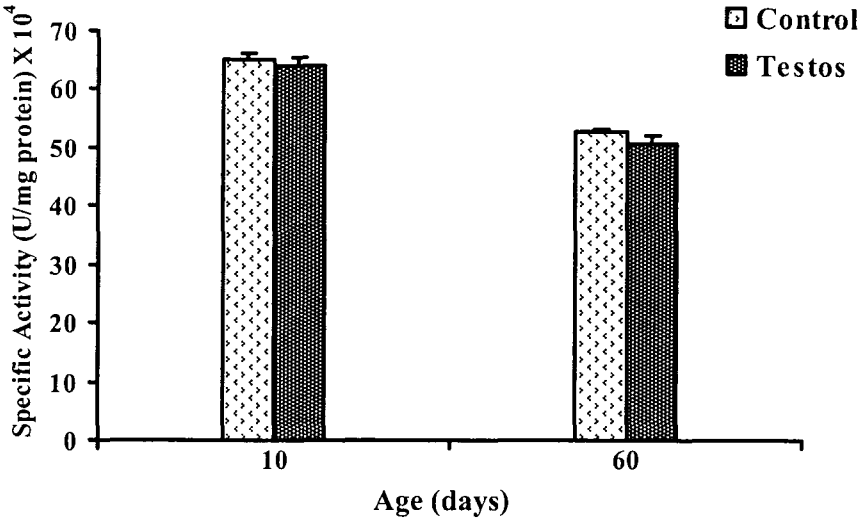


Fig. 49: Effect of testosterone (Testos) on the activity of proventriculus ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation.

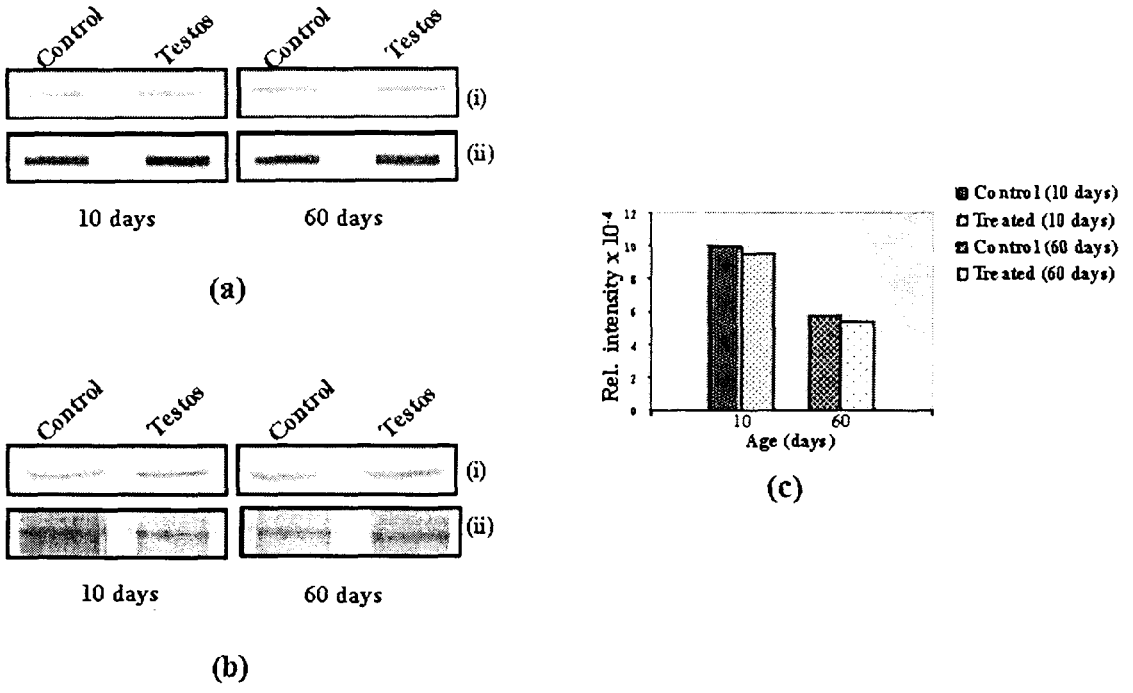


Fig. 50: (a) i. Slot blot analysis of ADA from proventriculus of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 μg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from proventriculus of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 μg protein) of cytosol containing ADA was loaded in each lane and processed for immoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.4.4. Small intestine- In the small intestine of chicken, testosterone was seen to have no significant effect on the activity of ADA in the two ages studied. [Table: 25; Fig. 51]. Slot and Western blots indicated that there was no change in the level of ADA protein as well [Fig. 52].

Small intestine				
ADA activity (U/mg Protein) x 10^3				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	19.85	18.59	21.67	20.36
SD	2.03	1.93	0.75	0.42
p		NS		NS
% (+/-)		NC		NC

Table 25: Effect of testosterone on the level of ADA activity in small intestine. SD, Standard deviation; *p*, Level of significance; % (+/-), Per cent increase or decrease; NS, Not significant; NC, No change.

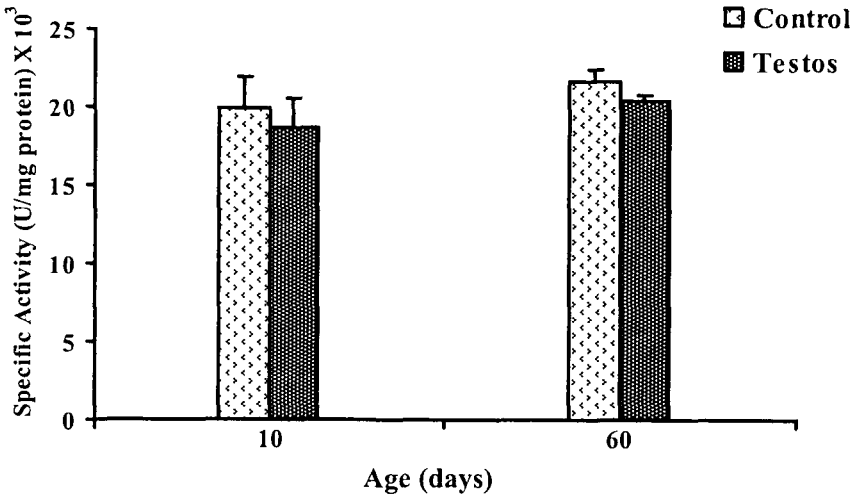
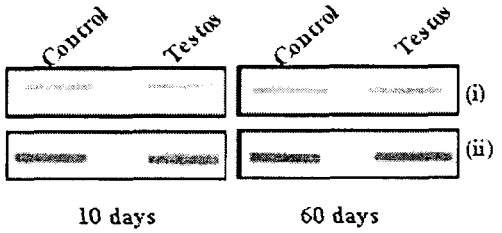
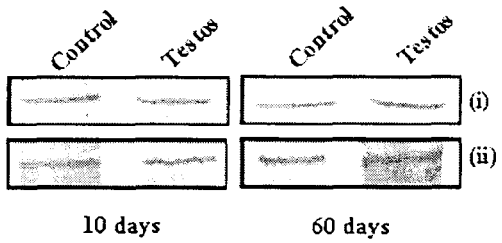


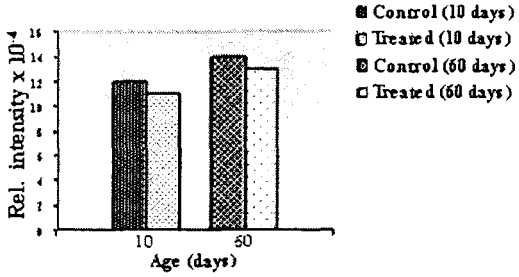
Fig. 51: Effect of testosterone (Testos) on the activity of small intestine ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation.



(a)



(b)



(c)

Fig. 52: (a) i. Slot blot analysis of ADA from small intestine of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 µg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from small intestine of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 µg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.2.4.5. Spleen- In the spleen, testosterone did not have any significant effect on the activity of ADA in the two ages studied. [Table: 26; Fig. 53] The results were confirmed by slot and Western blot analyses which showed no change in the protein of the enzyme [Fig. 54].

Spleen				
ADA activity (U/mg Protein) x 10^4				
Age (Days) →	**10**		**60**	
	Control	**Treated**	**Control**	**Treated**
Mean	23.88	10	25.13	60
SD	1.55	2.23	1.69	1.33
p		NS		NS
% (+/-)		NC		NC

Table 26: Effect of testosterone on the level of ADA activity in spleen. SD, Standard deviation; *p*, Level of significance; % (+/-), Per cent increase or decrease; NS, Not significant; NC, No change.

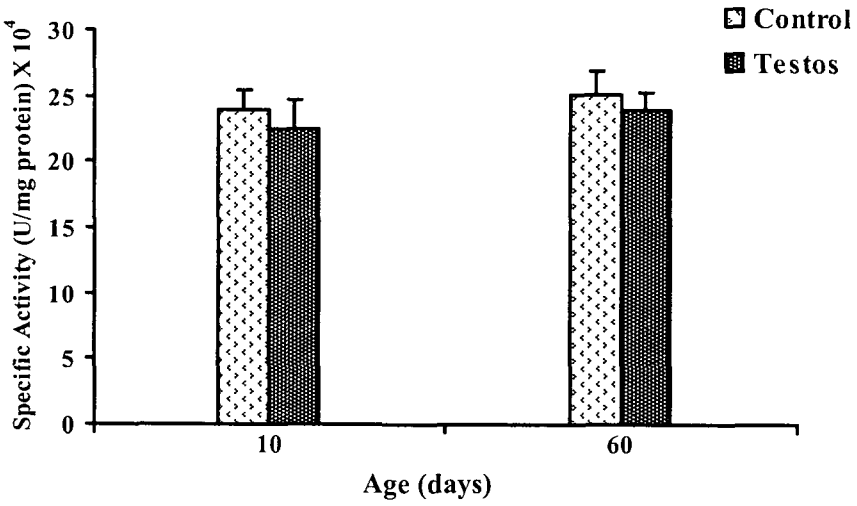


Fig. 53: Effect of testosterone (Testos) on the activity of spleen ADA. Values are expressed as mean from five chicken at each point. Bars represent standard deviation.

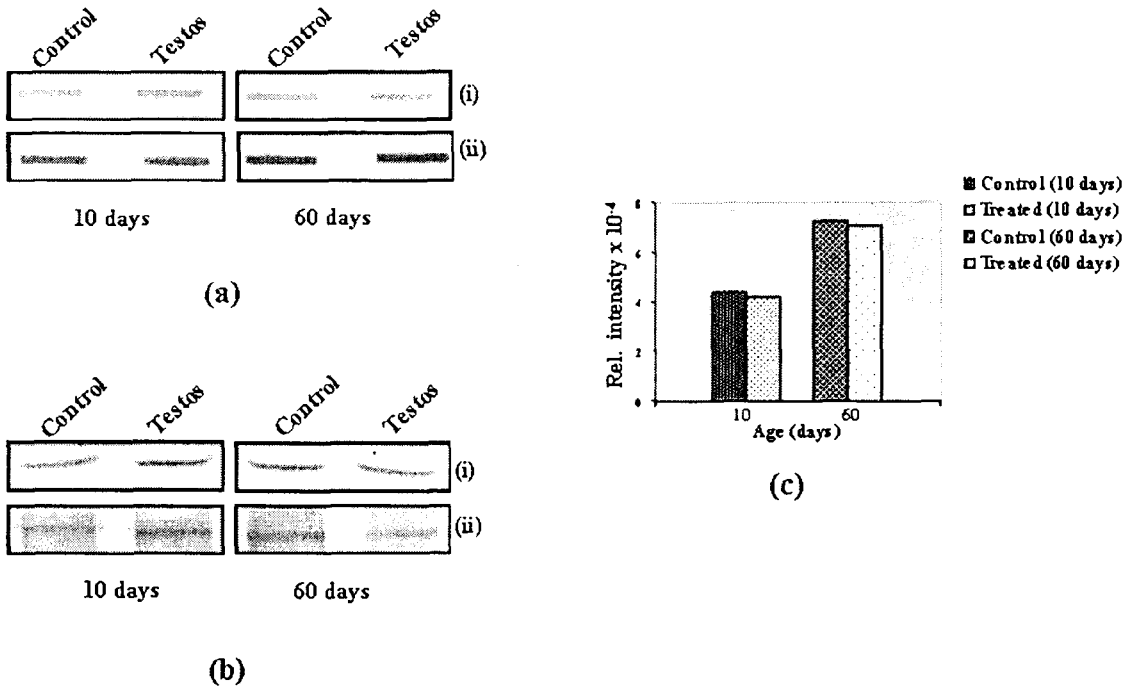


Fig. 54: (a) i. Slot blot analysis of ADA from spleen of chicken from day 10 and 60. (a) ii. Represents the total protein stained with India Ink as control. An equal amount (60 µg protein) of cytosol containing ADA was applied to each slot and processed for immunoblotting using human polyclonal ADA antibody. (b) i. Western blot analysis of ADA from spleen of chicken from day 10 and 60. (b) ii. The India ink stained Western blots for a major GIT protein of ~42 kDa on nitrocellulose membrane. An equal amount (50 µg protein) of cytosol containing ADA was loaded in each lane and processed for immunoblotting using human polyclonal ADA antibody. (c) The relative (Rel.) intensity of the slot blots determined by densitometric analysis (KDS-1 software).

3.3. IMMUNOPRECIPITATION

As the antibody used for slot and Western blotting of chicken ADA was of human origin, it was necessary to find the cross-reactivity of human anti-ADA antibody with chicken ADA. From the studies conducted, it was found that the cross-reactivity of human anti-ADA antibody to chicken ADA was ~40% [Fig. 55]

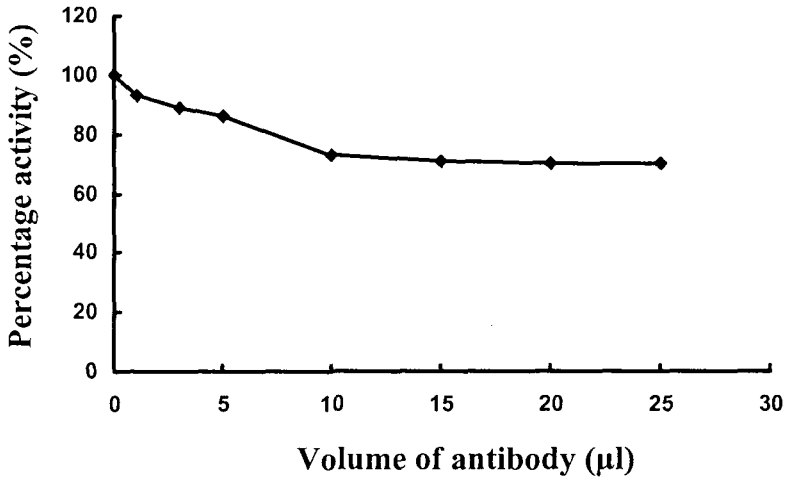


Fig. 55: Immunoprecipitation: Cross reactivity of chicken ADA with human anti-ADA antibody

3.4. ISOLATION AND PURIFICATION OF ADA

ADA was isolated and purified from small intestine of immature (1-day old) and mature (90-days old) chicken using the experimental procedures described in the materials and methods section. These two ages were chosen to find out if there were any differences in the kinetic as well as physicochemical properties of ADA from immature and mature ages. The summary of the purification tables and results have been outlined in tables 27 (1-day) and 28 (90-day). The degree of purification of ADA from the small intestine of immature and mature mice was 503- and 569-fold with a yield of 4% and 5%, respectively. ADA from both the ages got purified till homogeneity, as evident from its single band on gel electrophoresis.

