REGULATION OF GLUCOCORTICOID RECEPTORS DURING POSTNATAL DEVELOPMENT OF CHICKEN

Abstract

By

DANIEL NONGBRI
Department of Biochemistry

Submitted in partial fulfillment of the requirement of the Degree of
Doctor of Philosophy in Biochemistry

of

North-Eastern Hill University
Shillong, India
October - 2009

Thesis

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ABSTRACT

The lifespan of an animal may be broadly divided into three phases: developmental, reproductive, and senescence. All changes in the animal form and functions during development depend on unique set of genes, which are sequentially activated and repressed. Such changes determine which proteins are made, and where and when, during development. The duration of this phase may vary within certain limits and may be influenced by intrinsic factors such as hormones and other effectors, and extrinsic factors such as nutrition and stresses. Hormones coordinate cellular interactions which are of central importance during development of different tissues and organs.

Glucocorticoids, an essential class of stress-induced endogenous steroid hormones under control of the hypothalamic-pituitary-adrenal axis, affect virtually all tissues and regulate various biologically important functions, from metabolism, behaviour, immune response to growth and development in cell-specific manner. Most of the physiological actions of glucocorticoids are mediated by binding to a specific intracellular protein called glucocorticoid receptor (GR), which is ubiquitously distributed and acts as ligand-dependent transcription factor belonging to the superfamily of nuclear receptors. Nuclear receptors are still being found, and more than 300 sequences have been reported. Many are important transcriptional regulators involved in widely different physiological functions such as the control of embryonic development, cell differentiation, and metabolic homeostasis.

As lipophilic molecules, glucocorticoids cross the cell membrane readily to interact with GR which resides in the cytoplasm in association with heat shock proteins and other chaperonic and immunophilic proteins. Upon hormone binding, the protein complex dissociates due to conformational change in the GR, a process termed 'activation' or 'transformation'. Activation of GR facilitates nuclear import where they dimerize and then bind specifically to glucocorticoid response elements (GREs), enhancing transcription or suppress transcription when it binds to negative GREs (nGREs). GR also modulates gene transcription via cross-talk with other transcription factors such as activator protein-1, nuclear factor-κB, members from the signal transduction and activator of transcription family.

Numerous studies have been made on the age-related changes in GR concentration, activation/transformation and their binding to nuclear chromatin in different mammalian tissues during postnatal development. It has also been demonstrated that changes in physicochemical properties of hepatic GR occur as a function of age. However, information on the changes in the level and in the physicochemical properties of GR during avian development is scarce. The present study describes the changes in the level of GR and also in the physicochemical properties of GR during postnatal development. Conformational

changes in the chromatin organization during postnatal ages which may be involved in glucocorticoid regulation of gene expression in developing chicken are also examined.

Studies on the binding of [^{3}H]dexamethasone to glucocorticoid receptor

Using [^{3}H]dexamethasone binding studies, our data show a change in the glucocorticoid receptor concentration in a tissue- and age-specific manner during the postnatal development of chicken. In the liver, the receptor level reaches a peak value by day 5 of postnatal age, which is significantly higher than the values observed in other age groups. In the kidney, the receptor concentration is maximum in the early postnatal age (0-day), and thereafter shows a gradual decline. The observed increase in receptor concentration at day 5 in liver and day 0 in kidney could be due to the increase in the receptor concentration *per se* or an increase in the receptor affinity for the hormone. To ascertain the above possibilities, slot blot analyses were performed and results indicate the increase in GR concentration at day 5 and day 0 in liver and kidney, respectively. Scatchard analysis of the binding data also confirmed the above findings. Moreover, there is no age-associated alteration in the dissociation constant (K_d) values in both the tissues. The glucocorticoid receptor concentration in the cardiac muscle (heart) is constant in the early postnatal ages up to day 30. By day 60, the receptor level declines and the level at day 90 is similar to that at day 60 of postnatal age. Slot blot analyses of the receptor preparation and the intensity of the slot bands confirm a decline in receptor protein level at day 60 compared to day 0 of postnatal age. In skeletal muscle, the glucocorticoid receptor level reaches the peak value by day 10 of postnatal age, which is significantly higher than the value observed at day 5 of postnatal age. The receptor level then declines at day 60 of postnatal age and the receptor level at day 90 is similar to that at day 60 of postnatal age. Slot blot analyses and the intensity of the slot bands show that the receptor protein level is higher at day 10 compared to day 0 of postnatal age. In the cerebral hemisphere, the receptor levels are quite low compared to the other tissues studied, however, the receptor level does not elicit any significant change in all the ages studied. Slot blot analyses and subsequent analyses of the intensity of the slot bands corroborate the above findings.

Studies on the activation of [^{3}H]dexamethasone-receptor complexes

DNA-cellulose binding assays both in liver and kidney show that the activation of [^{3}H]dexamethasone-receptor complexes both by heat and salt are significantly higher as compared to the unactivated receptor complexes. However, the degree of activation is similar for hepatic glucocorticoid receptors obtained from 0- and 30-day old chicken. The

results indicate no postnatal difference in the *in vitro* activation of the hormone-receptor complexes under the conditions mentioned above. In the kidney too, the thermal as well salt activation of [^{3}H]dexamethasone-receptor complexes were similar to that in liver since there was no difference in the magnitude of *in vitro* activation of the hormone-receptor complexes in the two ages studied. However, the fold of activation of [^{3}H]dexamethasone-receptor complexes both by temperature and salt is slightly lower (2-2.3 fold) compared to the liver. Nuclear binding assays in both the liver and kidney indicate that the thermal as well as salt activation of [^{3}H]dexamethasone-receptor complexes is similarly observed in both the age groups studied as seen using DNA-cellulose binding assay. However, in contrast to DNA-cellulose binding assay, nuclear binding of both the thermally- and salt-activated glucocorticoid-receptor complexes is significantly higher in immature (0-day) compared to that of mature (30-day) chicken. Nuclear exchange (cross-mixing) assays in both liver and kidney revealed that the higher magnitude of activation in 0-day old chicken is not due to the [^{3}H]dexamethasone-receptor of the two age groups studied but because of the differences of the nuclear properties. The sensitivity of both liver and kidney nuclear chromatin digestion by DNase I shows that 0-day old chromatin reflects a less condensed chromatin as compared to that of 30-day old, hence allowing greater binding to [^{3}H]dexamethasone-receptor complexes at this age of lifespan.

Studies on inhibition of activation of [^{3}H]dexamethasone-receptor complexes

Various exogenous and endogenous agents were used to inhibit the receptor activation process by heat and salt. Molybdate was found to be a more potent inhibitor compared to tungstate. Also, cadmium and oleic acid were found to be potent inhibitors of both heat and salt activation of GR. However, the magnitude of activation inhibition by these inhibitors remains the same at the two ages studied, indicating that the mechanism(s) of activation inhibition does not get altered during these ages of chicken.

Studies on the physicochemical properties of glucocorticoid receptors

Studies on determine the physicochemical properties of glucocorticoid receptors did not reveal any difference in the hepatic receptor from 0- and 30-day old chicken. Gel filtration analyses indicated the molecular weight and stoke radii values of ~255 kDa and ~5.65 nm, respectively for the unactivated glucocorticoid receptor complexes. For the thermally activated glucocorticoid receptors the values were ~86 kDa and 3.28 nm, respectively. It is also evident from our study that the elution pattern from DE-52 did not reveal any charge differences in the glucocorticoid receptors in the two ages studied. The unactivated

glucocorticoid receptor complexes from both the age-groups eluted at ~248 mM KCl, whereas the activated receptors eluted at a salt strength of ~100 mM KCl. These findings reveal no differences in the physical and chemical properties of the glucocorticoid receptors during postnatal development of chicken.

From these studies, it is concluded that the glucocorticoid receptor concentration and not the hormone binding affinity alters during postnatal development. In addition, an increased nuclear binding of both the heat- and salt-activated receptors is due to the more relaxed chromatin organization in immature animals as compared to mature ones, where chromatin is more compact. Molybdate, tungstate, cadmium and oleic acid were found to be inhibitors of heat and salt activation of GR in liver and kidney. However, the magnitude of activation inhibition by these inhibitors remains the same at the two ages studied, indicating that the mechanism(s) of activation inhibition does not get altered during these ages of chicken. Physicochemical properties studied remain unaltered during postnatal development. The observed changes in the level and activation binding of GR as well as chromatin organization may contribute to functional alterations in glucocorticoid actions which may provide a better adaptation to the changing demands made upon animals during development and also be responsible for tissue's responsiveness to glucocorticoids during such phase of animal's lifespan.

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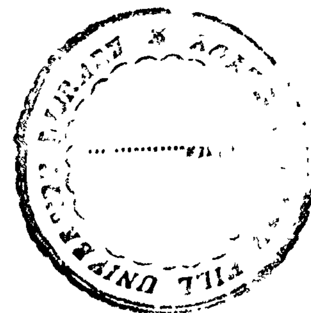

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I, **Daniel Nongbri**, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else,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

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

Daniel Nongbri
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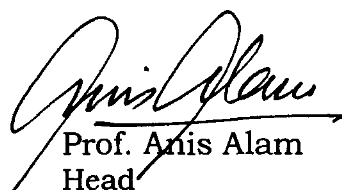

Prof. Anis Alam
Head
Dept. of Biochemistry
Head
Department of Bio - Chemistry
N.E.H.U, Shillong

Prof. R. Sharma
Supervisor
Dept. of Biochemistry
Prof. R. Sharma, Ph D , FNASc
Dept of Biochemistry
North Eastern Hill University
Shillong - 793022

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ACKNOWLEDGEMENTS

To begin with, I thank God for all his blessings in my journey towards the completion of my thesis.

I would like to thank my supervisor, **Prof Ramesh Sharma** for allowing me to work under him. His guidance, approachability and encouragement helped me a lot in understanding the problems and approaching them in a befitting manner. It was his selfless efforts, inspiration, affection and encouragement that made all possible. His patience and insight has made me realize and understand life in a broader perspective.

My thanks also goes to the Heads of the Department, **Prof. A. N. Rai, Prof. Lalthantluanga, Prof Anis Alam**, for allowing me to use all the facilities of the Department during the entire period.

I also would like to convey a deep sense of gratitude to all the other faculty members of the Department like **Prof. R. N. Sharan, Dr. D. Syiem, Dr. A. K Singh, Dr. P. K Ambasht, Dr. M. B. Syiem, Dr. S. Bhan and Mr T. Tripathi** for all the valuable suggestions and help.

A sincere note of appreciation is expressed for **Prof. B. B. P. Gupta,** Dept of Zoology, NEHU, for allowing me to use the liquid scintillation counter and for offering suggestions at times.

The non-teaching staff of the Department, **Mr. M. B. Singh, Mr. B. Laloo, Mrs. Y. Kharkongor, Mrs. Pushpa, Mr. Kushal, Mr. Ponderly, Mr. Kharir** and all the others also deserve a special note of gratitude for always being of help whenever required. I would also like to thank **Dr. Marbaniang, Dr. Joshi and Dr. Synchar** of Regional Poultry Farm, Kyrdemkulai, for their help. A special note of appreciation is deserved by **Pynkup Syiem and Deliver Kharumnuid** who has helped with a lot computational work.

The lab that I have spent so much time in has been a really wonderful place especially because of the friendly environment. I would like to thank my seniors **Dr. Debipreeta Dutta** and **Dr. Danswrang Goyary** for their help and valuable suggestions. A special note of thanks goes to **Piyali, Kitlangki, Preeticia** and **Banteiskhem** for a helpful as well as joyous atmosphere being created at all times. My juniors **Wanai** and **Teikur** have also been there at times to add to the environment. **Rennie, Yashmin, Meriyani, Jignasa, Amrita, Natasha, Banri, Khet, Norna** and all the other research scholars of the Dept. also need to be thanked.

It would be incomplete if I do not thank **Dr. C. Massar, Sir Gareth, Manbha, Hunhameshisha, Bah Ban, Goutam, Arun, Sanjoy, Gyan, Panna, Don, Pramod, Pothang, Thianglallal, Thomas, Arches, Filbert, Eva, Bala, Euginia & Jose** for being encouraging and helpful at all times.

My parents and family members have supported me at all times and it was their faith and belief in me that has guided and re-instilled my belief in myself.

Dated : 26th October, 2009 Daniel Nongbri

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ABBREVIATIONS

ACTH	: Adrenocorticotropic hormone
ATP	: Adenosine 5'-triphosphate
AVT	: Arginine vasotocin
BSA	: Bovine serum albumin
CBG	: Corticosterone binding globulin
CPM	: Counts per minute
DBD	: DNA binding domain
DNA	: Deoxyribonucleic acid
DNA-cellulose	: Deoxyribonucleic acid Cellulose
DNase I	: Deoxyribonuclease I
DTT	: DL-Dithiothreitol
EDTA	: Ethylenediaminetetraacetic acid
GPCR	: G-protein-coupled recptor
GR	: Glucocorticoid receptor
GRE	: Glucocorticoid responsive element
HBD	: Hormone binding domain
Hsp	: Heat shock protein
kDa	: Kilo Dalton
LBD	: Ligand binding domain
PUFA	: Polyunsaturated fatty acid
R_S	: Stokes radius
Tris	:Tris(hydroxymethyl)-amino methane

INTRODUCTION

Lifespan is a period that starts from the conception till death. It is a continuous process where at one end is the development and growth, and at the other end is the deterioration of functions or senescence or aging. The lifespan of an organism is the sum of deleterious changes and counteracting repair and maintenance mechanisms that respond to damages (Johnson *et al.*, 1999). It is broadly divided into three phases: developmental (growth), reproductive (adulthood) and senescence (aging) (Kanungo, 1994). Some authors have divided lifespan into two periods: prenatal (before birth) and postnatal (after birth), taking into account the characteristic anatomical, physiological and biochemical features at each stage. The prenatal period encompasses the embryonic and fetal stages, whereas the postnatal period includes neonatal, infancy, adulthood and old age (Timiras, 1994).

Developmental phase

The developmental phase includes an increase in the number and size of the cells, their differentiation to perform specialized functions and formation of organs. Several new proteins appear during this period and the levels of protein change as cells differentiate and organs form, indicate changes in the expression of corresponding genes. The sizes of organs increase leading to the increase in the size of the organism and its functional abilities, the most important of which is the ability to reproduce (Kanungo, 1994).

Reproductive phase

This phase is very important to the organism as it confers reproductive ability to them and therefore is important in the perpetuation and evolution of the species. The reproductive ability could be achieved by specialized structures and functions that have evolved during natural selection. Several genes that play an important role in the development and maintenance of reproductive ability are now expressed, e.g., the genes that code for FSH (follicle stimulating hormone) and LH (luteinizing hormone) in vertebrates, and for ovalbumin, vitellogenenin, and lysozyme for egg formation in egg-laying vertebrates (Kanungo, 1994).