Table 27: Purification chart of ADA from immature (1-day old) chicken

STEPS	Total Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific activity (U/mg) protein	Purification fold	Yield (%)
Crude extract	80	8526.32	322.5	0.04		100
27,500 x g Supernatant	75	2694.2	142.3	0.05	1	44
$(NH_4)_2SO_4$ (40%-70%)	23	1032.46	68.4	0.07	2	21
Dialysis	27	20.08	33.3	1.7	43	10
Sephadex G-100	14	15.23	29.6	1.94	49	9
Dialysis	16	2.3	16.8	7.3	183	5
DEAE-Cellulose	**6**	**0.6**	**12.1**	**20.1**	**503**	**4**

Table 28: Purification chart of ADA from mature (90-day old) chicken

STEPS	Total Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific activity (U/mg) protein	Purification fold	Yield (%)
Crude extract	80	8998.65	185.2	0.02		100
27,500 x g Supernatant	73	2598.02	156.5	0.06	3	85
$(NH_4)_2SO_4$ (40%-70%)	20	998.74	76	0.08	4	41
Dialysis	24	21.05	25.4	1.21	61	14
Sephadex G-100	12	16.05	21.6	1.35	68	12
Dialysis	15	3.6	12.1	3.36	168	7
DEAE-Cellulose	**7**	**0.8**	**9.1**	**11.37**	**569**	**5**

The elution profiles of immature (1-day) and mature (90-day) intestinal ADA on Sephadex G-100 are depicted in Fig. 56 and 58 respectively, whereas on DEAE-cellulose ion exchanger can be seen in Fig. 57 and 59. Both immature and mature small intestinal ADA was seen to be eluted at the same elution volume on Sephadex G-100 column as well as at the same ionic strength of 0.24 M NaCl from DEAE-cellulose column.

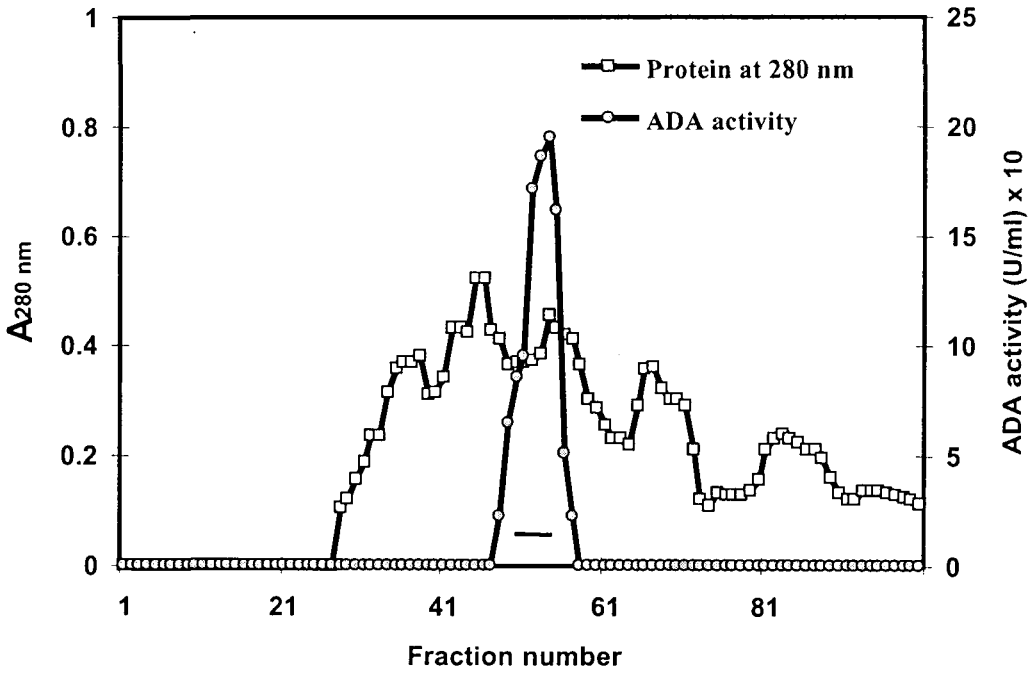


Fig. 56: Elution profile of intestinal ADA from 1-day old chicken through Sephadex G-100. Fractions were monitored at 280 nm for proteins and assayed for ADA activity. The dark horizontal line indicates the fractions pooled for further studies.

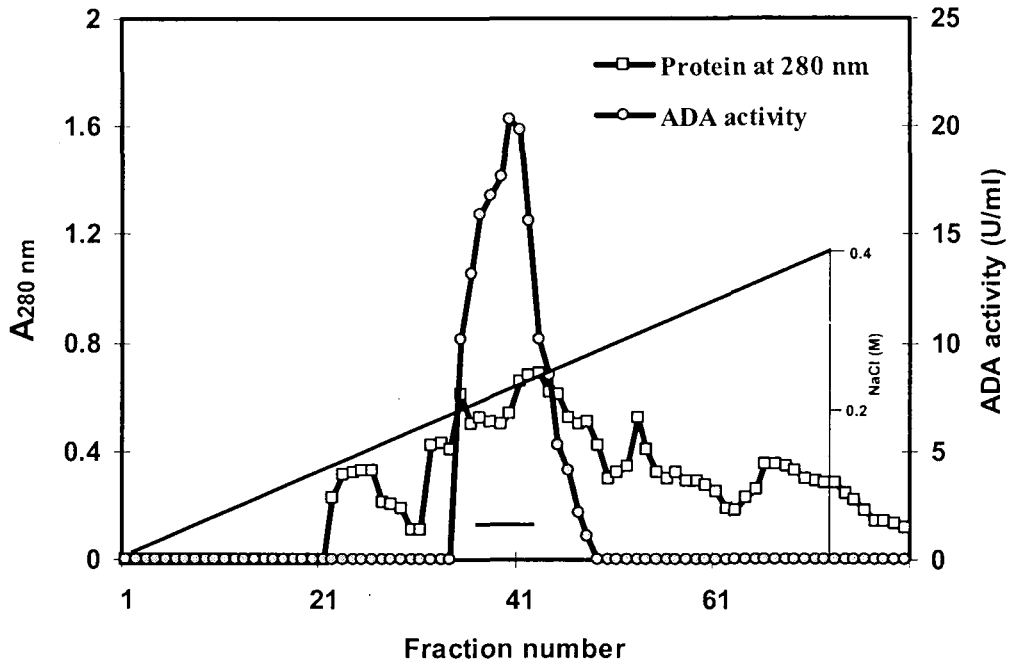


Fig. 57: Elution profile of intestinal ADA from 1-day old chicken through DEAE- cellulose ion exchanger column. Fractions were monitored at 280 nm for proteins and assayed for ADA activity. The inclined and horizontal lines indicate the linear gradient of sodium chloride from 0-0.4 M and the fractions pooled for further studies, respectively.

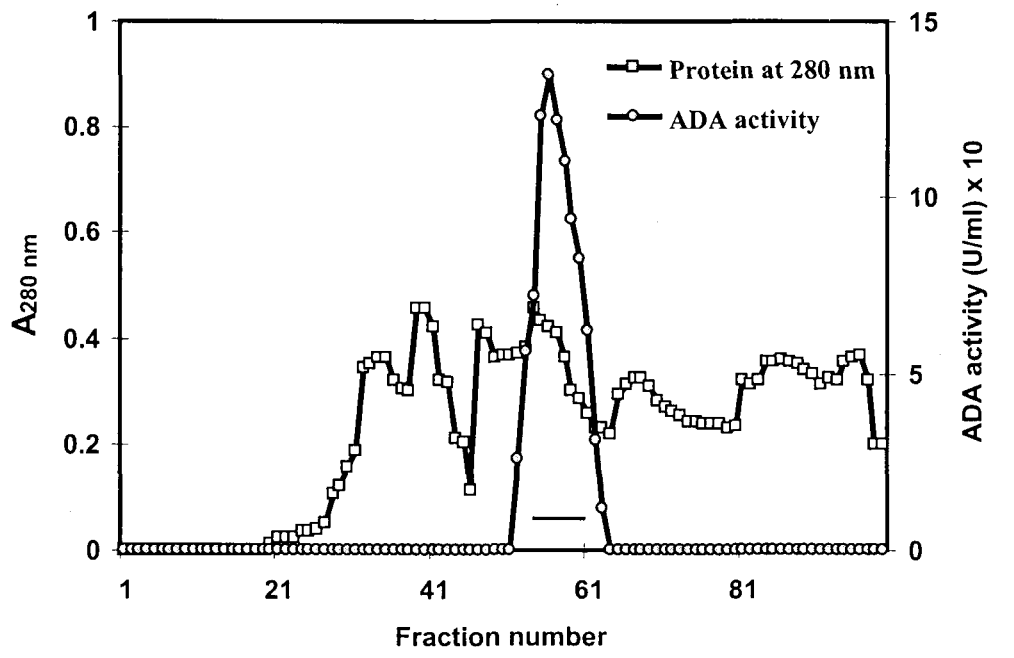


Fig. 58: Elution profile of intestinal ADA from 90-day old chicken through Sephadex G-100. Fractions were monitored at 280 nm for proteins and assayed for ADA activity. The dark horizontal line indicates the fractions pooled for further studies.

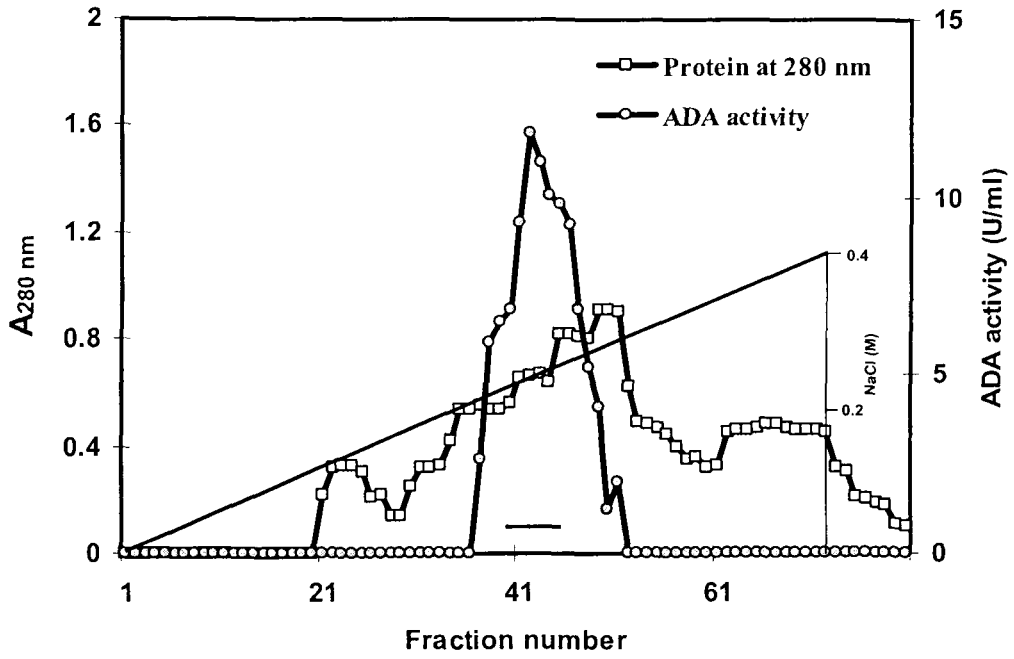


Fig. 59: Elution profile of intestinal ADA from 90-day old chicken through DEAE- cellulose ion exchanger column. Fractions were monitored at 280 nm for proteins and assayed for ADA activity. The inclined and horizontal lines indicate the linear gradient of sodium chloride from 0-0.4 M and the fractions pooled for further studies, respectively.

3.5. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) AND WESTERN BLOT ANALYSIS OF PURIFIED ADA

Purified small intestinal ADA from immature and mature chicken was electrophoresed on native and SDS polyacrylamide gel.

The native PAGE pattern of purified ADA from intestine of immature and mature chicken showed a single band on individual lane at a similar level from the origin of migration as seen in Fig. 60, indicating that both intestinal ADA have been purified till homogeneity and contain similar charge. SDS-PAGE also showed a single band of molecular weight 100 kDa, as shown in Fig. 61. To identify the bands for each purified ADA, Western blotting was performed from SDS gel and a single band was detected in the Western blotting for both immature and mature ADA (Fig. 62).

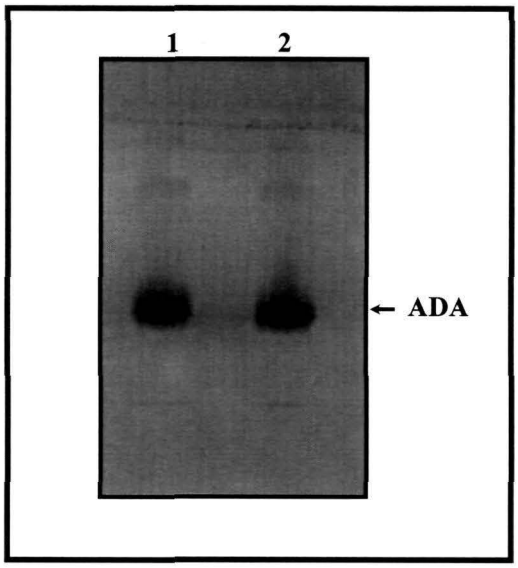


Fig. 60: Native polyacrylamide gel electrophoresis of purified ADA from intestine of 1-day (lane 1) and 90-day (lane 2) old chicken.