Senescent phase

It is a characteristic of all organisms whereby the functional abilities of all organs and the organisms decrease. Senescence is the accumulation of changes responsible for the sequential alterations that accompany advancing age and the associated progressive increases in the chance of disease and death (Upton, 1977). An important feature of senescence is the loss of reproductive ability. The basic cause of physiological deterioration occurring with advancing age in an organism has not yet been elucidated. Probably alterations in informational biomolecules like DNA, RNA, and proteins form the basis of such physiological changes. Because aging is a multifactorial process, it is likely that a number of different mechanisms are responsible for age-associated alterations and that different individuals exhibit different pattern of aging depending upon their specific inheritance and interaction with the environment (Thakur *et al.*, 1993).

Hormones in growth and development

Development and growth may be viewed as a series of multiple but integrated processes which are directed by chemical messengers or hormones. Hormones are signaling molecules for communication between cells. In birds, as in mammals, growth and development appears to be under an intricate control mechanisms which involve endocrine, paracrine and autocrine signaling cascades. A number of hormones have long been recognized as playing major roles in the control of growth. These include growth hormone (GH), glucocorticoids, thyroid hormones and the sex steroids. The effect of GH on growth is now accepted to be largely via the mediating factors, insulin like growth factors (IGF) I and II. In addition, other growth factors exert profound effects on growth and development. These growth factors include the transforming growth factors (TGF), epidermal growth factor (EGF), and the nerve growth factor (NGF). TGF-β has been found to exert profound effects on chondrocytes in leg long-bone of chicken (O'Keefe *et al.*, 1988). TGF-β acts to increase the synthesis of proteoglycan and non-collagen proteins and to inhibit collagen synthesis by avian chondrocytes. According to Scanes *et al.* (1990), hormones exert different effects depending on developmental stage and/or physiological and environmental conditions. Thus, hormones might: i) initiate development and differentiation of cells to organs ii) influence the rate of development of organ(s) iii) affect the supply of nutrients for growth and development iv) exert a homeostatic effect. Various hormones have homeostatic regulatory functions as well as influence growth and development. For example, insulin exerts an important role in chick embryo growth and development (de Pablo *et al.*, 1985).

Hormone receptors

Hormones exert their physiological effects by high affinity interactions with receptors located on the cell surface or intracellularly in the target cells. All water soluble signaling molecules (protein/peptide hormones, catecholamines, neurotransmitters and growth factors) act via cell-surface receptors. These receptors are transmembrane proteins that span the phospholipid bilayer of the cell membrane. The signaling molecule binds on the extracellular side of the receptor, which is thereby activated. Reception of the signal is synonymous with activation of the receptor for transmission of the signal across the cell membrane. Transmission of the signal implies specific communication with the effector protein, the next component of the signal transmission pathway on the inner side of the cell membrane. In this process, enzymatic activities can be triggered and/or the activated receptor engages in specific interactions with downstream signaling proteins. An intracellular signaling chain is set in motion, which finally triggers a defined biochemical response in a target cell. Most cell-surface receptors belong to one of the two classes: enzyme-linked cell-surface receptors and G-protein-coupled receptors (Nishizuka, 1992; La Marco and Vivanco, 1996).

G-protein-coupled receptors

Of the transmembrane receptors that receive signals and conduct them into the cell interior, the G-protein-coupled receptors form the largest single family. G-protein-coupled receptors (GPCRs), also known as seven-transmembrane domain receptors, 7TM receptors, serpentine receptors, and G-protein-linked receptors (GPLR), comprise a large family of transmembrane receptors that sense molecules outside the cell and activate signal transduction pathways and, ultimately, cellular responses. Approximately, 400 G-protein–coupled receptors (GPCRs) are known to mediate the effects of endogenous ligands and are the targets for about half of currently used prescribed drugs (Drews, 1996; Pierce *et al.*, 2002; Hill, 2006). The interaction of an agonist with the binding pocket of a GPCR induces a conformational change in the transmembrane-spanning segments. This results in its association with a G protein that leads to activation of a signal transduction pathway, resulting in the characteristic cellular response. GPCRs were conventionally thought to exist and act as monomers, but there is accumulating evidence that most GPCRs probably exist as dimers or even oligomers (Milligan, 2004; Bulenger *et al.,* 2005; Prinster *et al.*, 2005). Furthermore, different GPCRs may interact with each other, forming heterodimers. This has important implications for understanding cellular regulation and the action of agonists. Dimerization of GPCRs was first proposed by Agnati and colleagues in the 1980s, based on the finding of unexplained cooperativity between certain agonists and a larger-than-expected

molecular size of receptor proteins observed by gel electrophoresis. However, this idea received little attention until the last decade.

Enzyme-linked cell-surface receptors

Enzyme-linked cell-surface receptors were recognized initially through their role in responses to extracellular signal proteins that promote the growth, proliferation, differentiation, or survival of cells in animal tissues. Disorders of cell proliferation, differentiation, survival, and migration are fundamental events that can give rise to cancer, and abnormalities of signaling through enzyme-linked receptors. Like G-protein-linked receptors, enzyme-linked receptors are transmembrane proteins with their ligand-binding domain on the outer surface of the plasma membrane (La Marco and Vivanco, 1996). Instead of having a cytosolic domain that associates with a trimeric G protein, however, their cytosolic domain either has an intrinsic enzyme activity or associates directly with an enzyme. Six classes of enzyme-linked cell-surface receptors have so far been identified and these include:

i) Receptor tyrosine kinases: phosphorylate specific tyrosine residues on a small set of intracellular signaling proteins.
ii) Tyrosine kinase-associated receptors: associate with intracellular proteins that have tyrosine kinase activity.
iii) Receptor-like tyrosine phosphatases: remove phosphate groups from tyrosine residues of specific intracellular signaling proteins.
iv) Receptor serine/threonine kinases: phosphorylate specific serine and/or threonine residues on associated latent gene regulatory proteins.
v) Receptor guanylyl cyclases: directly catalyze the production of cyclic GMP in the cytosol.
vi) Histidine kinase associated receptors: activate a "two-component" signaling pathway in which the kinase phosphorylates itself on histidine and then immediately transfers the phosphate to a second intracellular signaling protein.

The extracellular signal proteins that act through receptor tyrosine kinases consist of a large group of secreted growth factors and hormones, e.g., epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), insulin, insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), macrophage colony stimulating factor (MCSF), and all the neurotrophins, including nerve growth factor (NGF).

Intracellular receptors

Intracellular receptors regulate gene expression in response to binding of small lipophilic molecules and are thereby involved in the control of diverse cellular processes. These proteins are ligand-activated transcription factors that are localized in the cytoplasm and/or in the nucleus. The ligands pass the cell membrane by simple diffusion and bind to the cognate receptors in the cytoplasm and/or in the nucleus. By binding to specific regions of target genes the ligand-bound receptor influences the transcription of these genes and thereby leading to a change in cognate gene expression. The naturally occurring ligands of intracellular receptors are lipophilic hormones, among which the steroid hormones, the thyroid hormone T3, and derivatives of vitamin A and D have long been known as central regulators of gene expression. These hormones play a significant role in metabolic regulation, organ function, and development and differentiation processes (Krauss, 2003). The first nuclear receptors to be characterized were those for the glucocorticoids, for estrogen, and for progesterone. Many other nuclear receptors could be identified based on common structural and functional features and these receptors are now grouped into a large superfamily with at least six different subfamilies (Robinson-Rechavi *et al.*, 2003). A number of receptors ('orphan receptors') have been identified for which the cognate hormone and their functions in the cell remain unknown. Several orphan receptors have been characterized, some of which have been suggested to control vital physiological and developmental process (e.g., Retinoic Z receptor, RZR; NGF-induced clone B, NGFI-B) (Krauss, 2003). Many orphan receptors have structural domains similar to that of members of steroid/nuclear receptor superfamily. Orphan nuclear receptors represent a tremendous opportunity in understanding and treating human diseases. Recently, Shi (2007) has highlighted recent advances in the use of orphan nuclear receptors and their potential as targets for drug discovery in diabetes, obesity, neurodegenerative diseases and other related disorders.

Retinoid receptors

The most important endogenous retinoid is all-trans-retinoic acid. Retinoids regulate a wide variety of essential biological processes, such as vertebrate embryonic morphogenesis and organogenesis, cell growth arrest, differentiation and apoptosis, homeostasis, vision, reproduction, bone development and hematopoiesis (Sporn *et al.*, 1994; Gudas *et al.*, 1994). The retinoic acid receptor (RAR) is a type of nuclear receptor (Germain *et al.*, 2006) which is activated by both all-trans retinoic acid and 9-cis retinoic acid (Allenby *et al.*, 1993). Retinoid is a term for compounds that bind to and activate retinoic acid receptors (RAR). Three different subtypes of RAR have been identified, RARalpha, RARbeta, and RARgamma in mammals, birds and amphibians (Chambon, 1994). Another nuclear receptor termed the

retinoid X receptor (RXR), has been identified (Mangelsdorf *et al.*, 1990) which also exists as three different subtypes RXRalpha, RXRbeta, and RXRgamma. RXR heterodimerizes with subfamily 1 nuclear receptors including constitutive androstane receptor (CAR), liver X receptor (LXR), farnesoid X receptor (FXR), peroxisome proliferator-activated receptor (PPAR), pregnane X receptor (PXR), retinoid acid receptor (RAR), thyroid hormone receptor (THR) and vitamin D receptor (VDR). RXR heterodimer in the absence of ligand is bound to hormone response elements complexed with corepressor protein. Binding of ligands to RXR results in dissociation of corepressor and recruitment of coactivator proteins which in turn promote transcription of the downstream target gene.

Vitamin D receptors

Calcitriol (1,25-dihydroxycholecalciferol) is the active form of vitamin D found in the body. It plays an important role in the maintenance of several organ systems. However, its major role is to increase the flow of calcium into the bloodstream, by promoting absorption of calcium and phosphorus from food in the intestine, and reabsorption of calcium in the kidneys. It is also necessary for bone growth and bone remodelling by osteoblasts and osteoclasts (van den Berg, 1997; Cranney *et al.*, 2007). These actions of viatmin D are mediated by its cognate receptor in the nucleus called vitamin D receptor (VDR) (Pike and Sleator, 1985). Upon activation by vitamin D, the VDR forms a heterodimer with the retinoid-X receptor and binds to hormone response elements on DNA resulting in expression or transrepression of specific gene products. Glucocorticoids are known to decrease expression of VDR which is expressed in most tissues of the body and regulate intestinal transport of calcium (Szpirer *et al.*, 1991).

Thyroid hormone receptors

Thyroid hormones (T_3 and T_4) are essential for development and differentiation of cells in humans and several other organisms (Brent, 2000). They increase the basal metabolic rate, affect protein synthesis, help regulate long bone growth, neuronal maturation and increase the body's sensitivity to catecholamines by permissiveness. These hormones also regulate protein, fat, and carbohydrate metabolism. The physiological actions of thyroid hormones are carried out by specific nuclear receptors called thyroid hormone receptors (THRs). T_3 binds to THR with higher affinity (10×) than T_4 and hence, is more biologically potent. THR belongs to the nuclear receptor superfamily and several isoforms of this receptor have been identified, THRα1, THRα2, and THRβ1, and THRβ2 (Glauser and Barakat, 1997; Flamant *et al.*, 2006).

Steroid receptors

The glucocorticoid receptor (GR), mineralocorticoid receptor (MR), estrogen receptor (ER), progesterone receptor (PR), and androgen receptor (AR) are classic members of the nuclear receptor superfamily, composing subfamily 3C. Members of this subfamily are among those receptors that were cloned the earliest, with the GR being cloned in 1985 and the MR, PR, and AR shortly thereafter (Hollenberg *et al.*, 1985; Arriza *et al.*, 1987; Misrahi *et al.*, 1987; Chang *et al.*, 1988; Lubahn *et al.*, 1988). Individually and in combination, these four receptors play pivotal roles in some of the most fundamental aspects of physiology such as the stress response, metabolism, immune function, electrolyte homeostasis, growth, development, and reproduction. Multiple signaling pathways have been established for all four receptors, and several common mechanisms have been revealed (Mangelsdorf *et al.*, 1995). These steroid hormone receptors also exemplify the tremendous capacity and precision of endocrine modulatory mechanisms. Patients carrying mutated receptors frequently experience severe complications, and transgenic animals lacking individual receptors frequently cannot reproduce and/or survive (Sato *et al.*, 2003; Sartorato *et al.*, 2004; Lin *et al.*, 2005; Matsumoto *et al.*, 2005). Temporally controlled tissue distribution patterns during developmental stages, reproductive phases, and disease conditions contribute to the diverse activities of these receptors.

Androgens control the development and maintenance of masculine characteristics in vertebrates by binding to androgen receptors. Androgens, which were first discovered in 1936, are also called androgenic hormones or testoids. They serve critical functions at different stages of life in the males (Katzung, 2004; Goodman *et al.*, 2006). During embryonic life, androgens virilize the urogenital tract of the male embryo, and their action is thus essential for the development of the male phenotype. The androgen receptor (AR), is a type of nuclear receptor (Lu *et al.*, 2006) which is activated by binding of either of the androgenic hormones testosterone or dihydrotestosterone (Roy *et al.*, 1999). Upon ligand binding, AR acts as transcription factor and regulates gene expression (Mooradian *et al.*, 1987).

Estrogens are the principle feminizing hormones involved in regulation of physiological functions, such as, secondary sexual development, maintenance of female phenotype and pregnancy. Estrogens readily diffuse across the cell membrane. Once inside the cell, they bind to and activate estrogen receptors which in turn up-regulate the expression of many genes. Recently, estrogens have been shown to activate a G protein-coupled receptor, GPR30 (Prossnitz *et al.*, 2007). There are two different forms of the estrogen receptor, usually referred to as ERα and ERβ which are the products of two different genes. Hormone activated estrogen receptors form dimers, and since the two forms are

coexpressed in many cell types, the receptors may form ERα (αα) or ERβ (ββ) homodimers or ERαβ (αβ) heterodimers (Li *et al.*, 2004).

Progesterone is the most important progestin in humans. It is synthesized in the ovary, testis, and adrenal gland from circulating cholesterol. Large amounts are also synthesized and released by the placenta during pregnancy. In the ovary, progesterone is produced primarily by the corpus luteum. Progesterone is involved in the female menstrual cycle, pregnancy (supports gestation) and embryogenesis of humans and other species. In addition to having important hormonal effects, progesterone serves as a precursor in the synthesis of estrogens, androgens, and adrenocortical steroids. Progesterone acts through the mediation of progesterone receptor (PR). PR is encoded by a single gene and it has two main forms, A and B that differ in their molecular weight (Gadkar-Sable *et al.*, 2005).

Mineralocorticoids are steroid hormones whose primary action is to promote salt and water balance. Activation of the mineralocorticoid receptor, upon the binding of its ligand aldosterone, results in its translocation to the cell nucleus, homodimerization and binding to hormone response elements present in the promoter of cognate genes. This results in the complex recruitment of the transcriptional machinery and the transcription into mRNA of the activated genes (Fuller and Young, 2005).