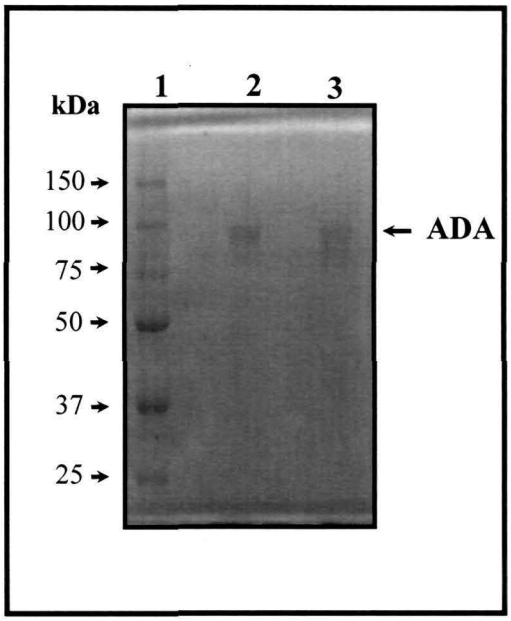


Fig. 61: SDS-Polyacrylamide gel electrophoresis of purified ADA from intestine of 1-day (lane 2) and 90-day (lane 3) old chicken. The molecular weight markers are exhibited in lane 1. The standard molecular weight markers are Biorad Precision Plus Protein^{TM} All Blue Standards.:

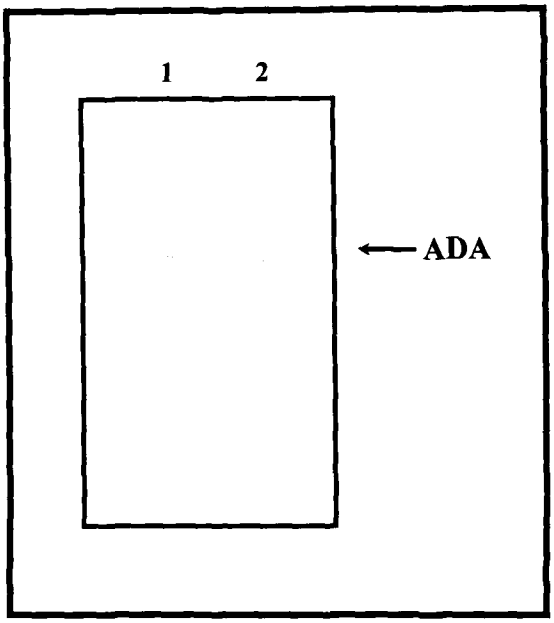


Fig. 62: Western blot of purified intestinal ADA from immature (lane 1) and mature (lane 2) chicken

3.6. MOLECULAR WEIGHT DETERMINATION OF THE PURIFIED ADA

Molecular weight of the purified ADA preparations from both immature and mature chicken intestine was estimated using Sephadex G-100 gel filtration and SDS-PAGE [Fig. 63 and 64, respectively].

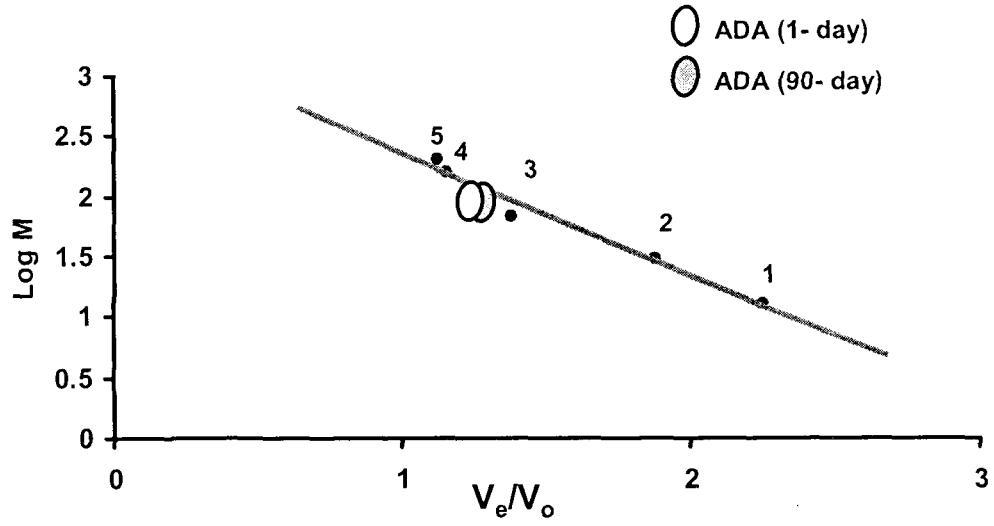


Fig. 63: Log molecular weight (M) versus V_e/V_o using Sephadex G-100 column for molecular weight determination of purified ADA from 1- and 90-day old intestine of chicken. The standard molecular weight markers were:

1. Cytochrome c (12.4 kDa)
2. Carbonic anhydrase (29 kDa)
3. Bovine serum albumin (66 kDa)
4. Alcohol dehydrogenase (150 kDa) and
5. β-amylase (200 kDa)

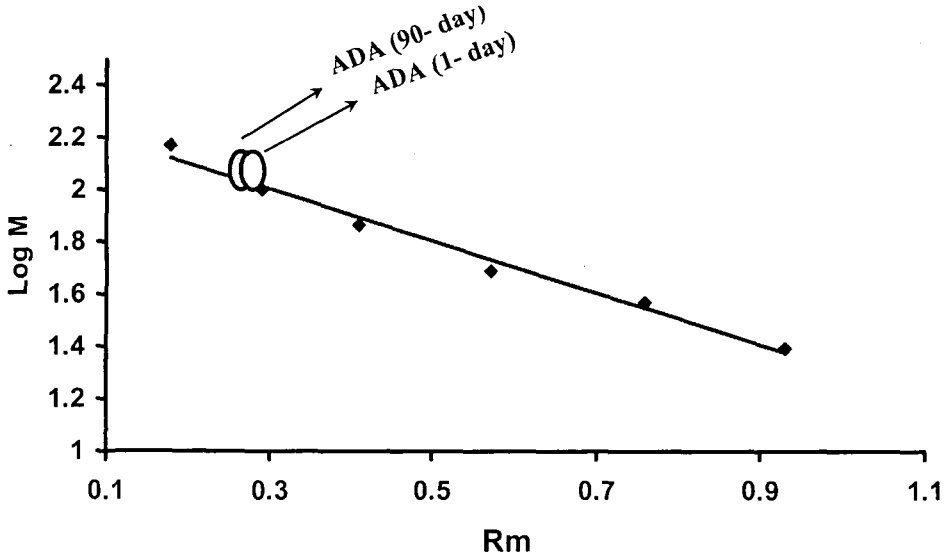


Fig. 64: Log molecular weight versus R_m (using SDS-PAGE) for molecular weight determination of purified ADA from 1- and 90-day old intestine of chicken. The standard molecular weight markers were the same as given in figure 61.

By both the methods, it was determined that purified intestinal ADA showed similar molecular weight. Using gel filtration analysis, the molecular weight of immature and mature ADA was estimated to be 100 kDa (Fig. 63). By SDS-PAGE, the molecular weight of immature and mature ADA was also found to be 100 kDa (Fig. 64).

3.7. KINETIC STUDIES OF PURIFIED ADA

Activities of the purified ADA from the small intestine of immature and mature chicken were studied at various concentrations of adenosine and 2'-deoxyadenosine using normal assay procedure. The Michaelis-Menten plots for the determination of K_m for adenosine are shown in Fig. 65 and 66.The Lineweaver-Burk plots are also shown as insets to the Michaelis- Menten plots. The computed K_m values of the immature and mature small intestinal ADA for adenosine were 33.3 μM and 34.2 μM, respectively. The Michaelis-Menten plots for the determination of K_m for 2' deoxyadenosine are shown in Fig. 67 and 68. The Lineweaver-Burk plots are also shown as insets. The computed K_m values of the immature and mature small intestinal ADA for 2'-deoxyadenosine were 14.3 μM and 14.3 μM, respectively. V_{max} values of immature and mature purified ADA for adenosine obtained from the same plots, were 22 μmol/min and 25 μmol/min, respectively, whereas the values determined using 2'-deoxyadenosine as substrate were 20 μmol/min and 18 μmol/min. The K_{cat} values of immature and mature purified ADA for adenosine estimated from the same plots, were 5.2 s^{-1} and 5.9 s^{-1}, respectively, whereas the values of K_{cat} estimated using 2'-deoxyadenosine as substrate were 4.2 s^{-1} and 4.0 s^{-1} (Table 29).

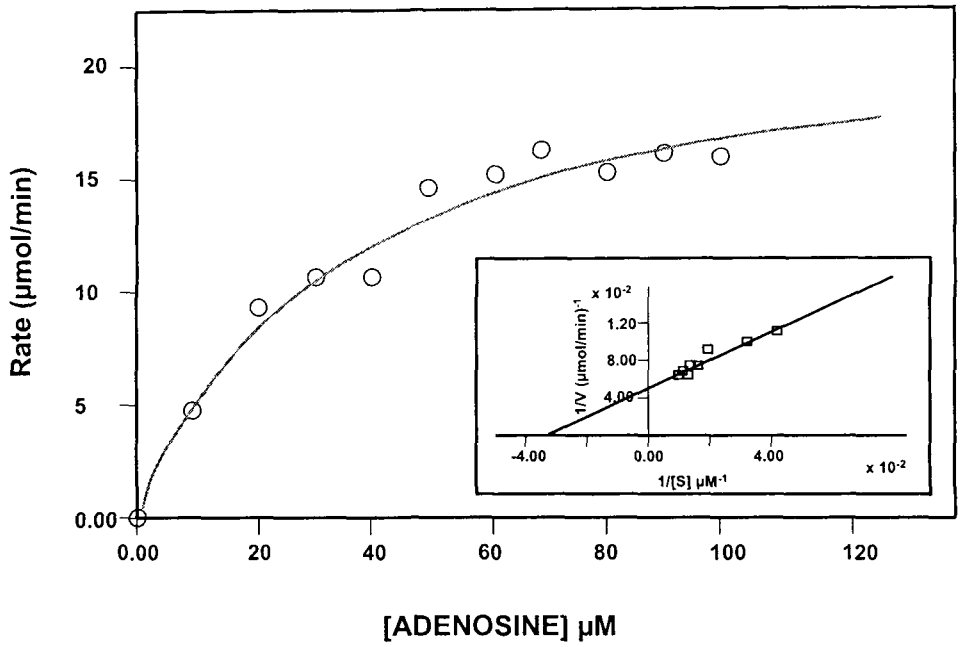


Fig. 65: Michaelis-Menten plot for purified intestinal ADA of immature (1-day) chicken using various concentrations of adenosine. Inset shows the Lineweaver-Burk plot. Data were computed and plotted in enzfitter programme (Sigma).

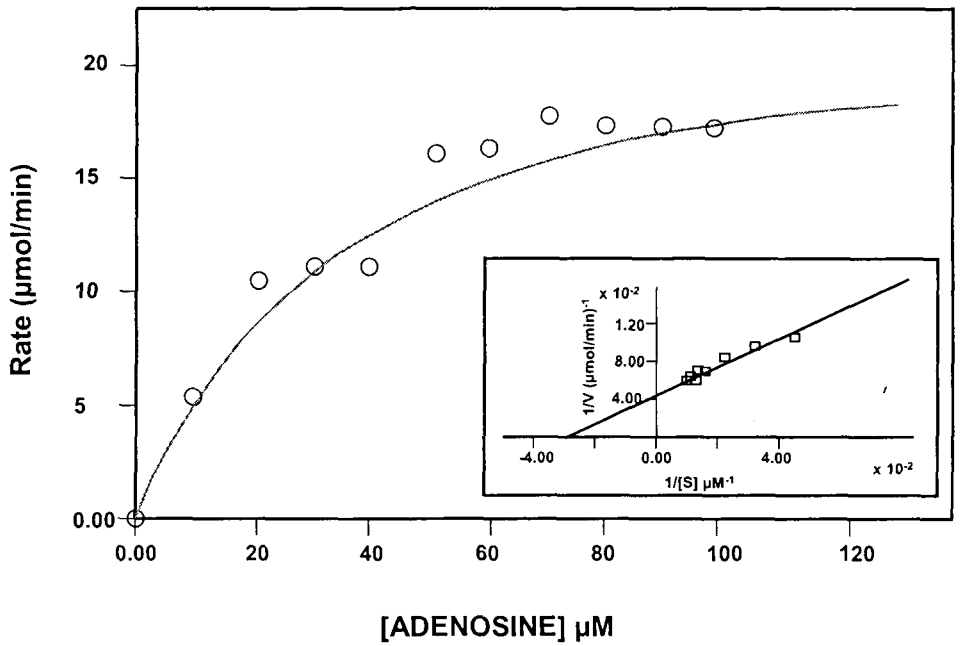


Fig. 66: Michaelis-Menten plot for purified intestinal ADA of mature (90-day) chicken using various concentrations of adenosine. Inset shows the Lineweaver-Burk plot. Data were computed and plotted in enzfitter programme (Sigma).

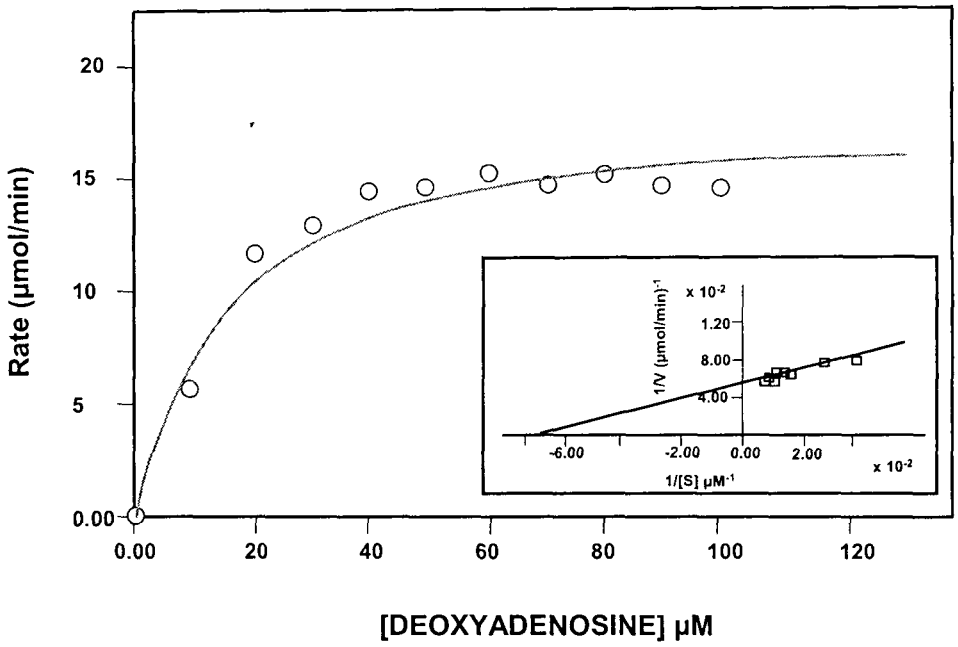


Fig. 67: Michaelis-Menten plot for purified intestinal ADA of immature (1-day) chicken using various concentrations of deoxyadenosine. Inset shows the Lineweaver-Burk plot. Data were computed and plotted in enzfitter programme (Sigma).