Most of the physiological actions of glucocorticoids (GCs) are mediated by binding to a specific intracellular receptor protein called GR; this receptor mediation could serve as a functional definition of glucocorticoid's effect. GCs may also exert nontranscriptional effects by binding to corticosteroid-binding globulin (CBG) that has bound to specific cell-surface CBG receptors in target cells (Maitra *et al.*, 1993).

Physiological effects of glucocorticoids

The fact that all cells possess the glucocorticoid receptor (GR) suggests that glucocorticoids (GCs) have a number of profound and diverse effects on physiologic systems. These include glucose metabolism, immune functions, programmed cell death, electrolyte balance, behaviour, memory, fetal development, and many other functions. Cortisol in human and corticosterone in rodents and birds are the major glucocorticoids that act through the GR to regulate homeostasis, stress responses and adaptation in developing animals (Munck *et al.*, 1984; Bodine and Litwack, 1990). GCs also play an important role in development and aging processes (Kalimi, 1984; Sharma, 1988). GCs are also used pharmacologically in treatment of wide range of rheumatic and many other inflammatory diseases. They also find

widespread use in chemotherapeutic regimes in patients with leukemias, lymphomas, and other cancers due to their critical role in the induction of apoptosis, but little is known about the effects of GCs on the growth and chemosensitivity of common human lymphomas (Yudt and Cidlowski, 2002; Lu *et al.*, 2005). Knowledge of the importance of GCs inspired many workers to gained better understanding of GCs. In the late 1940's, GCs were introduced into clinical medicine and the researchers primarily involved were awarded the Nobel Prize in medicine. Tadeusz Reichstein together with Edward Calvin Kendall and Philip Showalter Hench were awarded the Nobel Prize for Physiology on Medicine in 1950 for their work on hormones of the adrenal cortex which culminated in the isolation of cortisone. Lewis Sarett of Merck & Co. was the first to synthesize cortisone, using a complicated 36-step process that started with deoxycholic acid, which was extracted from ox bile.

Glucocorticoids exert a vast array of physiological functions via the GR. They are important regulators of carbohydrate, protein, and fat metabolism (Katzung, 2004; Goodman *et al.*, 2006). In the fasting state, glucocorticoids stimulate gluconeogenesis and glycogen synthesis via a variety of mechanisms including increasing the production of enzymes critical in gluconeogenesis, stimulating the release of amino acids from muscles, promoting insulin resistance in the peripheral tissues, and inhibiting adipokines such as adiponectin. These processes protect glucose-dependent tissues such as the brain and heart during starvation. Glucocorticoids also profoundly modulate immune responses by regulating the activity of peripheral leukocytes, by suppressing the production of cytokines and chemokines, and by changing the lifespan of immune cells. In addition, glucocorticoids are critical for the functions of the central nervous system (CNS), digestive, hematopoietic, renal, and reproductive systems.

Glucocorticoid actions are summarized below.

- *Metabolic effects: glycogen metabolism, glycogenesis, peripheral glucose utilization, lipid metabolism.*
- *Effects on musculoskeletal and connective tissues: bone and mineral metabolism, skeletal muscle, and connective tissue.*
- *Effects on fluid and electrolyte homeostasis.*
- *Neuropsychiatric and behavioral effects.*
- *Gastrointestinal effects.*
- *Developmental effects.*
- *Further actions: antiinflammatory and antiallergic effects.*

HPA axis

The hypothalamic-pituitary-adrenal (HPA) axis, also known as the limbic-hypothalamic-pituitary-adrenal axis (LHPA axis), is a complex set of direct influences and feedback

interactions among the hypothalamus, the pituitary gland and the adrenal glands. The adrenal glands of birds, like those in mammals, are next to the kidneys. The cortical and medullary components are intermingled and this constitutes a major characteristic of avian adrenal medulla (Vestergaard and Willeberg, 1978). In mammals, however, the medulla always occupies the central portion and remains encircled by the adrenal cortex with its three concentric zones. The interactions among these organs constitute the HPA axis, a major part of the neuroendocrine system that controls reactions to stress and regulates many body processes, including digestion, the immune system, mood and emotions, sexuality, and energy storage and expenditure.

A wide variety of species, from the most ancient organisms to humans, share components of the HPA axis. In birds, as in mammals, glucocorticoid release is under direct control of hypothalamic hormones that stimulate the release of adrenocorticotropic hormone (ACTH). These hypothalamic hormones are called secretagogues since they cause the secretion of ACTH from the pituitary. A suite of secretagogues, with corticotropin-releasing factor (CRF), arginine vasotocin (AVT), and mesotocin (MT) being primary, elicit ACTH secretion. All three secretagogues are present in the median eminence of the hypothalamus of birds (Mikami and Yamada, 1984; Mikami, 1986; Ball *et al.*, 1989) and are known to stimulate ACTH secretion both *in vitro* (Castro *et al.*, 1986) and *in vivo* (Romero *et al.*, 1998a; Westerhof *et al.*, 1992). Each of these secretagogues acts via the hypothalamic-pituitary portal blood. There are some reports that AVT is the primary secretagogue in birds. In some studies AVT was found to be more effective than CRF at inducing ACTH released both *in vitro* (Castro *et al.*, 1986) and *in vivo* (Westerhof *et al.*, 1992; Romero *et al.*, 1998a). CRF, however, is still important for ACTH release in birds (Carsia *et al.*, 1986; Carsia, 1990; Romero *et al.*, 1998a; Romero *et al.*, 1998b). AVT and CRF when combined produce a synergestic effect i.e. producing a greater response than use alone (Vale *et al.*, 1983; Antoni, 1993). In mammals, the relative importance of each secretagogue often depends on the stressor being applied (Antoni, 1993; Whitnall, 1993; Romero and Sapolsky, 1996). Although ACTH is the primary stimulus leading to corticosterone secretion from the adrenals, some other factors could also regulate corticosterone secretion. Sodium (Redondo *et al.*, 1988), somatostatin (Cheung *et al.*, 1988a), growth hormone (Cheung *et al.*, 1988b), and other peripheral hormones (Harvey and Hall, 1990), can regulate ACTH under some circumstances. These observations do not give a clear insight whether these factors regulate corticosterone release directly from the adrenals or indirectly by modulating either ACTH or ACTH secretagogue function. Furthermore, adrenal tissue can also change its affinity and sensitivity to ACTH, primarily through changes in ACTH receptors (Carsia, 1990).

Stresses whether physical (e.g. infection, thermal exposure, dehydration) or mental (fear, anticipation) activate the hypothalamus to release corticotropin releasing hormone (CRH). The CRH is released into the closed hypophyseal portal circulation, stimulating the pituitary to release ACTH. ACTH is released into the blood where it travels to the adrenals, to induce the synthesis and secretion of glucocorticoids. Released glucocorticoids have a negative feedback effect on the hypothalamus and pituitary that inhibits CRH and ACTH secretions. Disruption of HPA axis is known to contribute to a number of stress related disorders. For example, increased cortisol (hypercortisolism) has been seen in patients with major depressive disorder (Gold and Chrousus, 2002; Juruena *et al.*, 2004), and decreased cortisol (hypocortisolism) has been observed in people with post-traumatic stress disorder (PTSD), post infection fatigue and chronic fatigue syndrome (Demitrack *et al.*, 1991; Crofford *et al.*, 2004; Jerjes *et al.*, 2006). While it is not clear if dysregulation of the HPA axis is a primary or secondary effect of these disorders, there is evidence that stress-related disorders are influenced by early life adverse experiences that affect the neural architecture and gene expression in the brain. Childhood events such as severe infection, malnutrition, physical, sexual and emotional abuse are associated with many chronic illnesses later in life (Turner-Cobb, 2005).

Glucocorticoids are crucial for survival since even mild noxious stimuli can cause death in adrenalectomized rats (Darlington *et al.*, 1990b). Although adrenalectomy completely removes glucocorticoids, thereby removing any permissive effects of basal concentrations, small amounts of replacement glucocorticoids fail to rescue these animals (Darlington *et al.*, 1990a). This implies that glucocorticoid levels above maintenance levels are required to survive noxious stimuli. The importance of glucocorticoids for survival, however, immediately suggests that they play a fundamental role in the Darwinian fitness of an animal. Long-term glucocorticoid exposure, however, can lead to various deleterious effects, including neuronal death (Sapolsky, 1992). Stressed mothers lay eggs with high yolk corticosterone concentrations (Hayward and Wingfield 2004; Saino *et al.*, 2005) and exposure to corticosterone in the yolk enhances HPA function in Japanese quail (Hayward and Wingfield, 2003). Therefore, a balance must be maintained between needing glucocorticoids to survive a noxious stimulus and modulating glucocorticoid secretion to prevent deleterious exposures. Several avian species can seasonally modulate corticosterone release (Wingfield *et al.*, 1992; Wingfield *et al.*, 1997). What induces these changes, be it photoperiod, temperature, food availability, etc., is not clear yet. The findings, however, suggest that both the internal physiological factors (molt) as well as the external environmental factors are equally important in regulating seasonal corticosterone levels (Romero, 2001). Even factors that change behaviour, such as the presence of chicks leading

to parental care, can also modulate corticosterone levels (Wingfield *et al.*, 1992). Regulation of corticosterone levels may be attributed to the HPA axis because there are various levels in the HPA axis that might be subjected to seasonal change (Romero, 2001). The first level is at the adrenal where the adrenal tissue either can be exposed to a lower ACTH signal coming from the pituitary or can lose sensitivity to that ACTH signal. The second level is at the pituitary, where the corticotrophs that release ACTH either can be exposed to a lower secretagogue signal coming from the hypothalamus, or can lose their sensitivity to the secretagogue signal. The third level is at the hypothalamus, where there are at least three possibilities for a regulatory mechanism: fewer secretagogues could be released, the primary secretagogue could be shifted from a more potent (e.g. AVT) to a less potent secretagogue (e.g. CRF) or secretagogue releasing cells could be less sensitive to inputs from higher brain centers. Finally, seasonal changes in corticosterone negative feedback could regulate corticosterone secretion. Birds possess a negative feedback system similar to those of mammals (Dallman *et al.*, 1992).

Corticosteroid binding globulin (CBG)

The adrenal cortex of mammals and the chromaffin cells in adrenals of birds synthesize and release glucocorticoids into the blood circulation under the precise control of the HPA axis (Holmes *et al.*, 1991; Miller and Tyrrel, 1995). In plasma, these hormones are bound to corticosteroid binding globulin, also called transcortin, with high affinity. The bound form functions as a circulating reservoir of hormones that keeps a supply of free hormones available to tissues. CBG also enhances the half-life of the hormone. CBG has been found in every avian species that has been studied (Wingfield *et al.*, 1984; Silverin B, 1986). Corticosterones are released from CBG on reaching the target cells and because of its lipophilic nature can diffuse readily through the plasma membrane and bind to its cognate receptor.

Glucocorticoid receptor (GR)

The biological action of glucocorticoids is mediated by the activation of intracellular GR. GRs are expressed in almost all cell types and their density varies from 2,000-30,000 binding sites per cell (Adcock, 2000). The GR belongs to the superfamily of steroid/thyroid/retinoid acid receptor proteins that function as ligand-dependent transcription factors (Evans, 1988; Beato *et al.*, 1995; Bamberger *et al.*, 1996; Karin, 1998). Cloning of a full-length cDNA for the chicken GR shows that it encodes 772 amino acids and shares high homology with that of

```
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[Homo           MDSKESLTP-GREEN-PSSVLAQERGDVMDFYKTLRGGATVKVSASSPSLAVASQSDSKQ 58
[Gallus         MDSKELLKSSDQDETRKNALMSTKGGIVMDFHPPFRGGASAQAFVSAAPLPVSSQSDSAQ 60
                ***** *     ::*     :::     * ***:: .:****:.:...*:. : .:**:** *

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                ****: ****  :*******::********.**:*:*  *: *.    :      :* * :*

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[Rattus         IDEN-LLSPLAGEDDPFLLEGDTNEDCKPLILPDTKPKIKDTGDTILSSPSSVALPQVKT 297
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                ****************************:*******.:*********:*************:

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Sequence alignment of GR protein from rat (accession no. NP_036708), mouse (NP_032199), human (accession no. CAJ65924) and chicken (accession no. ABB05045). Sequences were retrieved from NCBI database and all the sequences were aligned using ClustalW software from EBI server.

the human (73%), mouse (73%), rat (71%), rabbit (72%), and trout (51%) sequences (Kwok *et al.,* 2007). Multi Sequence Alignment (MSA) data of GR from rat (accession no. NP_036708), mouse (accession no. NP_032199), human (accession no. CAJ65924) and chicken (accession no. ABB05045) by clustalW program from European Bioinformatics Institute (EBI) server also show that chicken GR shares high homology with that of human (73%), mouse (74%), and rat (74%) sequences.

Cytoplasmic GR are bound to protein complexes that include two subunits of the heat shock protein 90 (hsp90) which act as molecular chaperones, and FK-binding proteins, that protect the receptor and prevent its nuclear localization by covering the sites on the receptor that are needed for transport across the nuclear membrane into the nucleus (Wu *et al.,* 2004). There are also evidences of the presence of other associated proteins incuding a 59 kDa immunophilin protein, chaperones (such as p23 and *src*), several kinases of the mitogen-activated protein kinase (MAPK) signaling system and various other inhibitory proteins (Tai *et al.,* 1992; Truss and Beato, 1993; Pratt, 1998; Almawi and Melemedjian, 2002; Wikstrom, 2003). It appears that hsp90 is necessary for ligand binding to GR and may facilitate the proper folding of the GR into an optimal DNA binding conformation (Picard *et al.*, 1990). Hormone binding initiates the release of the chaperone proteins from the GR, allowing dimerization and translocation of the receptor to the nucleus. In the nucleus, the GR binds to glucocorticoid response element (GRE) and can either activate or repress transcription depending on the context of the target promoters. In addition, the GR cross-talks with other transcription factors, such as nuclear factor-κB (NF-κB) and AP-1, to repress their gene activation activities via protein-protein interaction (Beato *et al.*, 1995; Mangelsdorf *et al.*, 1995; McKay and Cidlowski, 1999; De Bosscher *et al.*, 2000). Such GR-mediated repression has been postulated to be one of the molecular bases for anti-inflammatory and immunosuppressive activities of glucocorticoids.

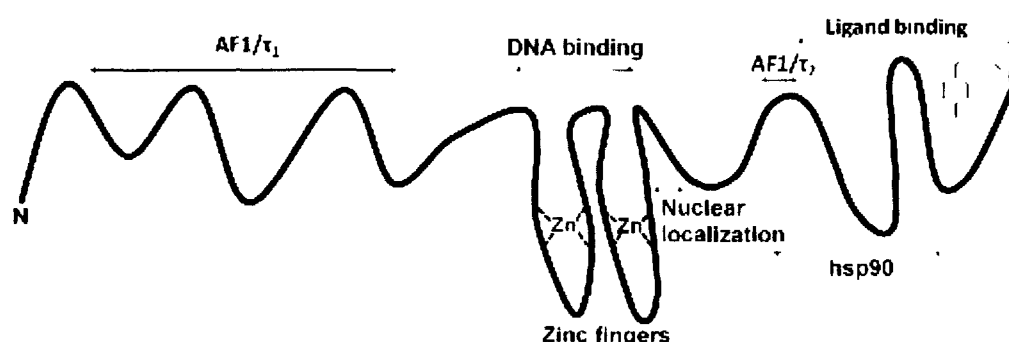
Isoforms of GR

Two human isoforms of GR have been identified, termed GR-α and GR-β, which originate from the same gene by alternative splicing of the GR primary transcript (Hollenberg *et al.*, 1985; Encio and Detera-Wadleigh, 1991; Oakley *et al.*, 1997). GR-α is the predominant isoform of the receptor and the one that shows steroid binding activity (Hollenberg *et al.,* 1985). Its expression has been reported in various tissues and cell types (de Castro *et al.*, 1996; Oakley *et al.,* 1999; Honda *et al.,* 2000). GR-β differs from the GR-α in its carboxy terminus, where the last 50 amino acids of GR-α is replaced by a nonhomologous 15-amino acid sequence (Pujols, 2002). GR-β does bind to glucocorticoids nor transactivate target genes (Hollenberg *et al.,* 1985; Oakley *et al.,* 1996; Hecht *et al.,*1997). The expression of GR-β, both at the mRNA and protein level, seems to be much lower than that of the GR-α

(Oakley *et al.*, 1996; Dahia *et al.*, 1997; Hecht *et al.*, 1997; Oakley *et al.*, 1997; Honda *et al.*, 2000). Although the physiological significance of GR-β is still unknown, an overexpression of GR-β has been reported in glucocorticoid resistant diseases, such as asthma (Leung *et al.*, 1997; Hamid *et al.*, 1999; Sousa *et al.*, 2000), ulcerative colitis (Honda *et al.*, 2000), chronic lymphocyte leukemia (Shahidi *et al.*, 1999) and nasal polyposis (Hamilos *et al.*, 2001). The role of GR-β is not very clear yet but it was found that when GR-β is more abundant than GR-α, it acts as a dominant negative inhibitor of GR-α activity (Bamberger *et al.*, 1995; Oakley *et al.*, 1996) through a mechanism that mostly involves the formation of transcriptionally impaired GR-α-GR-β heterodimers (Oakley *et al.*, 1999). GR-β, as well as GR-α, binds to hsp90, but GR-β-hsp90 complexes are less stable than those of GR-α-hsp90 complexes (Oakley *et al.*, 1999). Alternative splicing event leading to GR-β is minimally activated in most cells and tissues, therefore, GR-β seems unlikely to have any inhibitory effect on GR-α function (Pujols *et al.*, 2002).

Modular domains of GR

The GR consists of three different domains with various functions: an N-terminal activation function-1 domain (AF-1) having transactivation functions, a DNA-binding domain (DBD) that implies a zinc-finger motif and a ligand-binding domain (LBD) containing α-helices which are involved in the formation of the hydrophobic ligand-binding pocket (Giguere *et al.*, 1986; Wikstrom, 2003). The C-terminal part of the LBD is termed the AF-2 helix, and it plays essential role in transactivation with co-activators. Among nuclear receptors, AF-2 helix is extremely conserved whereas AF-1 is quite variable. The three-dimensional structure of isolated domains of several different nuclear receptors has revealed conserved structural motifs for ligand and DNA binding.



MODULAR STRUCTURE OF GLUCOCORTICOID RECEPTOR

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Ligand binding domain

Ligand binding to GR confers conformational alterations in the receptor that is critical for its biological activity. The LBD also exhibits a conserved structure consisting of 10-12 α helices that fold into an anti-parallel helical sandwitch consisting of a central core of helices positioned between helix bundles on either side (Wurtz *et al.,* 1996). This three layered structure creates a wedge shaped hydrophobic cavity in which the ligand is suitably placed. The C-terminal of most α helix (helix 12) extends away from the LBD core in the absence of ligand and undergoes a significant repositioning in response to binding hormone by folding against the core of the LBD. This repositioning of helix 12 seals the entry site of the binding cavity and creates a hydrophobic surface on the LBD that is recognized by coactivators. Other functional determinants have been identified within the broader three domains. In addition to providing a pocket for binding ligand, the LBD contains determinants for binding heat shock proteins (hsps) and immunophilins (Pratt and Toft, 1997), dimerization in the absence of DNA (Glass, 1994) and a ligand-dependent transcriptional activation domain, termed activation function AF-2 or *tau2* (Danielian *et al.*, 1992; Parker, 1995). The very end of AF-2, sometimes called the AF2-core, serves as a direct molecular switch that recruits coactivator proteins and activates the transcription of target genes when flipped into the active conformation by hormone binding (Feng *et al.*, 1998).

DNA-binding domain

The central part of the receptor is the DBD, which also participates in receptor dimerization, nuclear translocation, and transactivation. The structural motif of the DBD is two zinc fingers formed by the coordination of four cysteines to one zinc atom. Site-directed mutagenesis demonstrated that seven of eight cysteines are essential for receptor function (Severne *et al.*, 1988). The major grove of the DNA double helix has been shown to be a contact area (Scheidereit *et al.,* 1986). In particular, the region spanning the carboxy terminal of the first zinc finger, the P box, is believed to be involved in the specificity of the binding to DNA (Umesono and Evans, 1989). The second carboxy terminal zinc finger is also required for DNA binding (Danielsen *et al.,* 1989). Five amino acids at the amino terminal base of the second zinc finger comprise the D-box, which is involved in the homodimerization of the GR by interacting with the equivalent part of the other DBD in a GR homodimer (Umesono and Evans, 1989; Dahlman-Wright *et al.*, 1991). The amino acids responsible for the nuclear localization, the nuclear localization signal (NLS), are adjacent to the second zinc finger. 2D-NMR of free GR in solution and GR bound to DNA revealed the three-dimensional structure of the DNA binding domain (Hard *et al.,* 1990; Luisi *et al.,* 1991). In the DBD a second dimerization domain is present that is dependent on DNA binding. DNA-dependent

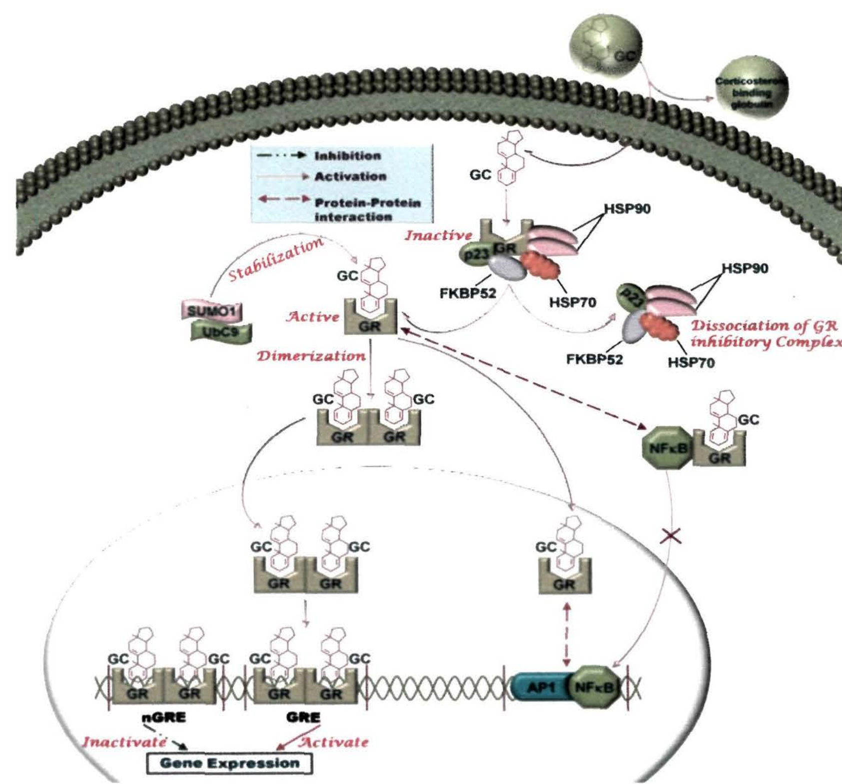
dimerization has the dual role of stabilizing the receptor-DNA complex and acting as a molecular ruler for orientation of the receptor dimer with respect to appropriately spaced DNA response elements (Dahlman-Wright *et al.*, 1991; Freedman, 1992; Zilliiacus *et al.*, 1995).

N-terminal modulatory domain

The N-terminal end of GR is highly variable in sequence and contains the transcription activation function (AF1 or *tau*1) motif. This motif along with other motif, AF2 in the C-terminal end, presumably modulate target genes by interacting with components of the basal transcription machinery, and/ or with coactivators or other transcription factors, largely in a cell- or tissue-specific context. AF1/*tau*1 has been shown to be critical for target gene specificity (Dahlman-Wright *et al.,* 1995). In the estrogen receptor (ER), this region is also known to be regulated by nonendocrine pathways, involving, for example, protein kinases, which are often responsible for cell signaling (Rogatsky *et al.*, 1998; Tremblay *et al.*, 1999). O'Malley's group has presented evidence showing that a particular RNA, called the steroid receptor RNA activator (SRA), selectively interacts with this AF-1 region and acts as a coactivator (Lanz *et al.*, 1999). Kato's group discovered RNA helicase, which is a novel coactivator that bridges the ER and SRA (Watanabe *et al.*, 2001). Moreover, this region, as well as the LBD, has been suggested to be targeted by a member of vitamin D receptor interacting protein (DRIP)/thyroid hormone receptor-associated protein (TRAP) cofactors (Rachez *et al.*, 1998).

Glucocorticoid action mechanism

At the cellular level, glucocorticoid actions are mediated by an intracellular protein, the GR. Unliganded GR resides in the cytoplasm as a multiprotein complex comprising of heat shock protein 90 (hsp90) which binds as a dimer to the C-terminal domain, hsp70, hsp56, p59 immunophilin, FKBP52 and the small p23 phosphoprotein. Binding of hormone releases GR from the complex and the GR specifically binds to and modulate the activity of target gene promoters and is, therefore known as ligand-regulated transcriptional regulatory protein. Glucocorticoids act on the target cells with a mechanism that may be summarized in the following steps:



GLUCOCORTICOID ACTION MECHANISM

i) *Since glucocorticoids are lipophilic, they enter the target cell by passive diffusion through the plasma membrane and bind non-covalently to the high affinity cytoplasmic GR to form hormone-receptor complex.*

ii) *The hormone-receptor complex undergoes activation/transformation, a process that involves conformational changes leading to dissociation of chaperone proteins which results in unmasking of the receptor nuclear localization signal and subsequent nuclear translocation of the activated hormone-receptor complex.*

iii) *Inside the nucleus, the activated hormone-receptor complex dimerizes and interacts with DNA by targeting specific nucleotide palindromic sequences termed glucocorticoid response element (GRE), which could be positive or negative (nGRE) of the target genes.*

iv) *The GR can render chromatin accessibility to other transcription factors.*

v) *The GR can affect other signal transduction cascades through mutual protein-protein interactions with other transcription factors and ultimately, generate cellular response(s) in a non-genomic manner.*

Glucocorticoid actions can be divided into genomic and non-genomic effects mediated by glucocorticoid receptors (Pratt, 1998; Almawi and Melemedjian, 2002; Buttergeit and Scheffold, 2002; Adcock and Lane, 2003; Wikstrom, 2003). Non-genomic glucocorticoid activities can be subclassified further to three modes of action: cytosolic GR mediated non-genomic effects; non-specific non-genomic effects (for example, physical interactions with

the plasma membrane at high glucocorticoid concentrations); and effects that are considered to be mediated by membrane-bound GR (Buttergeit and Scheffold, 2002; Spies *et al.*, 2006).

Genomic mechanism of glucocorticoid action

Glucocorticoids are lipophilic; hence they could easily pass through the plasma membrane of target cells. Glucocorticoids produce their effects on responsive cells by activating GR to directly or indirectly regulate the transcription of target genes. The inactive GR is able to bind to different glucocorticoids with high affinity. The number of genes per cell directly regulated by glucocorticoids is estimated to be between 10 and 100, but many genes are indirectly regulated through an interaction with other transcription factors and coactivators (Barnes, 2006). Formation of the activated hormone-bound GR complex results in the dissociation of the associated proteins from the GR under physiological intracellular condition, a process termed as activation or transformation (Tsai and O'Malley, 1994; Pratt and Toft, 1997; Almawi and Melemedjian, 2002). This glucocorticoid-receptor complex is then translocated to the nucleus where it binds as a homodimer to specific DNA binding sites called glucocorticoid responsive elements, GREs (Almawi and Melemedjian, 2002).