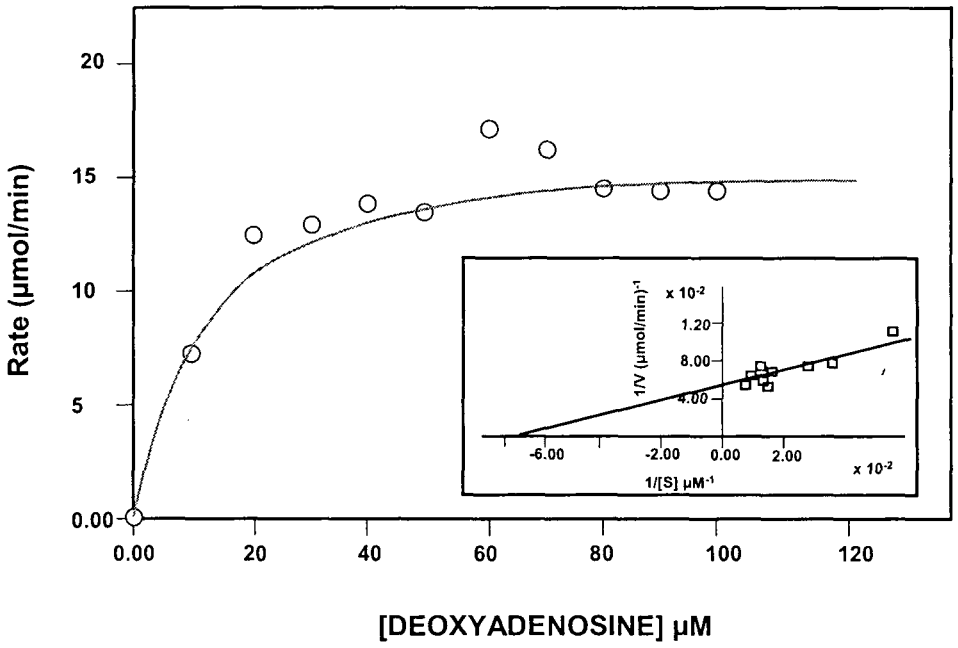


Fig. 68: Michaelis-Menten plot for purified intestinal ADA of mature (90-day) chicken using various concentrations of deoxyadenosine. Inset shows the Lineweaver-Burk plot. Data were computed and plotted in enzfitter programme (Sigma).

Table 29: Summary of the kinetic data of purified ADA from 1- and 90-day old chicken

SUBSTRATES	PARAMETERS	1-DAY	90-DAY
ADENOSINE	K_m (μM)	33.3	34.2
	V_{max} (μmole/min)	22	25
	K_{cat} (sec^{-1})	5.2	5.9
2' DEOXYADENOSINE	K_m (μM)	14.3	14.3
	V_{max} (μmole/min)	20	18
	K_{cat} (sec^{-1})	4.2	4.0

K_i of purified ADA from immature and mature chicken was determined using various concentrations of purine riboside as an inhibitor, at two fixed concentrations of adenosine, in order to study the modes of inhibition and also the change as a function of age. Dixon's plots for purine riboside inhibition of immature and mature purified intestinal ADA (as given in Fig. 69 and Fig. 70) indicated that the mode of purine riboside inhibition of both ADA was competitive, since the lines of intersect at the two concentrations of adenosine were on the negative side of the plot. The estimated K_i values for immature and mature intestinal ADA were found to be 6 μM and 7 μM, respectively.

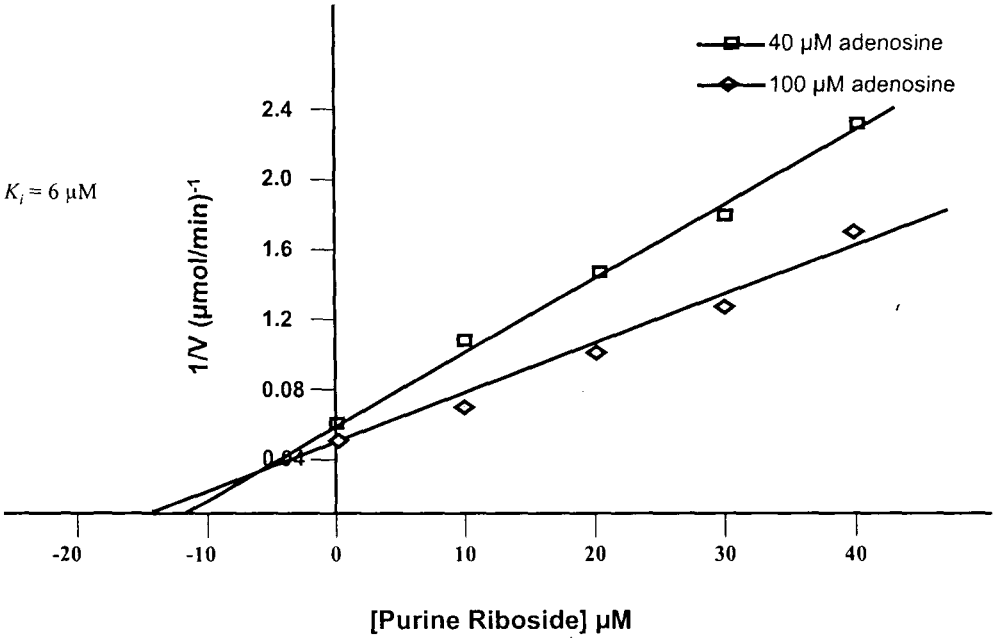


Fig. 69: Dixon's plot for purine riboside inhibition of immature (1-day) chicken intestinal ADA. Data were computed and plotted using enzfitter programme (Sigma).

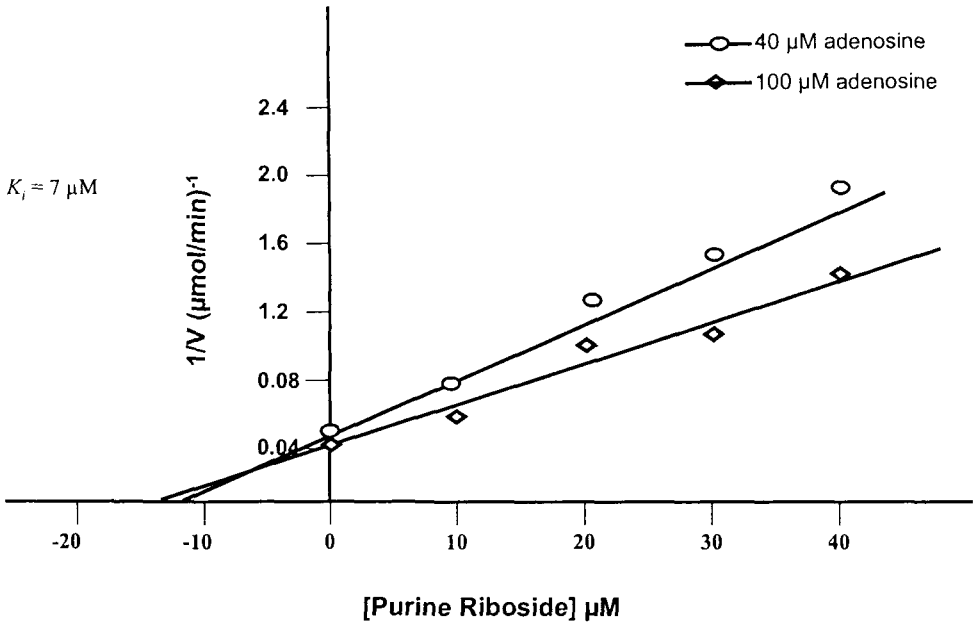


Fig. 70: Dixon's plot for purine riboside inhibition of mature (90-day) chicken intestinal ADA. Data were computed and plotted using enzfitter programme (Sigma).

3.8. pH STABILITY OF ADA

Purified ADA preparations from immature and mature chicken were pre-incubated with buffers having different pH from 4 to 9 and assayed thereafter using normal procedure. The results were expressed as percentage of activity, taking the activity at pH 5.5 as 100 % (Fig. 71). It was observed that both the ADA were stable at similar range of pH profile. About 94% of the activity of both the enzymes of the two ages was retained after preincubating at pH 9 for 1 hour, at pH 4 about 50% of the activity was lost in the purified ADA of both ages.

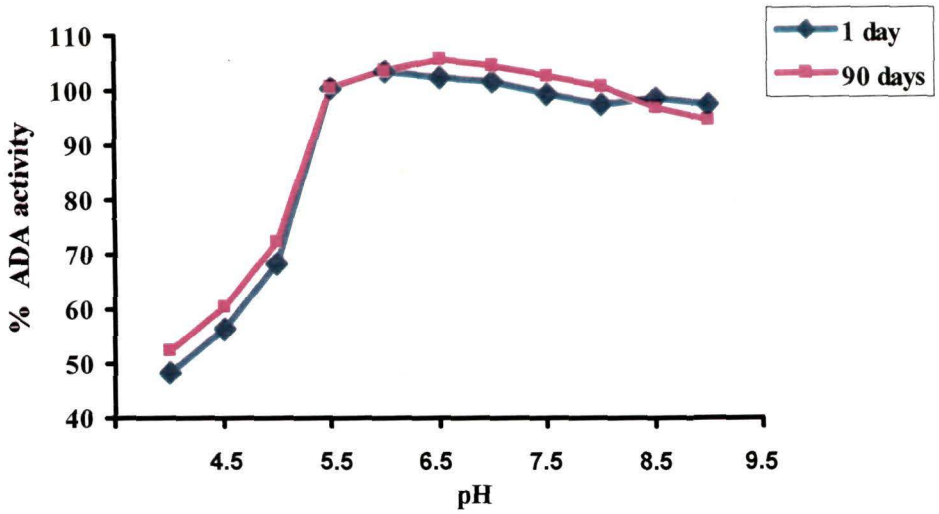


Fig. 71: pH stability profile for both immature (1- day) and mature (90- day) purified chicken intestinal ADA. Results are expressed as percentage of activity, taking the pH at 5.5 as 100%.

3.9. TEMPERATURE STABILITY OF ADA

Purified ADA from immature and mature chicken was assayed at different temperatures from 25°C to 70°C. The results were expressed as percentage of activity taking the activity at 25°C as 100% (Fig. 72). Data showed that ADA from both immature and mature chicken showed stability till 55°C.

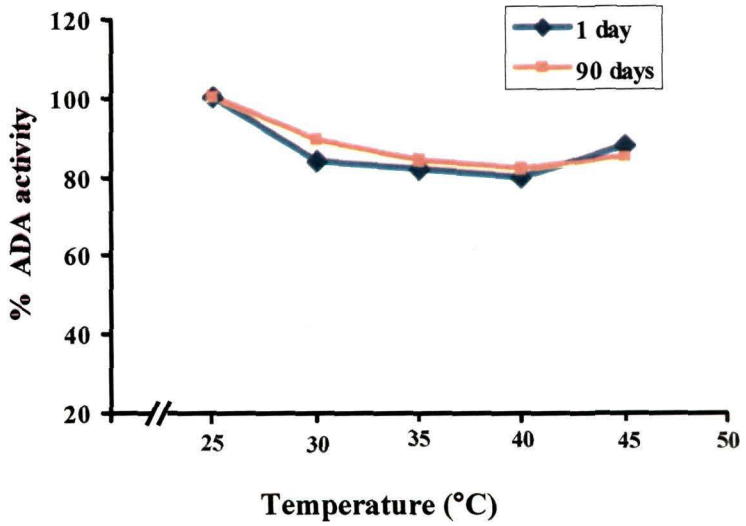


Fig. 72: Temperature stability profile for both immature (1- day) and mature (90- day) purified chicken intestinal ADA. Results are expressed as percentage of activity, taking the activity of ADA at 25°C as 100%.

3.10. UREA DENATURATION OF ADA

Inactivation studies using different concentrations of urea on purified intestinal ADA of both immature and mature chicken were performed (Fig. 73). Both purified ADA gets 50% inactivation (IC_{50}) at 0.5 M urea. This indicated that both immature and mature small intestinal ADA of chicken were similarly susceptible to urea denaturation.

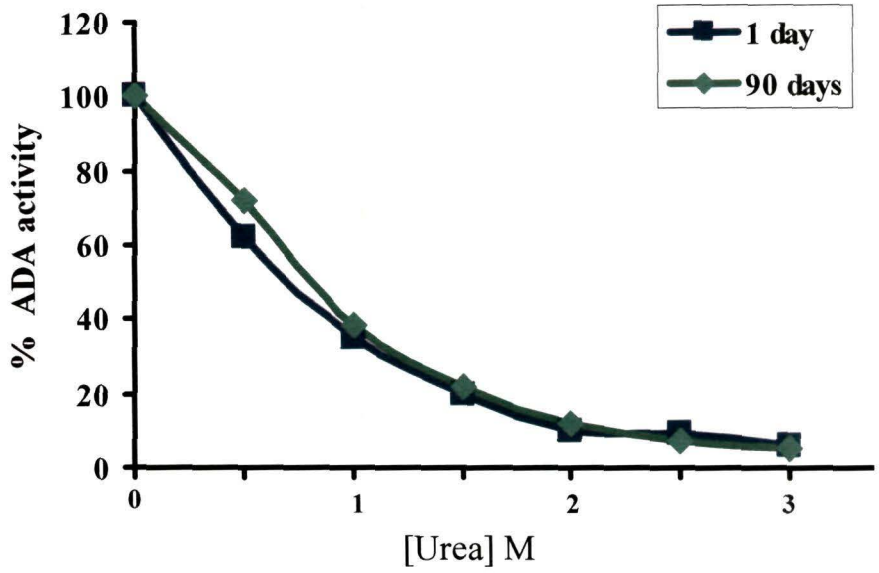


Fig. 73: Inactivation profile for both immature (1- day) and mature (90- day) purified chicken intestinal ADA at various concentrations of urea. Results are expressed as percentage of activity, taking no urea as 100 %.

3.11. STUDIES ON THE EFFECTS OF VARIOUS MODULATORS ON ADA

The effects of various modulators were studied on the activity of purified ADA of both ages. The activities of purified intestinal ADA from immature and mature chicken were studied in the presence of dithiothreitol (DTT), β-mercaptoethanol, 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Ca^{2+}, Mg^{2+}, Hg^{2+} and caffeine using normal ADA activity assay procedure.

The data showed that dithiothreitol, β-mercaptoethanol and DTNB all inhibited the activity of purified ADA albeit to a similar degree in both the immature and mature ages. The extent of inhibition by these sulfhydryl modifying agents was in the range of 25-35%. Among the divalent cations, Ca^{2+} was found to be less inhibitory (15%) to ADA followed by Mg^{2+}

4. Discussion

(50%) and then Hg^{2+} (88%). In addition to these, caffeine was also found to be inhibitory to ADA activity to the extent of 45% [Fig. 74].

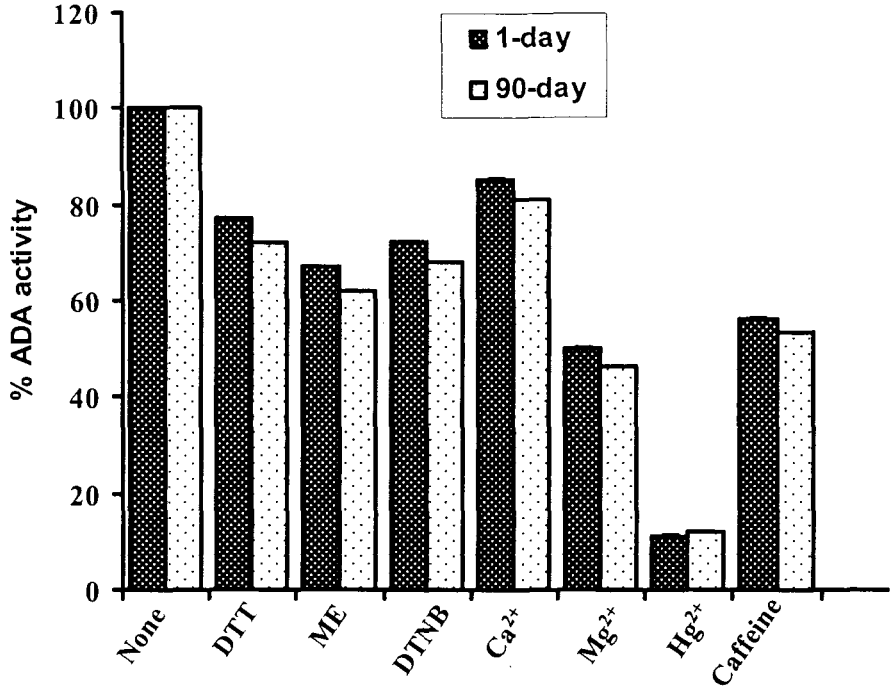


Fig. 74: Effects of various modulators on activity of purified ADA from the small intestine of immature (1-day) mature (90-day) chicken. DTT, ME, DTNB, Ca^{2+}, Mg^{2+} and Hg^{2+}indicate dithiothreitol, β-mercaptoethanol, 5, 5'-dithiobis-(2-nitrobenzoic acid), calcium, magnesium and mercury respectively. Results are expressed as percentage taking no modulators as 100%.