Activation of GR

Once glucocorticoids enter the target cells they interact with its cognate receptor. The GR undergoes a process of activation or transformation whereby the heat shock proteins and other associated proteins dissociate from the GR and the nuclear localization signals are unmasked (Pratt and Toft, 1997). Transformation of steroid receptors has been described to occur under physiological conditions (Munck and Foley, 1979). The existence of GR as inactive complex in the cytoplasm in association with Hsp's is to facilitate the folding of the LBD into a high-affinity steroid binding conformation. The activation of GR is a crucial step in GC action, as it is a rate-limiting step for nuclear or chromatin binding (Bamberger *et al.*, 1996). Activation of the glucocorticoid-bound receptor can be made to occur by several factors like heat, salt, dialysis, gel filtration and elevated pH (Milgrom *et al.*, 1973; Moudgil *et al.*, 1986). The transformed 4S receptor exhibits increased binding affinity for isolated nuclei, chromatin, DNA-cellulose, phosphocellulose and ATP-sepharose (Milgrom *et al.*, 1973; Kalimi *et al.*, 1975). Numerous theories, including dephosphorylation of the receptor protein itself or an ancessory protein (Leach *et al.*, 1979; Barnett *et al.*, 1980), glucocorticoid receptor phosphorylation (John and Moudgil, 1979), dissociation of a low molecular weight inhibitor or modulator (Sekula *et al.*, 1981), limited proteolysis (Miller *et al.*, 1981), and simple subunit dissociation (Vedeckis, 1983) have all been proposed as possible mechanisms

involved in activation. Additionally, activation process may be highly regulated and involve several of these processes occurring either simultaneously or sequentially. Thus, insight with respect to the mechanism(s) of this obligatory step is essential in order to expand our understanding of how hormonal information is transmitted within the target cell. Inhibition of activation process by various inhibitors may provide some understanding about the involvement of any functional group or chemical moiety in the activation process. Nishigori and Toft (1980) first reported inhibition of PR transformation by molybdate. Subsequently, both heat and salt transformation of cytosolic GR were inhibited by molybdate (John and Moudgil, 1979). Tungstate and vanadate were also active in causing inhibition of transformation (Murakami, 1982). The effect of molybdate was reversible, and it was effective only when added before transformation; that is, addition of molybdate after transformation did not influence DNA binding of activated receptor. Studies by Raaka *et al.* (1985) on the effect of molybdate on receptor transformation in intact cells revealed reduced nuclear accumulation of GR after steroid treatment. Polyunsaturated fatty acid inhibits *in vitro* activation of hepatic glucocorticoid receptor in mice (Ranhotra and Sharma, 2004).

Translocation of GR

The nuclear import of the GR is one of the key control points in the regulation of glucocorticoid hormone action. In general, protein transport from the cytoplasm to the nucleus involves nuclear localization signals (NLS), i.e., short peptide sequences that are necessary and sufficient for the nuclear localization of their respective proteins (Gorlich *et al.*, 1995; Nigg, 1997). The nuclear import of the GR is mediated by NL1, a stretch of basic amino acids at the immediate C-terminal end of the receptor DBD, and a second significantly less characterized NLS in the ligand binding domain, NL2 (Picard and Yamamoto, 1987). NL1 of the GR is bipartite, and confers constitutive nuclear localization of the receptor (Picard and Yamamoto, 1987). In contrast, NL2 acts as a dominant negative NLS in the absence of ligands (Picard and Yamamoto, 1987). It is believed that the GR shuttles between the cytoplasm and the nucleus, and subcellular localization of the GR is determined by equilibrium of nuclear import and export. The GR translocates to the nucleus in a ligand- and energy-dependent manner, and nuclear export of the GR also requires adenosine triphosphate (ATP) (Madan and DeFranco, 1993; Tang *et al.*, 1997; Yang *et al.*, 1997). Within the nucleus, the hormone-bound GR binds to the palindromic DNA sequences, called glucocorticoid response elements (GREs), exclusively as a homodimer (Glass, 1994). After binding to DNA, the GR is considered to communicate with basal transcription machinery, interacting with or without other transcription factors and coactivators, and ultimately regulating the target gene (Onate *et al.*, 1995; Katzenellenbogen *et al.*, 1996).

Glucocorticoid response elements (GREs)

Activated hormone-bound GR complex forms a homodimer and binds to GRE in the 5'-upstream promoter region of the glucocorticoid responsive genes. The way in which activated GR seeks out the small number of GREs in approximately 100 000 genes is not very clear yet, but recent findings suggest that the GR dimer binds non-specifically to DNA then attaches to another strand of DNA before dissociating from the first site of attachment. This is repeated until a high-affinity GRE site is encountered (Lieberman and Nordeen, 1997). Binding of GR dimer to GRE changes the rate of transcription, resulting in either induction or repression of the gene. Binding of ligand-activated glucocorticoid receptor to positive GRE results in induced synthesis of anti-inflammatory proteins (e.g. lipocortin 1, IκB), but also regulator proteins that are important for metabolism (for example, enzymes that are involved in gluconeogenesis), thereby exerting many different effects on the cellular, organ and organism level (Ctahn *et al.,* 2007). This process, which is mediated via positive GREs, is termed 'transactivation'. The consensus sequence for GRE binding is the palindromic 15-bp sequence GGTACAnnnTGTTCT (where n is any nucleotide). The negative GREs on the other hand, have a more variable DNA sequence. Transcription of genes can be inhibited by glucocorticoids by direct interaction of ligand-activated GR with negative GREs, such as the pro-opiomelanocortin, α-fetoprotein and prolactin gene (Sakai *et al.,* 1988; Drouin *et al.,* 1993). Glucocorticoids act as anti-inflammatory agents by suppression of transcription of inflammatory genes, like interleukin (IL)-1 and IL-2, via negative GREs (Beato, 1989; Falkenstein *et al.*, 2000).

The exact role of negative GREs in mediating glucocorticoid-induced effects in cellular systems remains largely unclear. Transcription factors can be displaced from their positive GRE, through direct protein-protein interaction between transcription factors and ligand-activated GR. Monomers of ligand-activated GR complex directly or indirectly interact with transcription factors, such as activator protein 1 (AP1), nuclear factor-κB (NF-κB) or interferon regulatory factor (IRF-3) to cause 'transrepression' (Reily *et al.,* 2006). These transcription factors are involved in regulating the expression of pro-inflammatory genes. In this way, glucocorticoids inhibit nuclear translocation and the function of several pro-inflammatory mediators, including cytokines (e.g. IL1, IL2, TNF-α, IFN-γ) and prostaglandins. Repression of gene transcription can also take place by interaction of GR dimer with GRE which overlap with TATA box as seen in the osteocalcin gene (Meyer *et al.*, 1997). Most genes that are repressed by glucocorticoids have no GRE. Apart from these simple GRE, there are 'composite' GRE that do not share these GRE sequences, but depend on the presence of other transcription factors that bind to DNA (Miner and Yamamoto, 1991). GR also binds to less well-defined regions of DNA and regulate promoters that contain no

obvious GRE sequences. Other transcriptional factors that bind in the vicinity of GRE may have a powerful influence on the glucocorticoid inducibility on that particular gene.

GR coactivators

A series of proteins which interact with the LBD converge on a family of related proteins that are collectively termed as p160 coactivators. The p160 family of proteins, termed steroid receptor coactivators (SRCs), consist of three closely related members. SRC-1 (or F-SRC-1 or NCoA- 1) was the first p160 coactivator identified as a protein that interacts with AF-2 of human PR (Onate, 1995). Mouse glucocorticoid receptor-interacting protein-1 (GRIP-1), identified by yeast two hybrid as a GR interacting protein, and the human ortholog TIF-2 (transcriptional intermediary factor-2) constitutes the SRC-2 class of p160 proteins (Voegel *et al.*, 1996; Hong *et al.*, 1997). The SRC-3 class was cloned independently by several groups and was termed co-integrator-associated protein (p/CIP), receptor-associated coactivator-3 (RAC3), activator of thyroid receptor (ACTR), thyroid receptor-activator-1 (TRAM-1) or AIB 1(amplified in breast cancer 1) (Anzick *et al.*, 1997; Chen *et al.*, 1997; Li *et al.*, 1997; Takeshita *et al.*, 1997; Torchia *et al.*, 1997). Interestingly, AIB1 was isolated as an expression sequence from an amplified chromosomal region (20q) in breast cancer suggesting its overexpression may have a role in breast cancer (Anzick *et al.*, 1997). A distinct structural feature of the p160 coactivators is the presence of multiple LXXLL (Where L is leucine and X is any amino acid) signature motifs which comprise determinants for direct interactions with the nuclear receptor LBD (Xu *et al.*, 1999). The p160 coactivators do not themselves appear to have DNA binding activity but are recruited to promoters of steroid responsive target genes via protein-protein interaction with nuclear receptors. They bind directly to a broad range of nuclear receptors in a manner dependent on hormone agonist and the integrity of AF-2. Initial evidence for a biological role of p160 in nuclear receptor function *in vivo* has been provided by disruption of the SRC-1 gene in mice. Mice bearing a homozygous deletion of SRC-1 exhibit partial resistance to multiple hormones including estrogen, progesterone, androgen and thyroid hormone (Xu *et al.*, 1998; Weiss *et al.*, 1999). SRC-1 null mutant female mice exhibit reduced growth and development of the mammary gland and uterus in response to estrogen and progesterone and males show reduced growth and development of the prostate and testis in response to androgen. SRC-1$^{-/-}$ mice, however, are viable and fertile and were reported to overexpress SRC-2 suggesting that different members of the p160 coactivator family have overlapping redundant functions (Xu *et al.*, 1998).

Mechanism of action of p160 coactivators

Chromatin has a repressive effect on transcription that limits accessibility of the general transcriptional machinery (RNA polymerase II and associated general transcription factors) to DNA. Disruption of chromatin structure to alleviate this repressive effect can be mediated by two general pathways: acetylation of core histones and ATP dependent remodeling by the yeast SWI-SNF protein complex and its vertebrate homolog. It has been known for some time that transcriptionally active chromatin is correlated with hyperacetylation of core histones and that hypoacetylation correlates with inactive regions of chromatin. Acetylation of positively charged lysine residues in the N-terminal tails of core histones decreases the affinity of histones for DNA and destabilizes mononucleosome and higher order nucleosomal structure (Pazen and Kadonaga, 1997). Nuclear receptors are capable of recruiting at least three different classes of coactivators that possess intrinsic histone acetylase activity (HAT) including p160 (SRC-1 and SRC-3), cAMP-responsive element binding protein (CBP) and the CBP associated factor p/CAF (Chen *et al.*, 1997). CBP (and closely related p300) was initially identified as a coactivator of CREB (Kwok *et al.*, 1994) and p/CAF, which has significant homology to yeast Gcn5, was initially defined as a protein with HAT activity that interacts with CBP (Yang *et al.*, 1996). Insight into a possible mechanism of p160 coactivation came with the finding that SRC-1 is capable of interacting with the C-terminus of CBP/p300, and together they can coactivate transcription synergistically (Yao *et al.*, 1996). In addition, CBP/p300 itself interacts with the nuclear receptors in a ligand dependent manner, again through the AF-2 domain (Kamei *et al.*, 1996). CBP/p300 and p160 coactivators both possess intrinsic histone acetylase (HAT) activity and therefore may be acting in concert to remodel chromatin and therefore render it accessible to basal transcription machinery.

Cross-talk between signaling pathways

In addition to ligand-dependent activation, extracellular signaling molecules such as peptide hormones, growth factors, and cytokines communicate with their intracellular targets through surface receptors. They activate signal transduction pathways that finally lead to the regulation of gene expression mediated by transcription factors such as c-Fos, c-Jun, cAMP-responsive element-binding protein, and others. The mechanism usually involves phosphorylation of those transcription factors by kinases that are activated as a result of the ligand-receptor interaction at the cell surface. The nuclear receptors are also indicated as being targets of some, but not all, kinases involved in signal transduction, and the phosphorylation of nuclear receptors provides an important mechanism for cross talk

between signaling pathways (Freedman, 1999; Shao and Lazar, 1999). Multiple kinase pathways have been implicated in the modulation of nuclear receptor-mediated gene regulation: cAMP-dependent protein kinase, casein kinase, glycogen synthase kinase (GSK), c-Jun kinase, cyclin-dependent kinases (CDKs), and mitogen-activated protein kinases (MAPKs) (Freedman, 1999; Shao and Lazar, 1999). All aspects of receptor function can be regulated by kinases, including DNA binding and dimerization, transcriptional activity, interaction with cofactors, and ligand binding. In the case of the GR, Ser246 (Krstic *et al.*, 1997) Ser224 and 232 (Krstic *et al.*, 1997) and Thr171(Rogatsky *et al.*, 1998) all of which are located in the AF-1 region, are indicated as being targets of MAPK, CDKs, and GSK-3, respectively.

In a physiological and/or pharmacological context, it should be noted that many effects of glucocorticoids are achieved not only by activation, but also by the inhibition of target gene expression (Reichardt *et al.*, 1998). This is particularly true for the anti-inflammatory and immunosuppressive effects of glucocorticoids that involve the negative transcriptional regulation of proinflammatory genes (Cato and Wade, 1996). This mode of regulation is distinct from the positive regulation and does not necessarily involve the interaction of the GR with GRE, but is achieved by the interaction between the GR and so-called negative GRE (nGRE) (Drouin *et al.*, 1993). On the other hand, the expression of many proinflammatory genes is positively regulated by a certain class of transcription factors, for example, AP-1 and NF-kB (Cato and Wade, 1996). The negative regulation of these genes by the GR is sometimes referred to as "cross-talk" between the GR and these transcription factors, or transrepression (McKay and Cidlowski, 1998). Numerous molecular mechanisms have already been presented to account for such mutually exclusive interactions between transcription factors; e.g., direct protein-protein interaction, the squelching of coactivators, and inhibition of the catalytic activity of enzymes that modulate the transcription factors. Moreover, it has recently been reported that the GRα could heterodimerize with other members of the nuclear receptor superfamily, including the GRβ (Oakley *et al.*, 1996) MR (Liu *et al.*, 1995) and AR (Chen *et al.*, 1997).

Rapid effects of glucocorticoids

It takes about 30 min for activation of GR, nuclear translocation of the GR complex, binding to GREs in the promoter regions of target genes and the initiation of transcriptional and translational processes leading to newly synthesized proteins. It usually takes hours or days before changes on cellular, tissue or organism level become evident. However, some of the immunosuppressive, anti-inflammatory and anti-allergic glucocorticoid effects occur too fast to be explained by the classical, genomic glucocorticoid action (Buttgereit and Scheffold,

2002; Cato *et al.*, 2002; Croxtall *et al.*, 2000; Falkenstein *et al.*, 2000; Hafezi-Moghhadam *et al.*, 2002). Rapid clinical effects have been observed when glucocorticoids are administered intra-venously or intra-articulary at high dose. Efforts over the past few years have led to the understanding of the mechanism of the rapid effects of glucocorticoids. Three different non-genomic mechanisms have been proposed to explain rapid anti-inflammatory and immunosuppressive glucocorticoid effects (Croxtall *et al.*, 2000; Falkenstein *et al.*, 2000; Buttgereit and Scheffold, 2002; Cato *et al.*, 2002; Hafezi-Moghhadam *et al.*, 2002). These include:

1) *Non-genomic effects mediated by the cytosolic GR*
2) *Non-genomic effects mediated by membrane-bound GR (mGR)*

Non-genomic glucocorticoid effects caused by cytosolic GR

Non-genomic glucocorticoid effects are mediated by cytosolic GRs after their interaction with glucocorticoids. As stated before, the unliganded GR is associated with numerous proteins like heat-shock proteins and several kinases (such as MAPKs). Binding of ligand to GR causes the dissociation of all the associated proteins and the nuclear translocation of the hormone-receptor complexes. The release of signaling molecules from the GR-multiprotein complex due to ligand binding, such as *src*, is considered to be responsible for rapid glucocorticoid effects (Croxtall *et al.*, 2000). Another observation relates to arachidonic acid which is an essential mediator of cell growth and many metabolic and inflammatory reactions. Release of arachidonic acid from membrane-associated phospholipids is controlled by different mediators (such as growth factors, adaptor proteins, MAPK and lipocortin 1) and can be inhibited by glucocorticoids by GR-dependent but transcription-independent mechanism (Croxtall *et al.*, 2000). These observations indicate that GR is not only important as a transcription factor, but is also involved in rapid non-genomic glucocorticoids-induced effects.

Non-genomic glucocorticoid effects caused by membrane-bound GR

Glucocorticoids have rapid effects on plasma and mitochondrial membranes. At high concentrations, glucocorticoids intercalate into biological membranes and therefore changes the physicochemical properties as well as activities of membrane-associated proteins (Buttgereit and Scheffold, 2002; Buttgereit *et al.*, 2004). These result in reduced calcium and sodium cycling across plasma membranes of immune cells, which is thought to contribute to rapid immune-suppression and a subsequent reduction of the inflammatory process (Buttgereit and Scheffold, 2002). Glucocorticoids also have direct effects on mitochondrial

membranes since their interactions with mitochondrial membranes enhance proton leaks which contribute to impaired ATP production (Stahn *et al.*, 2007). These rapid glucocorticoid effects on mitochondrial membranes may contribute to clinically relevant outcomes, since ATP is vital for housekeeping activities of immune cells as well as their specific effector functions, such as cytokine synthesis, migration, phagocytosis, antigen processing and antigen presentation (Stahn *et al.*, 2007).

In immune cells, non-genomic glucocorticoid effects involve a membrane-bound glucocorticoid receptor (Stahn *et al.*, 2007). The existence of this receptor has been shown for the first time in amphibian neuronal membranes and in lymphoma cells (Gametchu *et al.*, 1999). Buttergeit *et al.* (2004) showed the existence of membrane-bound GR on human blood mononuclear cells by using high-sensitive immunofluorescent staining. Studies on the overexpression of cytosolic GR does not show any increased in membrane-bound GR, therefore it is assumed that membrane-bound GR is a variant form of GR produced by differential splicing or promoter switching or by post-translational editing (Barthalome *et al.*, 2004). The origin, the detail mechanism of action and function of this receptor still remains unexplained. Stahn *et al.* (2007) reported that stimulation with liposaccharide increases the percentage of membrane-bound GR positive monocytes, which could be prevented by inhibiting the secretory pathway. Therefore, immunostimulation could be responsible for the up-regulation and trans-cellular transport of membrane-bound GR (Barthalome *et al.*, 2004). Clinical data revealed that in patients with rheumatoid arthritis there is a correlation with the increased number in membrane-bound GR positive monocytes and B-lymphocytes with different parameters of disease activity (i.e. higher disease activity scores correlate with increased number of membrane-bound GR positive cells) (Barthalome *et al.*, 2004; Buttgereit *et al.*, 2005). A similar increase in number of membrane-bound GR positive monocytes and B-lymphocytes was also observed in other diseases like ankylosing spondylitis and systemic lupus erythematosus (Tryc *et al.*, 2006; Spies *et al.*, 2006). These observations demonstrate that membrane-bound GR may play a role in the pathogenesis of chronic inflammatory diseases. Since glucocorticoid-induced membrane-bound GR mediated apoptosis has been reported (Gametchu *et al.*, 1999), the up-regulation of membrane-bound GR due to immunostimulation or in case of disease condition could be considered as a protective mechanism (Stahn *et al.*, 2007).

Although pharmacological evidence for a membrane corticosteroid receptor in birds is sparse, there is substantial behavioural evidence for their existence. Rapid ($<$20 min) behavioural effects of corticosteroids, consistent with a non-genomic mechanism of action, have been demonstrated in white-crowned sparrows and chickens. In captive white-crowned sparrows, corticosterone increases perch-hopping activity within 15 min (Breuner *et al.*,

1998; Breuner and Wingfield, 2000). The rapid actions of glucocorticoids are also supported by the work of Sandi and Rose. (1997) which reported the effect of corticosterone on memory formation in day-old chicks learning a passive avoidance tasks. Chicks trained on a strong-aversion task showed an increase in circulating corticosterone, and retained the aversion 24 h later. In contrast, chicks trained on a weak-aversion, showed no elevation of corticosterone during the trial, and showed no aversion 24 h later. However, intracranial administration of corticosterone during the weak-aversion learning trial could retain the aversion 24 h later. In a similar experiment, corticosterone had to be injected before, or just before 15 min for the learning trial to be effected. These data supported that corticosterone has a rapid effect on memory formation in birds as well.

It is evident from the foregoing literature that the glucocorticoid-mediated signal transduction process is very important in the growth, development and the maintenance of the overall homeostatic balance in higher organisms. The GR after activation by a ligand, elicits pleiotropic and conditional regulation of gene expression which may allow the fine tuning of cellular metabolic processes and stress responses.

The entire work was performed with the following objectives:

i) Determination of age- and tissue-specific endogenous level of GR at various postnatal ages (0-, 5-. 10-, 30-, 60- and 90-day) of chicken. The level of GR was confirmed by performing slot blot analysis using polyclonal rabbit anti-GR-antibody raised against amino acid sequence from the central region of GR. Apart from monitoring the changes in the level of receptors, the affinity of the receptor for the hormone may vary at different postnatal ages, hence, Scatchard analysis was performed to see if there is any change in the dissociation constant at selected postnatal ages of chicken.

ii) The activation of GR by heat and salt and its inhibition by molybdate, tungstate, cadmium and oleic acid, at two selected ages (0- and 30-day) and in two different tissues (liver and kidney) were studied, using DNA-cellulose and purified nuclear binding assays, to assess the age and tissue related changes. Nuclear binding assays were performed to determine whether the changes in binding properties of hormone-receptor complexes to purified nuclei was due to the receptor or changes in nuclear chromatin properties. DNase I digestion studies of the nuclear chromatin were done to ascertain the change in chromatin organization, which may influence tissue responsiveness to glucocorticoids.

iii) The physicochemical properties (molecular weight, stoke's radii and charge content) of the unactivated and activated GR were determined to see if there is any age-related change in these properties.

EXPERIMENTAL PROCEDURES

MATERIALS

Animals

Male chicken (Rhode Island Red, RIR breed) were locally purchased 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 various postnatal age groups (0-, 5-, 10-, 30-, 60- and 90-day) were used for experimentation. Institutional guidelines were followed during entire period of experimentation.

Chemicals and reagents

All chemicals used in the experiments, including biochemicals were of highest analytical grade and were obtained from the following companies.

Amersham Biotech, England: [1,2,4,6,7-^{3}H] dexamethasone (specific activity, 83 Ci/mmol).

Sigma Chemical Company, St Louis, USA: Non radioactive dexamethansone, Tris, sodium molybdate, sodium tungstate, cadmium chloride, calf thymus DNA, dimethyl sulfoxide (DMSO), oleic acid (C18:1), EDTA, dithiothreitiol, β-mercaptoethanol, Triton X-100, DNase I, diphenylamine, dextran T-70, DNA-cellulose, bovine serum albumin, molecular weight markers, Sephadex G-100, G-200, anion exchanger DE-52, nitrocellulose membrane (0.45 µm pore size), activated charcoal, and gel filtration molecular weight markers (MW 12.4-443 kDa).

Qualigens, India: Sucrose, glycerol, sodium chloride, potassium chloride, sodium hydroxide, hydrochloric acid, acetic acid, orthophosphoric acid, calcium chloride, magnesium chloride, potassium di-hydrogen phosphate and di-potassium hydrogen phosphate.

Bengal chemicals and Pharmaceuticals, India: Ethanol.

Whatman, England: Qualitative filter papers.
Merck, India: Acetaldehyde.
Sisco Research Laboratories, India: Scintillation cocktail-W
Bangalore Genei, India: Goat-antirabbit-IgG-ALP conjugate, Tween-20, BCIP/NBT.

Instruments and apparatus

Tissues were homogenized in Remi tissue homogenizer (model RQ-127A). All the centrifugations were done using Hitachi (Himac CR20B2 model) and Beckman Coulter (Allegra ™ 64R) high-speed refrigerated centrifuges. Absorbance measurements were done with a Hitachi U-2000 double beam UV/visible spectrophotometer, using glass and quartz cuvettes of 1 cm path length. Tissues were stored at -80ºC in Heto ultra freezer until use. pH meter was from Control Dynamics , India. All radioactive countings were done in Wallac 1409 liquid scintillation counter having an efficiency of 68 % for tritium. The slot blotting was performed on a Bio-Rad Bio-Dot® SF Micro filtration apparatus following the instructions given in the user's manual.

Buffers

All buffers were prepared in double distilled water and the pH adjusted at room temperature. Buffers used were as follows:

A. For assay of glucocorticoid receptor

0.25 M sucrose/ 10 mM Tris-HCl, pH 7.5/ 1 mM EDTA/ 10 mM Na_2MO_4/ 10% (v/v) glycerol / 1 mM DL-dithiothreitol/ 10 mM NaCl

B. For activation/ inhibition/ DNase I digestion studies

(i) 0.25 M sucrose/ 10 mM Tris-HCl, pH 7.6
(ii) 0.25 M sucrose/ 10 mM Tris-HCl, pH 7.6/ 0.5% (v/v) Triton X-100
(iii) 0.25 M sucrose/ 10 mM Tris-HCl, pH 7.6/ 4.2 mM $MgCl_2$

C. For gel filtration studies

(i) 100 mM KH_2PO4, pH 7.5/ 1 mM EDTA/ 1 mM β-mercaptoethanol/ 20 mM Na_2MO_4
(ii) 100 mM KH_2PO4, pH 7.5/ 1 mM EDTA / 1 mM β-mercaptoethanol/ 20 mM Na_2MO_4/ 300 mM KCl

D. Ion-exchange studies