Gradual change in size, shape, and function during an organism's life that translates its genetic potentials (genotype) into functioning mature systems (phenotype) is known as development. Growth and development are an intricate control of various processes. In mammals, development begins with fertilization, the process by which the male gamete, the sperm cell, and the female gamete, the oocyte, fuse to give rise to a diploid cell, the zygote. In aves, the sperms enter the oviduct of the female and travel through the various parts of the reproductive organs to reach the infundibulum. Once inside the infundibulum, these sperm remain live for one week or more, waiting for the eggs, which undergo the process of formation. The eggs are formed as yolk, in the ovaries of the hen. The chicken genome has a haploid content of 1.2×10^9 base pairs (bp) of DNA organized on 38 autosomes plus the Z and W sex chromosomes. Development of an organism involves several biochemical and physiological changes (Kirkwood, 1977). For a meaningful interpretation and understanding of the phenomena of development and/or aging, studies on enzyme levels and its regulation by hormones and other metabolites require the necessity to interpret changes in enzyme activities correlating to, as well as encompassing physiological changes of the tissues and the organism.

Biochemical reactions are catalyzed by specific proteins called enzymes, which catalyze specific reactions. They are responsible for specific functions during the initiation, duration and termination of various phases of the lifespan of an organism. A considerable quantity of literature is available describing changes in the activities of enzymes during development and aging. Some detail reviews in the subject are available (Kirkwood, 1977; Sharma, 1988a). Adenosine, the substrate for the enzyme worked upon, plays an important neuromodulatory role in the central nervous system, and adenosine deaminase is an important enzyme in the degradation of adenine nucleotides (Bellé *et al.*, 2009). Adenosine deaminase catalyzes the irreversible deamination of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine. It has been found that adenosine deaminase deficiency is a rare inherited disorder of purine metabolism that leads to a form of severe combined immunodeficiency (SCID) (Resta and Thompson, 1997). If no treatment is provided, the condition is fatal in the first year of life and therefore requires early intervention (Donofrio *et al.*, 1978). Several other diseases are manifested due to an increase or decrease in the enzyme level and hence are of interest for the studies undertaken. The activity of ADA is subject to changes depending upon the degree of

activity of the cell, *i.e*, whether differentiation or proliferation occurs (Franco *et al.*, 1998). In experiments performed with thymocytes and peripheral T-lymphocyte subpopulations, it has been shown that the cellular content of ADA diminishes as maturation occurs (Ma *et al.*, 1982; Massaia *et al.*, 1982).

A deficiency in the gene encoding the major enzyme is lethal in mouse and *Drosophila* and leads to severe combined immunodeficiency (SCID) in human. SCID is characterized by gross impairment of both the humoral and cell-mediated immunity and also by susceptibility to overwhelming fungal, bacterial and viral infections. The syndrome comprises a heterogeneous group of primary immunodeficiencies associated with various defects of the immune system involving both T- and B-lymphocytes, and sometimes natural killer cells. ADA deficiency accounts for almost half of the autosomal recessive forms of SCIDs (Lim and Elenitoba-Johnson, 2004). Studies have also shown that mutations in the adenosine deaminase gene are responsible for a form of severe combined immunodeficiency (SCID) caused by the lymphotoxic accumulation of ADA substrates, adenosine and 2′-deoxyadenosine (Cassani *et al.*, 2008). In these organisms, enzyme deficiency causes increased adenosine/deoxyadenosine concentration in body fluids and some organs. Elevated levels of adenosine and deoxyadenosine are toxic to certain mammalian and insect cells, and it was shown for mouse and human that it is a primary cause of pathophysiological effects. Data suggest that the major role of adenosine deaminases in various taxa is the protection of tissues against increased levels of adenosine and deoxyadenosine (Dolezelova *et al.*, 2005).

Abnormalities of ADA have also been observed in other diseases of immune system, like acquired immunodeficiency syndrome (Yoshino *et al.*, 2010), lymphomas (Husain *et al.*, 2007), leukemias (Hussein and el-Belbessy, 1998; Sauter *et al.*, 2008), anemias, hemolysis-associated pulmonary hypertension (Tofovic *et al.*, 2009), hepatitis (Kaya *et al.*, 2007), focal epithelial hyperplasia (Heck's disease) (Artac *et al.*, 2009), short limbed dysplasia (Ramanan *et al.*, 2000), psoriasis (Erbagci *et al.*, 2006) and jaundice (Lepore *et al.*, 1989). Although the main function of ADA is the development of the immune system in humans, it seems to be associated with the differentiation of epithelial cells and monocytes, neurotransmission, and maintenance of gestation (Moriwaki *et al.*,1999). Though ADA is present universally in almost

all tissues, its activity varies markedly among different tissues within different species. The activity level is also different during the pre- and postnatal development of organisms. In aves, adenosine is a physiological signal in neuronal differentiation of the CNS-derived catecholaminergic CAD cell line and suggests that adenosine signaling is involved in neural crest cell development *in vivo*. CAD is a variant of Cath.a, a CNS cathecholaminergic cell line, which expresses neuronal properties but lacks neuronal morphology (Bilodeau *et al.*, 2005). Adenosine is also a metabolic regulator of renal blood flow, capable of decreasing glomerular filtration rate (GFR), exerting immunosuppressive, antiproliferative and anti-inflammatory properties (Kocic *et al.*, 2002). Studies relating to developmental changes of adenosine deaminase in chicken are limited. In chicken embryonic neurons, deficiency of ADA is associated with an accumulation of adenosine and generation of neurotoxicity (Wakade *et al.*, 1995; Zhao *et al.*, 1999), whereas an accumulation of 2'-deoxyadenosine causes cell death in embryonic chicken sympathetic ganglia and brain (Zhao *et al.*, 1999). The levels of adenosine and 2'-deoxyadenosine are controlled by adenosine deaminase that catalyzes the irreversible breakdown of these metabolites (Fox and Kelley, 1978). The physiological consequences of these metabolites are well documented in individuals genetically deficient in this enzyme (Hershfield and Mitchell, 1995). In normal individuals, ADA appears to be present in high amounts in lymphoid tissues to maintain low concentrations of adenosine and deoxyadenosine permitting better lymphocyte survival. Both B and T lymphocytes are extremely sensitive to adenosine and deoxyadenosine (Pollok *et al.*, 1998). Elevated levels of deoxyadenosine often lead to cytotoxicity resulting from interference with deoxynucleotide metabolism (Hershfield and Mitchell, 1995). Adenosine functions as an extracellular signal transducer mediating a vast array of biological effects by interacting with specific cell surface receptors coupled to adenylate cyclase system. These receptors (A_1/A_2) regulate adenylate cyclase by inhibiting or stimulating and influencing the intracellular level of cAMP, respectively.

In chicken, the ADA gene is located on chromosome 20 and is approximately 64 kb in length. Sp1 protein is essential for both enhancer-mediated and basal activation of ADA promoter (Yeung *et al.*, 1985), suggesting a housekeeping role of ADA (Dusing and Wiginton 1994). In chick embryo fibroblasts, v-*jun* oncoprotein downregulates a family of direct target genes by binding to the DNA indirectly through Sp1/3 (Chamboredon and Castellazzi, 2005).

Our interest in the tissue distribution of ADA in various postnatal ages of chicken, as well as to study the effect of hormones and metabolites on its activity was kindled by the plethora of reports on ADA in mammals but somewhat limited information of the enzyme in avian systems. For a meaningful interpretation and understanding of the phenomena of development and/or aging, studies on the level of enzyme and its regulation by various hormones and metabolites require the necessity to interpret changes in enzyme activities correlating to, as well as encompassing physiological changes of the tissues and the organism.

The present work includes:

i. The study on the activity level of ADA at different postnatal ages and their tissue-specific patterns
ii. Regulation of ADA by glucocorticoids, thyroid and sex hormones as well as Bt_2cAMP, a membrane permeable analog of cAMP
iii. Purification of ADA from small intestine of immature and mature chicken and
iv. Study of the physico-chemical and kinetic characteristics of ADA from immature and mature chicken

4.1. NORMAL ENDOGENOUS LEVEL OF ADENOSINE DEAMINASE

ADA activity in the male chicken exhibits tissue specificity as well as postnatal developmental changes. The normal endogenous level of ADA activity (U/mg protein) is shown in figures 5-14 for esophagus, crop, proventriculus, small intestine and spleen, respectively, at various postnatal ages. The data indicate the highest level of activity in small intestine, followed by proventriculus, esophagus, crop and low activity of ADA in spleen. Our findings reveal that the normal endogenous level of ADA in the GIT of chicken is highest on the day of hatching. Among the regions of the GIT studied, the highest level of ADA activity is found in the small intestine followed by the esophagus, proventriculus and crop. In the esophagus, the activity is highest in day 1 and is seen to decrease at day 10 and shows a slow decrease thereafter. In the crop, ADA activity is highest in day 1 showing a decrease at day 10

and then no decline in the activity. The proventriculus is also seen to have a very high level of ADA activity at day 1 which shows a significant decline at day 30 and 90. Region specific studies indicate that in the small intestine, the level of activity is highest in day 1 followed by a sharp decrease at day 10 and then remains almost constant thereafter. Unlike all the tissues of GIT, the activity of ADA in the spleen is seen to increase maximally at day 30 after which there was a decline.

In all the studies, our interest was to find out if there was a change in the level of the protein of the enzyme for which slot blots [Fig. 6 a (i), 8 a (i), 10 a (i), 12 a (i), 14 a (i)] and immunoblots [Fig. 6 b (i), 8 b(i), 10 b (i), 12 b (i), 14 b (i)] were done for the two ages where differences were observed in almost all cases. The antibody used for Western and slot blots of chicken ADA was of human origin; hence, it was necessary to find the cross reactivity of human anti-ADA antibody with chicken ADA. Using BLASTP 2.2.19+, the homology between human (acc. no. NP000013) and chicken (acc. no. Q5ZKP6) ADA was found to be 66% (Altschul *et al.*, 1997). The cross-reactivity of the antibody used to chicken ADA was found to be ~40% [Fig. 55]. Representative data of the blots shows that the level of the protein of ADA is indeed seen to change or remain constant wherever applicable. The relative intensities of the slots [Fig. 6 (c), 8 (c), 10 (c), 12 (c), 14 (c)] were plotted using densitomertric analysis by using the KDS-1 software.

The avian gastrointestinal mucosal immune system has evolved with specialized features that reflect their role as the first line of defense on mucosal surfaces (Hyun *et al.*, 1996). High level of ADA on the day of hatching may ensure lower adenosine and better survival of lymphoid cells. The presence of high level of ADA activity in the upper alimentary canal probably serves a catabolic role to guard the chicken against dietary sources of adenosine which could exert unwanted physiologic effects. The newly hatched chicken is thus well disposed to face the onslaught of high sources of adenosine which accumulate during development. As both adenosine and 2'- deoxyadenosine are lymphotoxic, highest levels of ADA in the small intestine may maintain low cellular concentration of these metabolites and assist a better survival milieu for lymphocytes. The Western blot analyses of ADA protein at two selected ages of day 1 and 10 corroborate the activity decline at day 10 of ADA compared

to day 1 except proventriculus. Such findings clearly show that the higher activity of ADA level at day 1 is because of its protein level. It has earlier been reported that newly hatched chicken showed a significantly higher ADA activity in bursa tissue regardless of whether ADA activity is expressed per mg of wet tissue, per 10^9 cells or per mg tissue or cell protein (Ratech *et al.*, 1980).

It has been found that the murine gastrointestinal mucosa is heavily populated with dispersed aggregates of non-encapsulated lymphoid tissues, which mediate local immune responses at the mucosal surface and that the intestinal epithelium can serve as extra-thymic T-cell generative organ and may influence T-cell receptor (TCR) selection (Poussier *et al.*, 1992). As adenosine and 2'-deoxyadenosine are both lymphotoxic, highest level of ADA in these tissues, probably ensures a low level of these metabolites in these tissues, which thus serves a better survival milieu for lymphocytes. ADA may also have a nonimmunological role in the gastrointestinal physiology, where the presence of high levels of ADA in the gastrointestinal tract may shield the chicken against unwanted physiological effects of adenosine and 2'-deoxyadenosine, which have been seen to exert a broad range of potent physiologic effects (Chinsky *et al.*, 1990). This role has also been supported by previous studies in murine system, where it was shown that labeled nucleic acids fed to mice are broken down rapidly to nucleosides which are further catabolized in the intestine and liver. However, since we found no activity of ADA in liver, it is presumed that dietary adenosine is transported from the liver rather than being catalyzed by ADA. A previous study has shown that liver reserves adenosine and diverts it to other tissues (Pritchard *et al.*, 1975). A high ADA activity level during early postnatal development of chicken probably helps the tissues to cope with the increasing need for immunological competence, thus decreasing adenosine, which could otherwise exert unwanted physiologic effects.

4.2. HORMONAL REGULATION

The level of enzymes and their inducibility by certain hormones are age-related phenomena (Wilson, 1973; Sharma, 1988a). A lot of interest focusing on the mechanism of hormone regulation involving feedback control, synergistic, reciprocal interactions and cross-

talk in signal transduction has appeared in a number of reviews (Wada *et al.*, 1990; Sharma, 1993). However, a change in the activity might not be directly equated with a change in enzyme concentration and a number of other possible mechanisms affect enzyme induction and/or repression (Sharma, 1994).