10 mM KH_2PO_4, pH 7.5/ 1 mM β-mercaptoethanol/ 5 mM Na_2MO_4. For eluting the bound proteins, 0-400 mM KCl in the same buffer was used.

METHODS

[^{3}H]dexamethasone binding studies

Tissue preparation

Chicken were killed by decapitation at a fixed time of the day (12:00 h) to avoid any circadian variation. The animals were killed as soon as they were taken out of the cages to prevent adrenal stimulation. The required tissues were quickly excised, freed of fat and connective tissues and washed in chilled normal saline (0.9% NaCl). Tissues (liver, kidney, brain, heart and skeletal muscles) were then blotted dry and stored separately at -80°C until use. All the experimental procedures were carried out at 2-4°C, unless otherwise mentioned.

Preparation of cytosol

A 20% (w/v) homogenate of the minced tissues was prepared in chilled buffer A using a motor driven Potter-Elvejham homogenizer. The homogenates were then subjected to centrifugation at 2000 × g for 10 min at 2°C and the nuclei and other cellular debris were discarded. The supernatants were further centrifuged at 40,000 × g for 45 min at 2°C to obtain a clear fat free cytosol. The clear cytosols thus obtained were used for the assay of glucocorticoid receptors.

Determination of saturable [^{3}H]dexamethasone binding

To determine the concentration of [^{3}H]dexamethasone required to obtain maximum saturable binding, 100 µl aliquots of cytosol were incubated in 1.5 ml eppendorf tubes with 2.5-100 nM [^{3}H]dexamethasone. This provided the values for total binding (T). Parallel tubes containing 500-fold molar excess of unlabelled dexamethasone in addition to [^{3}H]dexamethasone were used to obtain the non-specific (NS) binding. All the tubes were incubated for 6 h in ice, with gentle vortexing of the tubes after every 30 min to ensure proper binding of the hormone to receptor. At the end of incubation period, the unbound hormone was removed by the addition of 50 µl of ice-cold dextran-coated charcoal (DCC, 4% activated charcoal + 0.4% dextran T-70 in buffer A) (Beato and Feigelson, 1972). The contents were mixed properly and after 10 min of incubation, the charcoal was pelleted by centrifugation at 2000 × g for 10 min. Hundred µl of the supernatant, devoid of any charcoal particle, was carefully pipetted out into scintillation vials and to it 4 ml of scintillation cocktail added. The contents were thoroughly mixed and the radioactivity measured in a Wallac 1409 liquid scintillation counter with an efficiency of 68% for tritium. The values for specific-binding of [^{3}H]dexamethasone at each concentration point were calculated as follows:-

Specific [^{3}H]dexamethasone bound= Total bound (T) – Nonspecific bound (NS)

The values, thus obtained were plotted against the respective concentration of [^{3}H]dexamethasone used and the curve provided the optimal concentration of [^{3}H]dexamethasone required to give maximum saturable binding.

Time kinetics for maximum binding

To obtain the time required for maximum saturable binding, assays were performed similar to that described above, except that [^{3}H]dexamethasone was used at a fixed concentration of 60 nM. The hormone-binding was allowed to occur for different periods of time- 0.5 to 10 h. Specific binding, at each time interval, was determined after removal of free hormone by dextran- charcoal method as described above. The data obtained were plotted against different time intervals and the curve provided the time required to obtain maximum saturable binding.

These two values, i.e., the saturable concentration of [^{3}H]dexamethasone and the time required to obtain maximum saturable binding were then use to determine the level of the glucocorticoid receptor in different tissues and at the different postnatal ages of chicken.

Determination of the glucocorticoid receptor level

Glucocorticoid receptor levels in various tissues of chicken and at different postnatal ages were obtained from 5-6 separate chicken of each age group. The assays were performed under identical conditions to minimize the effects of any external variation. Cytosols from different tissues of chicken were obtained as above, and used for determination of specific saturable binding of [^{3}H]dexamethasone at various postnatal ages. Radio receptor assay was performed using the method of (Kalimi *et al.,* 1983; Sharma and Timiras, 1987; Borbhuiya and Sharma, 1995; Ranhotra and Sharma, 2001; Dutta and Sharma, 2003; Nongbri and Sharma, 2007).

For total binding	For non-specific binding
100 µl Cytosol	100 µl Cytosol
20 µl [^{3}H]dexamethasone	20 µl [^{3}H]dexamethasone
(To give a final concentration of 60 nM)	
20 µl buffer	20 µl non-radioactive dexamethasone (Final concentration- 500-fold excess to that of [^{3}H]dexamethasone)

The contents were mixed by gentle vortexing and incubated for 4 h at 2°C, with vortexing at regular intervals to ensure proper binding. At the end of the incubation period, 50 µl of ice-cold dextran-coated charcoal (4% activated charcoal + 0.4% dextran T-70 in buffer A) was added to each tube and incubated for 10 min at 2°C to remove any unbound steroid (Beato and Fiegelson, 1972). The charcoal was then pelleted by centrifugation at 2000 × g for 10 min at 2°C. 100 µl of the charcoal free cytosol was carefully pipetted into scintillation vials

and 4 ml of cocktail-W added. The contents were thoroughly mixed and the bound radioactivity (CPM) measured in the liquid scintillation counter. Specific binding of [^{3}H]dexamethasone was calculated by substracting the radioactivity bound in the presence of 500-fold excess unlabeled dexamethasone (non-specific binding) from that bound in the presence of labeled dexamethasone alone (total binding) and expressed as fmol/ mg protein. The protein content in the final reaction mixture for each set of assay was determined according to Bradford's dye-binding method (Bradford, 1976) (Appendix I). Details of the conversion factor are given in Appendix II). The data obtained were statistically analysed to obtain the mean and the standard deviation values. The level of significance (p-value) between the data obtained for each age group was obtained using paired student's t-test.

Scatchard analyses

Scatchard analyses of the binding data were performed according to the method of Scatchard (1949). For each set of experiment, the tissues were pooled from 5-6 chicken of the same age group and the cytosol obtained as described above. The experimental protocol was similar to that described above except the assays were performed with increasing concentration of [^{3}H]dexamethasone (from 5-120 nM). Parallel tubes containing 500-fold molar excess of unlabelled dexamethasone to that of the respective [^{3}H]dexamethasone were prepared for each concentration point. Specific binding, $[S]_{bound}$ (i.e., total binding – non-specific binding) was determined for each concentration of [^{3}H] dexamethasone used. The total hormone concentration, $[S]_{total}$ added to each tube was also determined. Free hormone concentration, $[S]_{free}$ was obtained by subtracting $[S]_{bound}$ from $[S]_{total}$. The ratio of $[S]_{bound}/[S]_{free}$ was plotted against specific bound ($[S]_{bound}$) to give a linear regression plot. The intercept of the plot on the X-axis gave the maximal specific binding sites (B_{max}) and the slope gave the dissociation constant (k_d).

GR slot blot analyses

Polyclonal rabbit anti-GR-ab, raised against amino acid (406-422) sequence (SVFSNGYSSPGMRPDVS) from the central region of the rat-GR was a gift from Profs. N. Katunuma and H. Kido, Japan. The immunoreactivity with chicken GR was ascertained using immunoprecipitation of GR at various dilutions of rat antibody: and that gave nearly 55% cross-reactivity with chicken GR. The blotting was performed on Bio-Rad Bio-Dot® SF Micro filtration apparatus following the instructions given in the user's manual. Clear fat free cytosol was used for the slot blotting experiment. A nitrocellulose (NC) membrane was soaked overnight in ddH_2O for activation and proper binding. After placing the NC membrane in the slot blot apparatus, the slots used were rehydrated with ddH_2O for uniform binding. To each slot, 100 µl of cytosol adjusted to equal protein (50 µg) were applied and allowed to filter through the NC membrane by gentle vacuum. The NC membrane was then placed in

blocking solution (5% non-fatty milk in TBS) for an hour. It was washd in TTBS (20 mM Tris-HCl, pH 7.5/ 500 mM NaCl/ 0.05% Tween-20) twice and was kept for overnight incubation with the anti-GR-ab (1:500). After washing the membrane twice in TTBS, it was transferred to goat anti-rabbit IgG-alkaline phosphatase conjugate (1:3000) solution and kept for 3 h. The membrane was later washed twice in TTBS and finally in TBS (20 mM Tris-HCl, pH 7.5/500 mM NaCl) to remove the detergent. The substrate (BCIP/NBT) was added in TBS solution (1:1500) to the membrane and after the development of colour, the reaction was stopped by washing the membrane in ddH2O. It was then scanned using hp Scannerjet 7400 c. The net intensity of each band was ascertained using KDS-1D software (Kodak).

Activation studies

Activation studies were performed in two tissues, liver and kidney and at two postnatal ages, 0-and 30-day old male chicken. The magnitude of glucocorticoid receptor activation was judged using DNA-cellulose as well as purified nuclei binding assays.

Preparation of activated [^{3}H]dexamethasone-receptor complexes

Chicken were killed by decapitation and the tissues eviscerated, immediately, freed of all fat and connective tissues and rinsed in ice-cold saline. A 20% (w/v) homogenate of the tissues, pooled from 5-6 chicken of each group was prepared separately in Buffer B (i). The homogenates were centrifuged at 2000 × g for 10 min at 2°C to sediment the nuclei. The crude nuclear pellet was processed further to obtain purified nuclei. The supernatants were centrifuged at 40,000 × g for 45 min at 2°C and to the clear, fat free cytosols were added [^{3}H] dexamethasone to a final concentration of 60 nM. The contents were mixed by gentle vortexing and incubated for 4 hr. At the end of that period, 500 µl of chilled DCC [4% activated charcoal + 0.4% dextran T-70, prepared in the buffer B (i)] was added and the charcoal thoroughly dispersed in the cytosol by vortexing. After 10 min, the charcoal was pelleted and the supernatants pipetted into ice-cold test tubes. Aliquots of the cytosol, containing [^{3}H]dexamethasone-receptor complexes were subjected to the following treatments for 45 min to obtain the activated complexes, as described by Sharma and Timiras (1987):

- Incubated at 25°C to give thermally activated receptor complexes
- Incubated with 25 mM $CaCl_2$ at 0°C to give salt activated receptor complexes
- Incubated at 0°C to provide the unactivated hormone-receptor complexes

DNA-cellulose binding assay

The method of Kalimi *et al.*, (1975) was employed for DNA-cellulose binding assay. Commercially available DNA-cellulose (containing 3 mg DNA/g DNA-cellulose) was

suspended in buffer B (i) and allowed to stand overnight at 2°C. The mixture was then vortexed to give homogenous slurry, from which aliquots (100 µl) containing 100 µg DNA were transferred to 1.5 ml microcentrifuge tubes and a further 1 ml buffer B (i) was added. The cellulose suspension was pelleted by centrifugation at 2000 × g for 10 min at 2°C and the supernatant was discarded. Equal aliquots of activated and unactivated hormone-receptor complexes were added separately in duplicate to cellulose pellets and the mixture gently vortexed and incubated for 60 min at 0°C. The reaction mixtures were regularly vortexed to keep the cellulose in suspension. The reaction was stopped by addition of 1 ml ice-cold buffer B(i) followed by centrifugation at 2000 × g for 10 min at 2°C. The cellulose pellets thus obtained were washed twice with the same buffer. To the final pellet was added 1 ml of cocktail-W and transferred to scintillation vials, to which 3 ml cocktail was again added and the content was thoroughly mixed. The radioactivity bound in the pellet was counted and results were expressed as [^{3}H]dexamethasone-receptor complex bound to DNA-cellulose (CPM/100 µg DNA).

Nuclear binding assay

For nuclear binding assay, crude nuclear pellets obtained earlier were further processed (Eberhardt *et al.*, 1978). To the pellet was added 1 ml of ice-cold buffer B (ii) and the content gently homogenized at low speed and subsequently pelleted at 2000 × g for 10 min at 2°C. The pellet was then washed thrice in buffer B (i) followed by centrifuguration at 2000 × g for 10 min at 2°C and the final pellet thus obtained was suspended in buffer B(i) to give a homogenous slurry. Aliquots of which containing 100-150 µg of DNA were transferred to microcentrifuge tubes and washed in buffer B (i). Tubes were then subjected to centrifugation at 2000 × g for 10 min at 2°C to give the final purified nuclear pellet and the supernatant was discarded. Equal aliquots of unactivated and activated hormone-receptor complexes were added, in duplicate to the nuclear pellets and the content gently vortexed to keep the pellet in suspension for better interaction. Nuclear exchange assays (cross-mixing experiments) were also performed, wherein the heat (25°C for 45 min) and salt (25 mM $CaCl_2$ at 0°C) activated hormone-receptor complexes from 30-day old chicken were allowed to interact with the nuclei of 0-day old chicken and *vice-versa.* The nuclear binding was allowed to occur for 60 min at 2°C with regular vortexing for proper interaction. Subsequent processing of pellets and counting of bound radioactivity was performed as indicated for DNA-cellulose binding assay. The concentration DNA in the nuclear pellet was estimated according to the diphenylamine method of Burton (1956, 1968) (Appendix III). Finally, the results were expressed as [^{3}H]dexamethasone-receptor complex bound to nuclei (CPM/100 µg DNA).

DNase I digestion studies