4.2.1. *Effect of corticosterone*:

Our studies show that corticosterone significantly inhibits the ADA activity in all the regions of GIT except proventriculus, in an age- and region- specific manner (Fig. 15-24). In the esophagus, corticosterone significantly decreases the activity of ADA at day 10 (-23%), but more at day 60 (-41%). In the crop, corticosterone decreases the activity of ADA more at day 60 (-79%) than day 10 (-56%). In the proventriculus, corticosterone does not show any significant decrease at either of the two ages studied. In the small intestine, corticosterone decreases the activity of ADA at both day 10 (-36%) and day 60 (-41%). In the spleen, corticosterone decreases the activity of ADA at both day 10 (-32%) and day 60 (-40%). In all the tissues, the magnitude of inhibition is more pronounced at the later stage (day 60) of development in comparison to the younger age (day 10). Corticosterone inhibition of ADA activity may be correlated with the greater accumulation of adenosine and 2'- deoxyadenosine, which cause lymphotoxicity, suppressing immune responses (Mei *et al.*, 2002; Priebe and Nelson, 1991). Corticosteroids have potent immunosuppressive and anti-inflammatory effects. Although corticosteroids are an important weapon in the clinical arsenal for treating inflammatory episodes, the mechanisms underlying the actions and regulation of endogenous corticosteroids remain obscure. Susceptibility to diseases is more complex and requires the involvement of more than a single parameter (Harbuz *et al.*, 2003). Since corticosterone is immunosuppressive, it may be acting through the inhibitory action of ADA activity, leading to an accumulation of adenosine and 2'-deoxyadenosine, producing lymphotoxicity, leading to an immunosuppressive action (Singh and Sharma, 1995). The inhibition of ADA activity level by corticosterone was also ascertained using slot and Western blots analyses which confirmed the inhibition of ADA activity at ADA protein level. The immunosuppressive actions of corticosterone may thus control the host's immune response to a great extent (Lim *et al.*, 2007).

ADA converts adenosine to non- toxic inosine, protecting lymphoid cells against the cytotoxic effects of adenosine (Carson and Seegmiller, 1976).

As reported earlier, corticosterone inhibited ADA activity in a tissue-specific manner. The magnitude of inhibition was more pronounced at the later stage of chicken development (60-day) compared to a very young age (10-day) (Bhattacharjee and Sharma, 2009). This was comparable to the mice system, where previous work in our laboratory also demonstrated a greater inhibition of ADA activity at the later stages of mouse development (Singh and Sharma, 1995). The findings of age- and tissue-specific inhibition may be correlated to the differential adaptive role and maturation of corticosterone action mechanism, its receptor and post-receptor events (Kalimi *et al.*, 1988; Borbhuiya and Sharma, 1995). Pronounced inhibition at a later stage may be attributed to a greater maturation of corticosterone receptors, thus facilitating greater binding of the hormone to its receptor. Corticosterone is known to act by binding to specific intracellular receptors and by interaction of these hormone-receptor complexes to specific DNA sequences, called hormone responsive elements (HREs), thereby modulating the transcription of specific gene(s) (Yamamoto, 1985; Tsai and O'Malley, 1994). ADA level may be post-transcriptionally modified by corticosterone, and this possibility cannot be ruled out. If corticosterone inhibition is factual, then the removal of the endogenous source of corticosterone should be able to increase the level of ADA. Previously, in our laboratory, it has been shown that by adrenalectomy, i.e., by removal of the endogenous source of corticosterone, there indeed is an enhancement of the ADA level in mice. Thus, it proved that ADA inhibition is possibly under the tonic influence of circulating corticosterone (Singh and Sharma, 1995). The effect of single and long-term hydrocortisone intramuscular administration on adenosine deaminase activities in homogenate and subcellular fractions of the rat brain hypothalamus and hippocampus has been shown to undergo changes with the duration of hydrocortisone administration (Kononenko *et al.*, 1990). The inhibition of ADA activity level may result in significant attenuation of intestinal inflammation by corticosterone (Antonioli *et al.*, 2007), justifying its anti-inflammatory role other than the established mode via inhibiting production of inflammatory agents. These findings have also been supported by reports on suppression of ADA activity and its gene expression by dexamethasone, a synthetic glucocorticoid, in human

leukemic cells (Fernández-Mejia *et al.*, 1994) and also by tacrolimus, which is an antibiotic macrolide with immunosuppressant properties suppressing ADA activity (Pereira *et al.*, 2006).

4.2.2. *Effect of dibutyryl cAMP (Bt_2cAMP)*:

In search of a positive modulator of ADA, Bt_2cAMP, which is a membrane permeable analog of cAMP, was found to increase the activity of ADA in all regions of GIT studied except crop in an age- and region- specific manner. In the esophagus, dibutyryl cAMP significantly increases the activity of ADA at both ages, but more at day 60 (+67%) in comparison to day 10 (+42%). In the crop, dibutyryl cAMP is seen to have no significant effect at both day 10 and day 60. In the proventriculus, it increases the activity of ADA at both day 10 and day 60, but the magnitude of increase is more in day 60 (+29%) than day 10 (+21%). Similarly, Bt_2cAMP induces the activity of ADA in the small intestine at both the ages studied, but increases the activity more at a later stage of development, day 60 (+58 %) than at the young age, day 10 (+52%). Like the tissues of GIT, in the spleen too, Bt_2cAMP increases the activity of ADA at day 60 (+46%) but unlike the tissues of GIT, there was no effect at day 10. The induction of ADA activity in the GIT, by Bt_2cAMP, has been published (Bhattacharjee and Sharma, 2009). This is in agreement with the previous studies conducted on mice, which showed a stimulatory effect of Bt_2cAMP on ADA activity (Singh and Sharma, 1995). In the GIT, the activity level of ADA was found to be greatly enhanced at a later age (day 60) of chicken development when compared to the young age (day 10). This may be because of the differential expression of secondary messenger cascade at the later stage of GIT development. Bt_2cAMP is a well known second messenger for various protein and peptide hormones (Adashi and Resnick, 1986; Won *et al.*, 2004). The substrate adenosine influences the intracellular concentration of cAMP (Dalziel and Westfall, 1994). Adenosine receptors can be divided into major classes designated A_1, A_{2A}, A_{2B} and A_3, (Palmer and Stiles, 1995). These receptors are widely distributed throughout the organism's body. They are involved in many central and peripheral processes, including immunological and inflammatory responses (Marx *et al.*, 2001). Adenosine may inhibit or stimulate the amplifier enzyme, adenylate cyclase, depending upon the type of receptor adenosine binds to at a particular time. A_1 receptor inhibits adenylyl cyclase by interaction with an inhibitory GTP- binding protein G_i, while A_3 inhibits adenylyl

cyclase through $G_{i/o}$, whereas the A_2 receptor acts via stimulatory G_s to activate the enzyme. The findings reveal that adenosine may control its own intracellular level through cAMP that may induce the activity of ADA which might lower the increased level of adenosine and avoid the unwanted toxic effects of adenosine (Mohamedali *et al.*, 1993; Bhattacharjee and Sharma, 2009). As discussed earlier, deficiency of ADA leads to an accumulation of adenosine and 2'-deoxyadenosine. The pathogenesis of the immuno-insufficiency can be traced back to disturbances in the purine metabolism interfering with the mitogenically induced lymphocyte transformation and other lymphocyte functions, as determined by *in vitro* tests. Deoxyadenosine inhibits the ribonucleoside diphosphate reductase and synthesis of DNA. It also inhibits the enzyme S-adenosylhomocysteine (SAH) hydrolase which in turn leads to elevated levels of SAH that further inhibits methyltransferase reaction in which S-adenosylmethionine (SAM) acts as methyl group donor, thereby inhibiting DNA synthesis (Müller, 1983). cAMP, a second messenger historically viewed as a negative mediator of immune responses, was demonstrated to possess immunoenhancing activity (Koh *et al.*, 1995). Thus, cAMP increases the immune responses by stimulating ADA activity, lowering the level of adenosine and 2'-deoxyadenosine, to ensure better survival of lymphocytes (Singh and Sharma, 1995; Bhattacharjee and Sharma, 2009). Our findings are supported by the report of stimulating ADA of lymphoid organs by administration of caffeine, a phosphodiesterase inhibitor, enhancing cAMP levels (Bandyopadhyay and Poddar, 1994).

The immunoinducing role of cAMP may be because of stimulating activity of ADA, thereby decreasing the intracellular concentration of adenosine, ensuring a better environment for lymphocyte proliferation. The effects of cAMP are exerted by activating the enzyme cAMP-dependent protein kinase or by interacting with cAMP response element (CRE) of genes. The transcription factor cAMP response element-binding protein (CREB) binds to a DNA sequence known as CRE. The CREB is activated through phosphorylation by protein kinase A (PKA), but precisely how phosphorylation stimulates CREB function is unknown (Kwok *et al.*, 1994). The possibility of post-transcriptional and/or translational regulation of ADA by cAMP cannot be ruled out. Such induction of ADA activity level by Bt_2cAMP was also ascertained using slot and Western blots analyses that confirmed that ADA activity was induced at the protein level.

Bt_2cAMP increases the activity of ADA in almost all the tissues studied, except crop. We found that the activity was increases more at day 60 compared to day 10 (immature age). This finding is similar to the studies in mice system, conducted previously in our laboratory, where, the magnitude of stimulation was more pronounced at later stage of development. This age-specific Bt_2cAMP stimulation of ADA activity may be attributed to the differential expression of secondary messenger cascade at the later stage of development (Christoffersen *et al.*, 1973; Di Marco *et al.*, 1978).

4.2.3. *Effect of 3, 5, 3'-triiodothyronine (T_3)*:

It is well known that growth in poultry is regulated by thyroidal hormones. Extracellular adenosine inhibits DNA synthesis induced by TSH in FRTL-5 thyroid cells through the A_1 adenosine receptor-Gi system (Sho *et al.*, 1999). TSH promotes G_1 cyclin expression by inducing cAMP production. This cyclin expression then triggers cell cycle progression from the G_0/G_1 to the S phase. It is shown to increase adipose-tissue adenosine contents (Ohisalo *et al.*, 1987). Thyroid hormone is known to increase biogenesis, a response that is likely to enhance the ability of the cell to generate energy to meet associated increased metabolic demands (Hood *et al.*, 2000). Prompted by these findings, we studied the effect of T_3 on ADA activity in different tissues of chicken from two select ages day 10 and 60. However, in our course of study, there was no significant effect of T_3 on the activity of adenosine deaminase in the tissues studied (Fig. 35-44). The slot/Western blots also did not show any change in the level of the enzyme. This may be because of the physiology of humans and chicken being different, a difference in the receptor and post receptor events or the lack of the regulatory elements of thyroid hormone in the chicken ADA gene.

4.2.4. *Effect of testosterone*:

Studies indicate that sex steroids can influence the immune system. Self-administration of testosterone induced corticosterone levels in sham-operated rats (Macció *et al.*, 2005). It has been shown that corticosterone inhibited the ADA activity in the tissues studied at the two

postnatal ages (Bhattacharjee and Sharma, 2009). These results suggest that testosterone could modulate specific cellular immune responses and serum corticosterone levels leading to changes in the ADA level. Previous literature revealed that in humans, an aqueous extract of *Urtica dioica* increased testosterone which was capable of inhibiting ADA activity, used as one of the mechanisms in treating prostrate cancer patients. Investigation of possible effects of aqueous extract of *Urtica dioica* leaves on adenosine deaminase activity in prostate tissue from patients with prostate cancer revealed a significant inhibition on ADA activity of prostate tissue (Durak *et al.*, 2004). Thus, our interest arose in finding out the effect of testosterone on ADA activity in different tissues during postnatal development of chicken. To our surprise, no significant decrease in the activity level of ADA was produced in the tissues studied at the two postnatal ages studied. We postulate that there is no change observed probably due to the fact that the physiology of humans and chicken are different and there could be a difference in the sex steroid receptor and/or post-receptor events. There also could be a lack of the regulatory elements of testosterone in the ADA gene of chicken. The slot and Western blots analyses performed also indicated no change in the activity at even the protein level.

Thus, our studies concluded that the level of ADA is highest on the day of hatching and that the level is negatively and positively controlled by corticosterone and Bt_2cAMP in various regions of GIT, albeit more pronounced at later postnatal ages, depending on the maturation of steroidal and protein/peptide hormone signaling cascade during postnatal development of chicken for better metabolic adjustments in growing animals. Thyroid hormone and testosterone do not seem to have any significant effect on the activity of ADA at the two ages studied in the different tissues of chicken.

4.3. PURIFICATION AND PHYSICOCHEMICAL CHARACTERIZATION

Intestinal ADA from immature (1-day) and mature (90-day) chicken was purified by using identical procedures. ADA preparations from both the ages were passed through sephadex G-100 gel filtration and DEAE-cellulose column. The elution profiles indicated that both ADA, from immature and mature chicken, have similar molecular weights and ionic net charges. The purified preparations of ADA from the two ages were used to study their kinetic

properties using various substrates, inhibitors and ADA modulators, as well as their physicochemical properties to investigate any change as a function of age.

Purification and characterization of ADA has been done from different sources. From mammals, ADA has been purified from a number of organisms and from different tissues. Adenosine deaminase was purified from skeletal muscle of camel (*Camelus Dormedarius*) to homogeneity by using DEAE-Sephadex chromatography, ammonium sulfate precipitation, gel filtration and ion exchange chromatography (Alrokayan, 2002). Adenosine deaminase from the white and gray matter of the large hemispheres, cerebellum, medulla oblongata and pituitary anterior lobe has been isolated and purified from cattle brain (Sharoian *et al.*, 1994). Bovine brain adenosine deaminase was purified about 450 fold by salt fractionation, column chromatography on DEAE-cellulose (Lupidi *et al.*, 1992). Affinity chromatography has been used to purify adenosine deaminase from various sources: calf spleen, calf intestinal mucosa, chicken duodena and human erythrocytes (Rossi *et al.*, 1975). A double-stranded RNA-specific adenosine deaminase, which converts adenosine to inosine, has been purified to homogeneity from calf thymus. The enzyme was purified approximately $34x10^4$-fold by a series of column chromatography steps (O'Connell and Keller, 1994). Human erythrocyte adenosine deaminase has been purified approximately $8x10^4$-fold to apparent homogeneity using antibody affinity chromatography (Daddona and Kelley, 1977). Various forms of the cytosolic ADA have been identified in human tissues with different molecular weights (Van der Weyden and Kelley, 1976). Gel filtration of human thyroid extract with Sephadex G-200 revealed two molecular forms of adenosine deaminase differing in their molecular sizes (Jaroszewicz and Kowalczyk, 1995).

ADA has also been purified from other sources. It has been purified and characterized from *Penicillium politans* (Elshafei *et al.*, 2007). Adenosine deaminase was purified from *F. gigantica* through acetone precipitation and chromatography on CM-cellulose. Two forms of enzyme (ADAI, ADAII) were separated. ADAII was purified to homogeneity after chromatography on Sephacryl S-200 (Ali, 2008). Purification and characterisation of extracellular adenosine deaminase from *Streptomyces sp.* has been done (Jun *et al.*, 1991). The enzyme was purified from *Klebsiella sp.* to the homogeneous state by polyacrylamide gel

electrophoresis (Ling *et al.*, 1990). The protein binds with high affinity to a non-DNA conformation, which is most likely to the Z-DNA. The protein also has a binding site for double-stranded RNA (dsRNA). Peptide sequences from this protein show similarity to dsRNA adenosine deaminase, an enzyme that deaminates adenosine in dsRNA to form inosine (Herbert *et al.*, 1995). An extracellular adenosine deaminase was isolated from the culture supernatant of *Nocardioides sp.* J-326TK and purified 193-fold to homogeneity. It was a monomeric protein as judged by SDS/ PAGE (Jun *et al.*, 1994). ADA has also been purified from *Pseudomonas iodinum* (Sakai and Jun,1978).

Relatively little work has been done on the purification of the enzyme from the avian system. Nevertheless, it has been purified from a few different tissues as well as serum of chicken. Adenosine deaminase has been purified from egg yolk of chicken (Lopez *et al.*, 1990). Adenosine deaminase 1 (ADA1) has been purified from human and chicken liver. In humans, ADA1 was mainly purified concomitant with ADA-binding protein, dipeptidyl peptidase IV (DPP IV)/CD26 but, in chicken, only ADA1 without DPP IV was purified (Iwaki-Egawa and Watanabe, 2002). ADA activity is composed of two kinetically distinct isozymes, which are referred to as ADA1 and ADA2. ADA1 is widely distributed in many animals and well characterized. On the contrary, relatively little is known about ADA2, but ADA2 has been purified to homogeneity from chicken liver (Iwaki-Egawa *et al.*, 2004).

Our purified ADA from small intestine of immature (1-day) and mature (90-day) chicken showed a single activity peak by both gel filtration and DEAE-cellulose column, indicative of a single form of intestinal ADA from the two different ages of chicken, whose molecular weights and net charges were similar. The degree of purification achieved was 503 and 569 fold, with a yield of 4% and 5% respectively, for the two ages (day 1 and 90) of chicken. The results of purification have been summarized in tables 27 and 28. Gel filtration analysis of the specific activities of the enzyme of the two ages gave a single peak for ADA, demonstrative of a single form of the enzyme. Elution profile of the specific activities of this enzyme from the small intestine of both the groups exhibited the requirement of a similar ionic strength (0.2 M) of NaCl buffer. This indicated that there was no difference in the charge content of the enzyme molecule between the two age groups.

Using non-denaturing polyacrylamide gel electrophoresis (Fig. 60), the preparations of the purified enzyme from the small intestine of chicken of the two ages were electrophoresed. The gel was then stained with Coomassie Brilliant Blue (CBB). Staining of the gel after electrophoresis showed the presence of a single band, whose relative mobility was similar, exhibiting ADA in both the groups. This indicated that our ADA was purified to homogeneity and ADA of both ages possessed identical charges. The purified ADA preparations also gave a single band on SDS-PAGE (Fig. 61) with a molecular weight of 100 kDa. Thus, we conclude that the ADA purified does not have any subunits and is a monomeric protein. When Western blot (Fig. 62) was performed at the end of SDS-PAGE, it gave immunoreactivity to ADA antibody corresponding to the band obtained by SDS-PAGE. This further confirms our results of SDS-PAGE, and proves that the ADA is monomeric in nature. Gel filtration analysis of the purified ADA exhibited a molecular weight of both ADA as 100 kDa. This finding was consistent with the report of chicken liver ADA whose molecular weight was 100 kDa (Ratech *et al.*, 1981). The ADA from blood plasma of chicken also revealed a molecular weight of 100 kDa (De Boeck *et al.*, 1975).

These findings entail that intestinal ADA from immature and mature chicken is a single subunit structure with same molecular weight (100 kDa). The observation of this molecular weight of ADA was also observed in ADA2 which was purified to homogeneity from chicken liver, and the purified enzyme had a molecular mass of approximately 110 kDa on gel filtration (Iwaki-Egawa *et al.*, 2004).

4.3.1 *Kinetic Characterization:*

Kinetic studies on the purified ADA from the small intestine of both age groups were carried out to elucidate changes, if any, which might occur in the active site of the enzyme molecule during development. ADA from both ages (1-day and 90-day) showed a hyperbolic curve when the velocity was plotted against varying concentrations of both the substrates, adenosine and 2'-deoxyadenosine by using enzfitter programme (Perrella, 1988). The figures were drawn using Michaelis-Menten equation and Lineweaver-Burk transformation. From the

plots, it indicates that both the substrates do not exhibit allosteric effects on the enzyme activity. From the Michaelis-Menten equation and Lineweaver-Burk transformation, both the ADA show similar K_m for adenosine of 33.3 μM and 34.2 μM, respectively, for 1-day and 90-day old chicken. The computed K_m values of the immature and mature small intestinal ADA for 2'-deoxyadenosine are 14.3 μM and 14.3 μM, respectively. Analyses of the data indicated that there is no age-related difference in the affinity of ADA for adenosine as well as 2'-deoxyadenosine. However, both ADA from immature and mature chicken show more affinity towards 2'-deoxyadenosine than adenosine, indicating that 2'-deoxyadenosine is a better natural substrate, i.e., a lower concentration of 2'-deoxyadenosine is required to reach a similar reaction velocity. Thus, the higher catalytic activity of ADA for 2'-deoxyadenosine may help faster deamination of this metabolite since it contributes more to lymphotoxicity than adenosine (Blackburn *et al.*, 1996; Gakis *et al.*, 1998). Analysis of data indicates no significant difference between K_m, V_{max} and K_{cat} values of the enzyme for both substrates in the two age groups. There is also no significant difference observed in the K_i of immature and mature ADA for purine riboside. Purine riboside lacks a leaving group at C-6 and is an unreactive adenosine analogue, which is a potent reversible inhibitor of ADA (Kurz and Frieden, 1987). Hence, analyses of the data indicate that purine riboside is a strong competitive inhibitor of both ADA with a similar K_i of 6 μM and 7 μM, respectively. These values are quite similar to earlier reports on mice system (Mohamedali *et al.*, 1993; Sideraki *et al.*, 1996).

4.3.2. *ADA modulation studies:*

The pH stability studies indicate that ADA from immature and mature chicken is most stable in the broad range of pH from 5.5 to 8.0, after which slight instability started in both the ADA. Thus, it implies that the salt bridge contributed to both immature and mature ADA by the same degree. When the biochemical properties of adenosine deamination in soluble and membrane fractions of zebrafish (*Danio rerio*) brain were studied, the optimum pH for ADA activity was in the range of 6.0-7.0 in soluble fraction and reached 5.0 in brain membranes (Rosemberg *et al.*, 2008). This observation was further confirmed by the studies of urea denaturation where both the ADA requires almost the same concentration of urea to achieve 50% inactivation. Urea is a powerful protein denaturant as it disrupts the noncovalent bonds in

the proteins. The inactivation in the alkaline or in the urea solution could be derived from a slight deformation of the active site which could not hold the entire activity due to a change in the molecular form at sites distant from the active site, whereas, acidic pH was capable of producing a direct change in or around the active site. Earlier reports suggested that urea caused sequential change from native to intermediate topologies on the unfolding pathway (Shu and Frieden, 2004). Hence, it can be concluded that changes, if any, contributed by alkaline pH in the molecular form does not affect the active site of ADA as affected directly by acidic pH. We also conclude that changes in the molecular form of ADA caused by urea inactivation affect the active site of ADA to a large extent.

When purified ADA from immature and mature chicken was assayed at different temperatures, we observed that the enzyme was stable till 45°C, after which a drop in the activity was seen in both the ages, with almost all activity lost at 70°C. This finding was in line with purified adenosine deaminase from camel tick *Hyalomma dromedarii* (Mohamed, 2006). After 45°C, the rate of reaction started to decrease. This was because the increase in temperature after this did not increase the kinetic energy of the enzyme but instead disrupted the forces maintaining the shape of the molecule. The enzyme molecules were gradually denatured causing the shape of the active site to change. Temperatures above 65°C completely denatured the enzyme.

The effects of various modulators were studied on purified ADA of both ages. The activities of purified ADA from intestinal immature and mature chicken were studied in the presence of dithiothreitol (DTT), β-mercaptoethanol, 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Ca^{2+}, Mg^{2+}, Hg^{2+} and caffeine using normal ADA activity assay procedure. The data showed that DTT, β-mercaptoethanol, DTNB all inhibit the activity of purified ADA albeit to a similar degree in both the immature and mature ages. The extent of inhibition by these sulfhydryl modifying agents is in the range of 25-35%. Among the divalent cations, Ca^{2+} is found to be less inhibitory (15%) to ADA followed by Mg^{2+} (50%) and the Hg^{2+} (88%). In addition to these, caffeine is also found to be inhibitory to ADA activity to the extent of 45% (Fig. 74).

DTT is used to reduce the disulphide bonds of proteins and prevents intra- and intermolecular disulphide bonds between cysteine residues. In our experiments, DTT is inhibitory to the enzyme probably by reducing the disulphide bonds between active site cysteine residues. Our finding was in line with previous work done on ADA of chicken serum (Rokosu, 1983). β-Mercaptoethanol is observed to reduce the activity of the enzyme of both ages. β-Mercaptoethanol is also a well known denaturant which cleaves disulphide bonds, and adenosine deaminase is inhibited by thiol-modifying agents (Rokosu, 1983). Literature revealed that β-Mercaptoethanol in high concentrations inhibited the enzyme (Maguire and Meng, 2004). DTNB is used for the modification of free thiol groups in proteins. It rapidly forms disulphide bonds with the thiol and releases a thioate ion. Since the active site of adenosine deaminase has cysteine, DTNB may react with it and lead to a negative modification of the enzyme. Previous work on the effect of DTNB on ADA also revealed similar results (Rokosu, 1983). Divalent cations are electrophilic in nature and bind to the nucleophilic centres of the enzyme and modify its activity. For murine adenosine deaminase, metal ions bound at an additional site(s) have been found to inhibit the enzyme (Cooper *et al.*, 1997). Similarly, our studies show an inhibition of the activity of the enzyme by divalent cations to varying degrees. The maximum inhibition of enzyme activity is produced by Ca^{2+}, followed by Mg^{2+} and then Hg^{2+}. Mg^{2+} is an allosteric regulator of 5'-nucleotidase. It acts as a cofactor for various enzymes, a very important one being 5'-nucleotidase. While interacting with the enzyme, Mg^{2+} may bind using inner/outer space coordination, to either alter the conformation of the enzyme or take part in the chemical reaction (Cowan, 2002). Hg^{2+} modifies the enzyme activity through oxidation of thiol groups. It is a non competitive inhibitor of the enzyme. These findings have been found to be consistent with previous work done on the enzyme from different sources (Piggott and Brady, 1976; Ali 2008; Bellé *et al.*, 2009), as well as by previous report from our laboratory (Singh and Sharma, 2000). We find caffeine to also be inhibitory to the enzyme of both ages. Caffeine being structurally similar to adenosine may act as a competitive inhibitor (Fisone *et al.*, 2004). Also, like other methylated xanthines, caffeine is both a competitive nonselective phosphodiesterase inhibitor (Essayan, 2001) which raises intracellular cAMP, activates PKA, inhibits TNF-alpha (Deree *et al.*, 2008) and leukotriene (Peters-Golden *et al.*, 2005) synthesis, and reduces inflammation and innate immunity (Peters-Golden *et al.*, 2005).

For these reasons, caffeine probably was inhibitory to the purified enzyme, as was found previously in our laboratory in the mouse model too (Singh and Sharma, 2000).

From the findings embodied in this thesis, it is concluded that:

- ADA activity and its level expresses in a tissue- and age- specific manner during postnatal development of chicken to ensure its better suited physiological roles.
- Corticosterone inhibits ADA activity and shows a tissue- and age- specific pattern, indicating that ADA is under tonic inhibition by circulating corticosterone, correlating a differental adaptive role and maturation of corticosterone action mechanism, its receptor and post-receptor events.
- Bt_2cAMP is found to be stimulatory to the activity of ADA, thereby exhibiting immunoinducing role of cAMP.
- T_3 and testosterone do not produce any significant changes in ADA activity.
- Physicochemical and kinetic properties of purified ADA remain the same at both the immature and mature ages indicating that there is no alteration in these properties as a function of age in chicken during postnatal development.

These studies besides providing insight into the basic role of ADA during postnatal development of chicken also pave the way for using corticosterone and Bt_2cAMP in inhibiting and inducing, respectively, the activity of ADA in various ADA-related diseases as stated earlier.

5. Summary

Development is a continuous process by which organisms grow. The process of development onsets as soon as the fertilized egg begins to grow. It may be divided into two main periods, prenatal and postnatal. The prenatal development involves differentiation and encompasses three main stages- ovum, embryonic and fetal stages. Postnatal development begins with birth and continues into neonatal, infancy, childhood, adolescence and adulthood. Differentiation and development are programmed processes which occur due to sequential activation and repression of genes causing alterations in the levels of enzymes. The activities of several enzymes decrease and of several others increase as a function of age of an organism. Age leads to alterations in the levels of enzymes and their inducibility by certain hormones. The rate of synthesis of enzymes usually changes in response to changes in the extracellular environment. The regulatory mechanisms which control the level of enzymes at the biochemical level involve a change in the rate of synthesis and/or degradation and post-translational modifications. A number of hormones and growth factors exert an intricate but judicious control on the process of development in aves. The divergent morphology and physiology observed during development with many distinct complex and coordinated processes involve the control of the programmed circuits of gene expression. Organisms have certain genes which control specific events during development. Gene expression in organisms is regulated at essentially all possible levels- transcription, pre-mRNA processing, mRNA transport, mRNA stability, translation, and post-translational protein processing. The regulatory mechanisms with largest effects on phenotype have been shown to act at the levels of transcription and mRNA processing.

Adenosine deaminase (ADA; EC 3.5.4.4), a key enzyme participating in the purine salvage pathway, catalyzes the irreversible hydrolytic deamination of the substrates adenosine and 2'-deoxyadenosine to yield the products inosine and 2'-deoxyinosine, respectively. ADA is a well-characterized enzyme involved in the depletion of adenosine. It is essentially required for lymphocyte proliferation and differentiation. The physiological function of ADA is critical for controlling the levels of adenosine and 2'-deoxyadenosine in immunological, neurological and cardiovascular systems. ADA activity is widely distributed in human tissues and is highest in lymphoid tissues. In humans, rats and mice, the highest level of enzyme activity is found in

thymus, spleen, placenta, and in organs comprising the gastrointestinal tract, whereas low activity is found in muscle, lung and kidneys.

A correlation has been drawn with the failure of both B- and T-lymphocytes mediated functions due to deficiency of ADA because of different missense mutations in exon 4 of its gene. The absence of ADA in humans results in severe combined immunodeficiency (SCID), which is characterized by hypoplastic thymus, T lymphocyte depletion and autoimmunity. Abnormalities of this enzyme have also been reported in other diseases of immune system including AIDS, lymphomas, leukemias, anemia and several other unrelated disorders like short-limbed dwarfism, hepatitis and jaundice. Increased serum ADA activity has been reported in patients with liver diseases like chronic hepatitis and liver cirrhosis.

Various avian species provide models of evolution, development and differentiation, behaviour and ecology. Avian genomes appear to show relatively high levels of conservation, and the genome sequence and related information that are available for chicken provide added benefits for the genetic analysis of all wild and domestic birds. The immune system of chicken has the thymus, a paired lobulated gland along the neck of the chicken which is considered to be the source of T cells or small lymphocytes which mediate the rejection of hemografts, graft-virus-host reaction and delayed hypersensitivity. They also have the bursa of fabricus, an organ situated dorsally to the cloaca which is considered to be the source of B cells which form plasma cells and produce antibody. In chicken, the gene for ADA is located on chromosome 20 and is approximately 64 kb in length. Sp1 protein is essential for both enhancer-mediated and basal activation of ADA promoter.

Keeping in mind the role of ADA in the regulation of a wide array of physiological processes, the present work embodied in this thesis has been directed towards the following:

- To determine the normal endogenous activity level of ADA in different tissues of GIT (esophagus, crop, proventriculus, small intestine) and spleen at various postnatal ages (day 1, 10, 30, 60 and 90) of male chicken.

- To assess the role of hormones and their analogues like corticosterone (adrenalcortical hormone), testosterone (sex hormone), triiodothyronine (thyroid hormone) and a membrane permeable analog of cAMP, dibutyryl cAMP, on the activity of adenosine deaminase in different tissues of chicken at two specific postnatal ages (day 10 and 60) ascertained, and to postulate the effect of these hormones, if any, in modulation of ADA in a tissue- and age- specific pattern.

- To purify ADA from small intestine of two select age groups (day 1 and 90) of chicken and characterize its physicochemical and kinetic properties, with a view to analyze the alterations, if any, in these parameters of the enzyme during the course of development.

5.1. NORMAL ENDOGENOUS LEVEL OF ADA

ADA activity in the male chicken exhibits tissue specificity as well as age-related changes. The normal endogenous level of ADA activity (U/mg protein) has been ascertained for esophagus, crop, proventriculus, small intestine and spleen at various postnatal ages. The data indicate the highest level of activity in small intestine, followed by proventriculus, esophagus, crop and low activity of ADA in spleen. Our findings reveal that the normal endogenous level of ADA in the GIT of chicken is highest on the day of hatching. Amongst the regions of the GIT studied, the highest level of ADA activity is found in the small intestine followed by the esophagus, proventriculus and crop. In the esophagus, the activity is highest in day 1 and is seen to decrease significantly at day 10 and thereafter shows a slight decrease. In the crop, ADA activity is highest in day 1 showing a decrease at day 10 and then remains constant. The proventriculus is also seen to have a very high level of ADA activity at day 1 which shows a significant decline at day 30 and 90. Region specific studies indicate that in the small intestine, the level of activity is highest in day 1 followed by a sharp decrease at day 10 and then remains almost constant. Unlike all the tissues of GIT, the activity of ADA in the spleen is seen to increase maximally at day 30 after which there was a decline. In all the studies, our interest was to find out if there was a change in the level of protein of the enzyme for which the slot and Western blots were done for the two ages where differences were observed in almost all cases. Representative data of the blots show that the level of the protein

of ADA is indeed seen to change or remain constant for the ages studied. The avian gastrointestinal mucosal immune system has evolved with specialized features that reflect their role as the first line of defense on mucosal surfaces. High level of ADA on the day of hatching may ensure lower adenosine and better survival of lymphoid cells. Thus, a high ADA activity at early ages of chicken probably helps the tissues to cope with the increasing need for immunological competence, thus decreasing adenosine, which could otherwise exert unwanted physiologic effects.

5.2. HORMONAL REGULATION OF ADA

Our studies also show that corticosterone significantly inhibits the ADA activity in all the regions of GIT except proventriculus, in an age- and region- specific manner. In the spleen, corticosterone decreases the activity of ADA at both day 10 and day 60. The magnitude of inhibition is more pronounced at the later stage of chicken development (60-day) compared to a very young age (10-day). The findings of age- and tissue-specific inhibition may be correlated to the differential adaptive role and maturation of corticosterone action mechanism, its receptor and post-receptor events. Since corticosterone is immunosuppressive, it may be acting through the inhibitory action of ADA activity, leading to an accumulation of adenosine and 2'-deoxyadenosine, producing lymphotoxicity, leading to an immunosuppressive action. The inhibition of ADA activity level by corticosterone was also ascertained using slot and Western blot analyses which confirmed the inhibition of ADA activity at protein expression level. The immunosuppressive actions of corticosterone may thus control the host's immune response to a great extent. Pronounced inhibition at a later stage may be attributed to a greater maturation of corticosterone receptors and post-receptor events, thus facilitating greater binding of the hormone to its receptor and/or hormone-receptor binding to ADA gene promoter leading to inhibition of cognate gene expression.

Bt_2cAMP, which is a membrane permeable analog of cAMP, is found to increase the activity of ADA in all regions of GIT studied except crop. Like the tissues of GIT, in the spleen too, Bt_2cAMP increases the activity of ADA at day 60 but unlike the tissues of GIT, there was no effect at day 10. This stimulation is seen to be age and region specific. The substrate

adenosine influences the intracellular concentration of cAMP. Deficiency of ADA leads to an accumulation of adenosine and 2'-deoxyadenosine which are reported to be lyphotoxic. Thus, cAMP may increase the immune responses by stimulating ADA activity, lowering the level of adenosine and 2'-deoxyadenosine, to ensure better survival of lymphocytes. The immunoinducing role of cAMP may be because of enhancing the activity of ADA, thus decreasing the intracellular concentration of adenosine, which ensures an environment better suited for lymphocyte proliferation. In the GIT, the activity level of ADA was found to be greatly enhanced at a later age (day 60) of chicken development when compared to the younger age (day 10). This may be because of the differential expression of secondary messenger cascade at the later stage of GIT development. Such induction of ADA activity level by Bt_2cAMP was also reaffirmed using slot and Western blot analyses confirming that ADA activity is indeed induced at the protein expression level.

Thyroid hormones are known to decrease the activity of ADA in humans. However, in our course of study, there is no significant effect of thyroid hormone on the activity of adenosine deaminase in the tissues studied. The slot/Western blots also do not show any change in the level of the enzyme. This may be because of the difference in the physiology of humans and chicken, thyroid hormone receptors and post-receptor events or the lack of the regulatory elements of thyroid hormone in the chicken ADA gene.

Testosterone is also seen to decrease the activity of human ADA. But, no significant decrease in the activity level of ADA is seen in the tissues studied at the two postnatal ages, upon administration of testosterone. It could be due to a difference in the testosterone receptor and/or post-receptor events in human and chicken. There could also be a possibility of lack of testosterone/androgen regulatory elements (AREs) in the ADA gene of chicken. The slot and Western blot analyses performed also indicate no change in the activity at the protein expression level.

5.3. PURIFICATION AND PHYSICOCHEMICAL CHARACTERISATION OF ADA

Intestinal ADA from immature (1-day) and mature (90-day) chicken was purified using identical procedures. ADA preparations from both the ages were passed through sephadex G-100 gel filtration and DEAE-cellulose columns. The elution profiles indicated that both ADA, from immature and mature chicken, have similar molecular weights and ionic net charges. Gel filtration, PAGE and SDS-PAGE analyses indicate that both immature and mature ADA have similar molecular weight of 100 kDa, a similar overall charge and consist of a single molecular form. From the Michaelis-Menten equation and Lineweaver-Burk transformation, both the ADA show similar K_m for adenosine of 33.3 μM and 34.2 μM, respectively, for 1-day and 90-day old chicken. The computed K_m values of the immature and mature small intestinal ADA for 2'-deoxyadenosine are 14.3 μM and 14.3 μM, respectively. Analyses of the data indicate that there is no age-related difference in the affinity of ADA for adenosine as well as 2'-deoxyadenosine. However, both ADA from immature and mature chicken show more affinity towards 2'-deoxyadenosine than adenosine, indicating that a lower concentration of 2'-deoxyadenosine is required to reach a similar reaction velocity. Analysis of data indicates no significant difference between K_m, V_{max} and K_{cat} values of the enzyme for both substrates in the two age groups. There is also no significant difference observed in the K_i of immature and mature ADA for purine riboside. Hence, analyses of the data indicate that purine riboside is a strong competitive inhibitor of both ADA with a similar K_i of 6 μM and 7 μM, respectively. The pH stability studies indicate that ADA from immature and mature chicken is most stable in the broad range of pH from 5.5 to 8.0, after which slight instability starts in both the ADA. Thus, it implies that the salt bridge contributes to both immature and mature ADA by the same degree. The inactivation in the alkaline or in the urea solution can be derived from a slight deformation of the active site which cannot hold the entire activity due to a change in the molecular form at sites distant from the active site, whereas, acidic pH is capable of producing a direct change in or around the active site. When purified ADA from immature and mature chicken is assayed at different temperatures, the enzyme is stable till 45°C, after which a drop in the activity is seen in both the ages, with almost all activity lost at 70°C. This is because the increase in temperature after this does not increase the kinetic energy of the enzyme but instead disrupts the forces maintaining the shape of the molecule. Modulation studies on the activity of

ADA show that DTT, β-mercaptoethanol, DTNB all inhibit the activity of purified ADA albeit to a similar degree in both the immature and mature ages. The extent of inhibition by these sulfhydryl modifying agents is in the range of 25-35%. Amongst the divalent cations, Ca^{2+} is found to be less inhibitory (15%) to ADA followed by Mg^{2+} (50%) and the Hg^{2+} (88%). In addition to these, caffeine is also found to be inhibitory to ADA activity to the extent of 45%.

From the findings embodied in this thesis, it is concluded that:

- ADA activity and its level expresses in a tissue- and age- specific manner during postnatal development of chicken to ensure its better suited physiological roles.

- Corticosterone inhibits ADA activity in a tissue- and age- specific pattern, indicating that ADA is under the tonic inhibition by circulating corticosterone. Age-related difference in the magnitude of ADA inhibition is correlated to a differental adaptive role and maturation of corticosterone action mechanism, its receptor and post-receptor events.

- Bt_2cAMP is found to be stimulatory to the activity of chicken ADA, thereby exhibiting an immunoinducing role of cAMP.

- T_3 and testosterone fail to produce any significant change in ADA activity of chicken in either tissues or ages studied.

- Physicochemical and kinetic properties of purified ADA from small intestine of chicken remain the same at both the immature and mature ages indicating that there is no alteration in these properties as a function of age in chicken during postnatal development.

The studies compiled in the present thesis provide an insight into the basic role of ADA during postnatal development of chicken and also pave the way for using corticosterone and

Bt_2cAMP in inhibiting and inducing, respectively, the activity of ADA in various ADA-related diseases

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Curriculum Vitae

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Academic Qualifications:

Degree	Board/University	Year of passing	Division/Rank
M.Sc. (Biochemistry)	NEHU, Shillong	2003	I/2nd
B.Sc. (Biochemistry)	NEHU, Shillong	2001	I/3rd
HSSLC	MBOSE, Shillong	1998	I
SSLC	MBOSE, Shillong	1996	I

Experience:

1. Research Scholar: Department of Biochemistry, NEHU, Shillong: October 2005-Present

Technical experience:

- Rodent and avian non-survival surgery
- Intraperitoneal drug administration
- Polyacrylamide gel electrophoresis (PAGE), SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
- Agarose gel electrophoresis
- Gel filtration/Ion exchange chromatography
- Polyclonal antibody generation, immunoprecipitation, enzyme linked immunosorbent assay (ELISA)
- Protein purification, biochemical tests including enzymatic assays
- Sedimentation velocity, ultracentrifugation
- Western blotting, slot blotting
- PCR techniques

Computing skills:

- Microarray data analysis

- KDS 1 data analyzing software
- Basic Bioinformatics knowledge
- MS Office
- Enzfitter software
- NCBI Blast, ClustalW, Multalin analysis softwares, Molegro, MEGA, Prochek
- Windows XP, Windows 2000, Windows Vista
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2. Lecturer: Department of Biochemistry, St. Edmund's College, Shillong: August 2003-Present

Roles:

- Providing theoretical lectures at the undergraduate level
- Teaching topics like Biomolecules, Biophysical Chemistry, Nutritional Biochemistry, Physiology, Enzymology, Molecular Biology, Microbiology
- Conducting practicals for all the 3 undergraduate classes
- Examiner and scrutinizer for University undergraduate papers
- Teacher- in-charge for debate and quiz competitions, where role is training students for the same

Research Publication:

P Bhattacharjee and R Sharma (2009), Antithetical effects of corticosterone and dibutyryl cAMP on adenosine deaminase in the gastrointestinal tract of chicken during postnatal development, *Molecular and Cellular Biochemistry.* DOI: 10.1007/s11010-009-0045-1, 327: 79-86.

Symposiums, Conferences and Seminars Attended and Abstracts presented:

- **P Bhattacharjee** and R Sharma (2009) "Antithetical effects of corticosterone and N^6- 2'- O-dibutyryladenosine 3', 5'- cyclic monophosphate on small intestinal adenosine deaminase of chicken"; pp. 7, 2nd- 7th January, 96th Indian Science Congress meeting, NEHU, Shillong, Meghalaya

- **P Bhattacharjee** (2008) National seminar on "Toxicity of chemicals and their hazards with special reference to heavy metals" 23rd - 24th October; St Edmund's College, Shillong.

- **P Bhattacharjee** and R Sharma (2008) "Antithetical effects of corticosterone and N^6- 2'- O-dibutyryladenosine 3', 5'- cyclic monophosphate on small intestinal and splenic adenosine deaminase of chicken" at a National Seminar on "Advances in Medical and Microbial Biochemistry"; 13th- 14th March, Department of Biochemistry, NEHU, Shillong (Oral presentation)

- **P Bhattacharjee** and R Sharma (2007) "Corticosterone regulates adenosine deaminase of chicken in an age- and tissue- specific manner" in the 76th Annual Meeting of Society of Biological Chemists (India); 25th- 27th November, Department of Biochemistry, Sri Venkateswara University, Tirupati, Andhra Pradesh

- **P Bhattacharjee** and R Sharma (2007) "Dibutyryl c-AMP regulates adenosine deaminase of chicken in an age- and tissue- specific manner" at a Seminar on "Adaptation Biochemistry"; 22nd- 23rd March, Department of Biochemistry, NEHU, Shillong (Oral presentation)

- **P Bhattacharjee** and R Sharma (2006) on "Regulation of adenosine deaminase during postnatal development of chicken" in the 75th Annual Meeting of Society of Biological Chemists (India); 8th- 11th December, Jawaharlal Nehru University, New Delhi

- **P Bhattacharjee** and R Sharma (2006) "Tissue- and age-specific changes in adenosine deaminase of chicken" at a National Seminar on "Trends in Biochemical Research"; 31st March, Department of Biochemistry, NEHU, Shillong (Oral presentation).

Symposiums, Conferences and Seminars Attended:

- **P Bhattacharjee** (2008) Participated in a National Seminar on "Ageing in India with Special Reference to North East India"; 1st-2nd October, Indian Council of Social Science Research, North East Regional Centre, NEHU, Shillong.

- **P Bhattacharjee** (2006) "Biosafety measures with special reference to Cartagena Protocol", 27th - 28th August, Assam Agricultural University in collaboration with Department of Biotechnology, Jorhat, Assam.

Workshops Attended:

Training course on "*In Silico* Approach to genome analysis" from 5th- 11th February, 2009, conducted by the Bioinformatics Centre, NEHU, Shillong, funded by Department of Biotechnology, Ministry of Science and Technology, Govt of India

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Scholarships awarded:

- Recipient of **State Scholarship** for post-graduate studies (2001-2002).

- Recipient of **National Scholarship** at the Higher Secondary (1996-1997) and Undergraduate levels (1998-2000).