DNase I digestion studies were performed on the purified nuclei obtained from both liver and kidney of chicken of two postnatal ages (0- and 30-day), according to a modified method of Chaturvedi and Kanungo (1983). Heat activated (25ºC) [^{3}H]dexamethasone-receptor complexes from both the tissues and ages, were allowed to interact with their respective purified nuclei, as described above. After washing off the unbound hormone-receptor complexes, the pellets were incubated with DNase I in a total volume of 100 µl at 2°C for 45 min. The DNase I was dissolved in buffer B (iii) and used at a concentration of 150 units/100 µg DNA. The control tubes received 100 µl of buffer only (Dutta and Sharma, 2003). The nuclear pellets were properly mixed by gentle vortexing at regular intervals and the reaction was stopped by adding 1ml of buffer B (i) followed by centrifugation at 2000 × g for 10 min at 2ºC. The pellets were processed and the bound radioactivity determined as described above. The results were then expressed as % [^{3}H]dexamethasone-receptor complexes bound to nuclei. Control was taken as 100% bound.

Inhibition of activation studies

For inhibition of activation studies, hormone-receptor complexes were prepared from the liver and kidney of 0- and 30-day old chicken as described above. The magnitude of inhibition was assessed using DNA-cellulose and purified nuclei. Hormone-receptor complexes were subjected to heat and salt activation in the presence of molybdate (0-100 mM), tungstate (0-20 mM), cadmium (0-6 mM) and oleic acid (0-200 µM). These inhibitors were prepared as stock in buffer B (i), except for oleic acid (prepared in dimethylsulfoxide, DMSO) and added to aliquots of hormone-receptor complexes to give the final concentration as indicated. Control tubes received the buffer (minus inhibitors) and DMSO (instead of oleic acid). The binding assays, subsequent processing of the DNA-cellulose and nuclear pellets and the determination of bound radioactivity were performed as described above. Results were expressed as % inhibition. Controls (minus inhibitors) were attributed 0% inhibition.

Physicochemical characterization of glucocorticoid receptor

Physicochemical properties of glucocorticoid receptors were studied in the liver of chicken at two postnatal ages (0- and 30-day) to see if there is any age-related difference in various physicochemical parameters (molecular weight, stokes radius and net charge content) of the receptor. To determine the molecular weight and stokes radii of the unactivated as well as activated [^{3}H]dexamethasone-receptor complexes, gel filtration analyses were carried out using sephadex G-200 and G-100, respectively. To study the charge content, anion exchange chromatography of the unactivated and activated receptors were done. All the gel

filtration and ion-exchange chromatography procedures were carried out in the cold at 2-4°C, unless otherwise mentioned.

Gel filtration analyses of the unactivated receptor

The dry sephadex G-200 was suspended in distilled water and allowed to swell overnight at 25°C. The gel fines were thoroughly removed by repeated washing of the gel and decanting off the supernanant. A thick gel slurry was prepared and degassed for 1 h, this was then poured into a glass column (1.8 x 90 cm) kept in the cold. The gel, after being allowed to stand for some time, was equilibrated with buffer C (i), using a peristaltic pump attached to the lower end of the column. The buffer was pumped into the column at a flow rate of 20 ml/h and equilibration was continued till the pH of the eluent was same as that of the buffer used. After proper equilibration, the column was calibrated using [^{3}H]dexamethasone, blue dextran 2000 and standard molecular weight markers at a flow rate of 12 ml/h. [^{3}H]dexamethasone-receptor complexes from the liver of 0- and 30-day old chicken were prepared in buffer A as mentioned and the unbound hormone removed using dextran-coated charcoal. Aliquots (2 ml) from the two ages containing approximately the same radioactivity were loaded onto the column separately and eluted with buffer C (i) at 12 ml/h flow rate. Fractions of 2 ml were collected and 100 µl aliquots from each fraction transferred to scintillation vials, 4 ml cocktail-W added and the radioactivity counted for each fraction.

The elution volume of [^{3}H]dexamethasone provided the total gel volume (**V_p**) and that of blue dextran, the void volume (**V_o**). The distribution coefficient (**K_d**) and the available distribution coefficient (**K_{av}**) of the marker proteins and the unactivated receptors were calculated with the help of the following equations.

$$K_d = (V_e - V_o) / (V_i)$$

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

Where, **$V_i = V_p - V_o$**; **V_e** = elution volume of the marker proteins and the sample and **$V_t = \pi r^2 l$; r** = radius of the column and **l** = length of the gel bed. The apparent molecular weights and stokes radii of unactivated receptors, from both the ages were calculated (Andrews, 1970; Porath, 1963; Laurent and Killander, 1964) and plotted.

For molecular weight	**For stokes radii**
V_e / V_o Vs. log M	**$-(\log K_{av})^{1/2}$ Vs. R_s**
$M^{1/3}$ Vs. $K_d^{1/3}$	**$K_d^{1/3}$ Vs. Rs**

Gel filtration analyses of the activated receptor

The molecular weights and stokes redii of heat activated glucocorticoid receptors were analyzed on a sephadex G-100 column (3 x 40 cm). Gel was prepared and the column was

set and equilibrated with buffer C (ii) at a flow rate of 20 ml/h. After equilibration, the column was calibrated using [^{3}H]dexamethasone, blue dextran 2000 and standard low molecular weight markers, at a flow rate of 12 ml/h. The [^{3}H]dexamethasone-receptor complexes from the liver of 0- and 30-day old chicken were prepared in buffer B (i) and activated at 25°C for 45 min. Aliquots (2 ml) from the two ages containing approximately the same radioactivity were loaded onto the column separately and eluted with buffer C (ii). Fractions of 2 ml were collected and radioactivity in 100 µl, from each fraction counted. The apparent molecular weights and the stokes radii were determined as that for the unactivated receptors.

Ion exchange chromatography of unactivated and activated receptors

To determine the difference in the net charge content of unactivated and activated glococorticoid receptors from 0- and 30-day old chicken, anion exchange chromatography on DEAE-cellulose (DE-52) was done according to the procedure of Grandics *et al.,* 1984; Borbhuiya and Sharma, 1995). The ion exchange resin was suspended in 0.5 N HCl followed by extensive washing with distilled water to neutral pH. The resin was resuspended in 0.5 M NaOH followed by extensive washing with distilled water to neutral pH. A glass syringe of 5 ml capacity was used as a column. A thin film of dextran-coated charcoal ($\approx$2 mm) was layered at the bottom of the column. The washed resin was then gently poured over the charcoal layer and allowed to settle under pressure to give the gel bed a height of 3 cm. The resin was then washed extensively with buffer D, in the cold at a flow rate of 25 ml/h till the pH of the eluent equals that of the buffer. Unactivated [^{3}H]dexamethasone-receptor complexes, from the liver of both age groups were prepared in buffer B (i) and 2 ml of it loaded onto column. After sample application, the column was washed with 30 ml of buffer D to remove all the unbound proteins. The bound receptors were subsequently eluted with a 50 ml linear gradient of KCl (0-400 mM in buffer D), applied with the help of a gradient mixer at 25 ml/h flow rate. Fractions of 1 ml were collected and 100 µl from each fraction transferred to scintillation vials and 4 ml cocktail-W added. The radioactivity in each fraction was counted and from the elution plot, concentration of salt at which the receptor peak eluted was determined. A similar set of experiment provided the data for the heat activated receptors from both groups of animals.

Results obtained from the experiments are plotted as line and bar diagrams. Each data point represents the mean value ± standard deviation. In some cases, results are presented in a tabular form. Results obtained from two sets of data were statistically analyzed according to Student's *t*-test, with $P< 0.05$ taken as significant.

Glucocorticoid receptor binding studies

Concentration of [^{3}H]dexamethasone and the time required to obtain maximum saturable binding.

Fig. 1 depicts the kinetic of [^{3}H]dexamethasone binding by glucocorticoid receptors using increasing concentrations of [^{3}H]dexamethasone from 2.5-120 nM. The specific binding increases with increasing hormone concentration upto about 60 nM but, thereafter there is no significant increase in the binding of [^{3}H]dexamethasone by glucocorticoid receptors. This shows that maximum specific saturable binding was obtained at ~60 nM of [^{3}H]dexamethasone.

The time kinetics for [^{3}H]dexamethasone binding is shown in fig. 2. It is observed that the maximum saturable binding, using 60 nM [^{3}H]dexamethasone was attained by about 4 h of reaction time. Each data point in the figures represent the mean value obtained from four separate assays.

Glucocorticoid receptor level at various postnatal ages

The glucocorticoid receptor levels, expressed as fmol/ mg protein were determined using high-affinity [^{3}H]dexamethasone binding in the liver, kidney, cardiac muscle, skeletal muscle and the cerebral hemisphere at various postnatal ages (0-, 5-, 10-, 30-, 60- and 90-day) of male chicken. In the **liver**, the receptor level reaches a peak value by day 5 of postnatal age, which is significantly higher (+ 42%) than the value observed at day 0 of postnatal age. The receptor concentration values then show a gradual decline with age, i.e. – 25% at day 10 and – 18% at day 30 of postnatal age. The receptor levels at day 60 and 90 of postnatal age are similar to the values that of day 30 (Fig. 3). The slot blot analyses of receptor preparation (Fig. 4A), as well as the net intensity of the slot bands (Fig. 4B) confirmed the increased level of GR protein at day-5, as compared to day-0 of postnatal age. In the **kidney**, the receptor level is maximum in the early postnatal age (0-day). Thereafter, the receptor concentration declines by 18% at day 5 and remains unchanged up to day 30 of postnatal age. By day 60, the receptor level again decline by 16% and then remains unchanged up to day 90 of

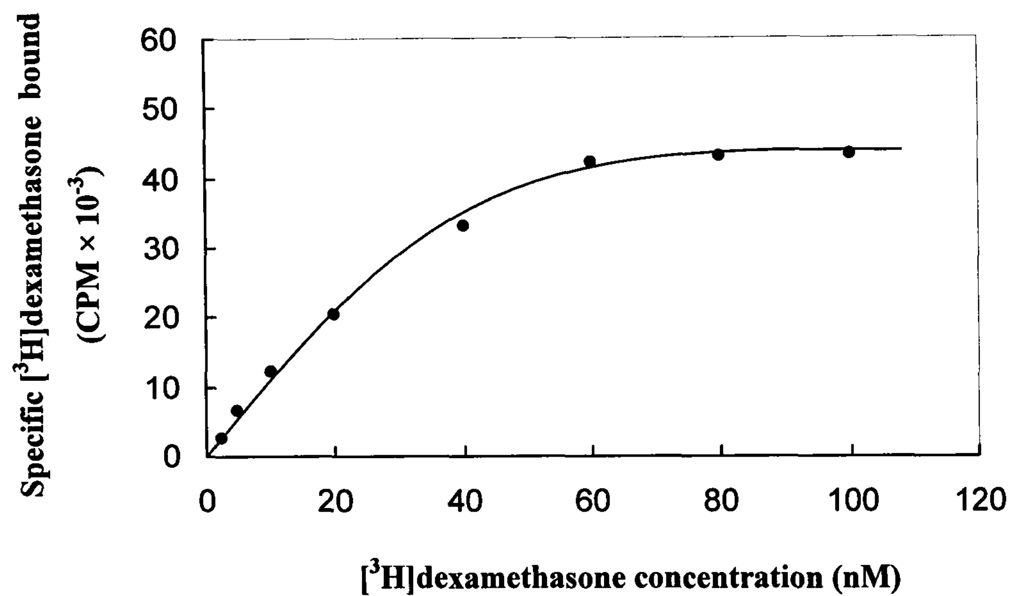


Fig. 1. Concentration dependence of [^{3}H]dexamethasone binding to glucocorticoid receptors in the liver of chicken. Aliquots of cytosol (100 μl) were incubated with increasing concentrations of [^{3}H]dexamethasone ± 500-fold excess non-radioactive dexamethasone for 6 hr at 2°C. Unbound hormones were removed by dextran coated charcoal (DCC) treatment as described in the Materials and Method section. Specific binding was calculated as the difference between total binding and that in presence of non-radioactive hormone. Each point represents the mean value of 4 separate set of experiments, each performed in duplicate.

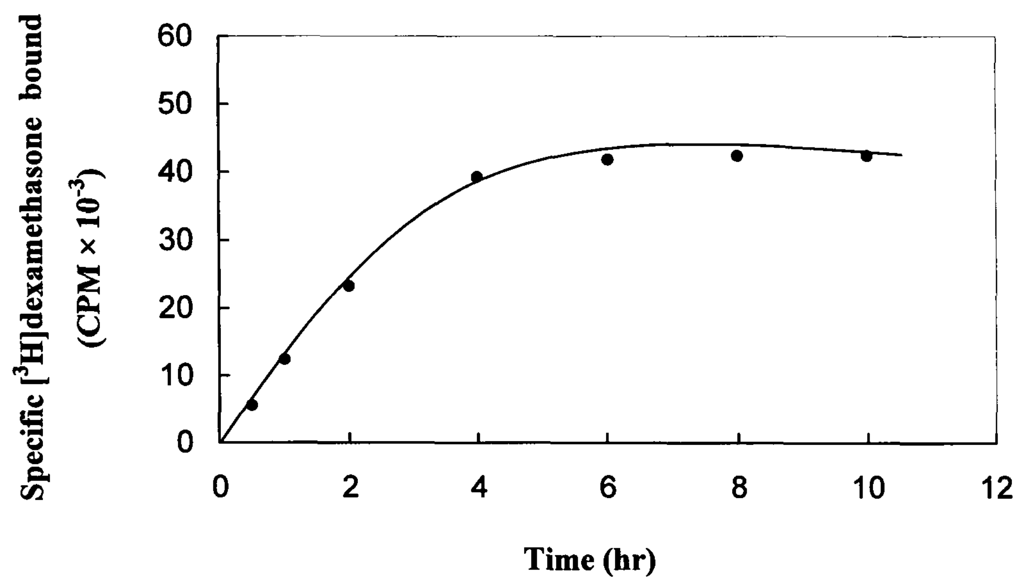


Fig. 2. Time kinetics of [^{3}H]dexamethasone binding to hepatic glucocorticoid receptors. Cytosols were incubated with 60 nM [^{3}H]dexamethasone ± 500-fold excess non-radioactive dexamethasone at 2°C. At the indicated intervals of time, specific binding was determined as mentioned in the Materials and Method section. Each data point is the mean value of 4 separate set of experiments.

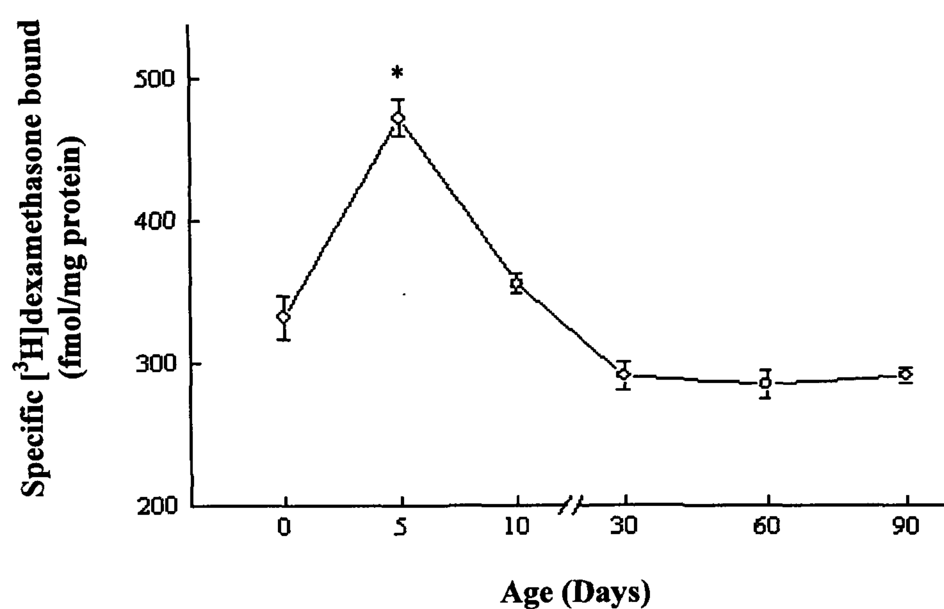


Fig. 3. Specific saturable binding of [^{3}H]dexamethasone in liver of chicken at different postnatal ages. Tissues fractionation and receptor assay conditions are described in the Materials and Methods section. Values are mean ± standard deviation from 4-5 chicken of each age group. * statistically significant as compared to other each groups

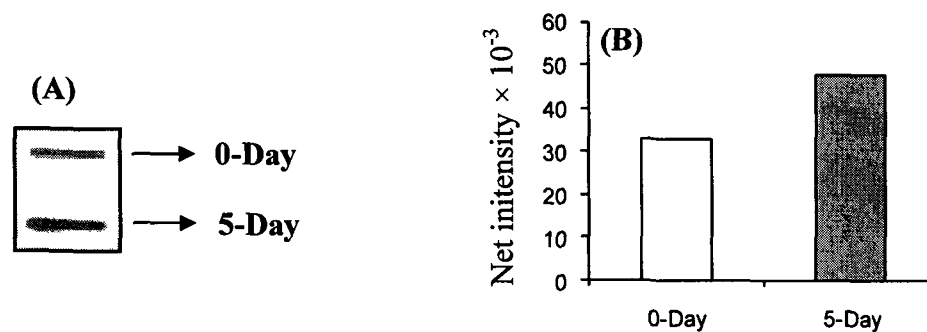


Fig. 4. Slot blot analysis of hepatic glucocorticoid receptor from 0- and 5-day old chicken (A). Net intensity of the slot blot (B). The details of experimental conditions are described in Materials and Methods section. Equal amount of liver cytosols containing GR from 0- and 5-day old chicken was applied to each slot and processed for immunoblotting using anti-GR antibody and anti-rabbit IgG-alkaline phosphatase conjugate. The net intensity of each band was ascertained using KDS-1D software (Kodak).

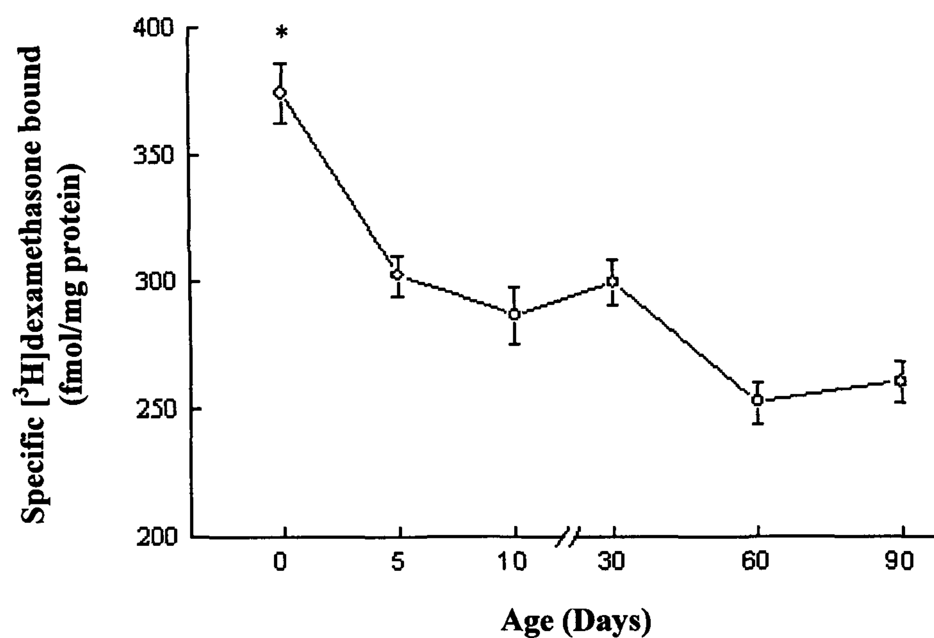


Fig. 5. Specific saturable binding of [^{3}H]dexamethasone in kidney of chicken at different postnatal ages. Tissues fractionation and receptor assay conditions are described in the Materials and Methods section. Values are mean ± standard deviation from 4-5 chicken of each age group. * statistically significant as compared to other each groups

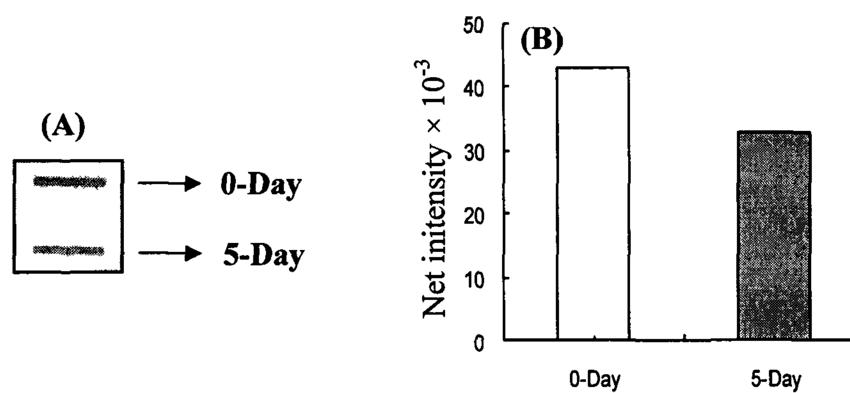


Fig. 6. Slot blot analysis of renal glucocorticoid receptor from 0- and 5-day old chicken (A). Net intensity of the slot blot (B). The details of experimental conditions are described in Materials and Methods section. Equal amount of kidney cytosols containing GR from 0- and 5-day old chicken was applied to each slot and processed for immunoblotting using anti-GR antibody and anti-rabbit IgG-alkaline phosphatase conjugate. The net intensity of each band was ascertained using KDS-1D software (Kodak).

postnatal age (Fig. 5). Slot blot analyses (Fig. 6A) and net intensity of the slot bands (Fig. 6B) corroborate the higher level of GR protein at day 0 in the kidney of chicken. The glucocorticoid receptor concentration in the **cardiac muscle** (heart) is constant in the early postnatal ages up to day 30. By day 60, the receptor level declines by 36% and the level at day 90 is similar to that at day 60 of postnatal age (Fig. 7). Slot blot analyses of the receptor preparation (Fig 8A) and the intensity of the slot bands (Fig. 8B) confirm a decline in receptor protein level at day 60 compared to day 0 of postnatal age. In **skeletal muscle,** the glucocorticoid receptor level reaches the peak value by day 10 of postnatal age, which is significantly higher (+ 69%) than the value observed at day 5 of postnatal age. The receptor level then declines by 41% at day 60 of postnatal age. The receptor level at day 90 is similar to that at day 60 of postnatal age (Fig. 9). Slot blot analyses (Fig. 10A) and the intensity of the slot bands (Fig. 10B) show that the receptor protein level is higher at day 10 compared to day 0 of postnatal age. In the **cerebral hemisphere,** the receptor levels are quite low compared to the other tissues studied, however, the receptor level does not elicit any significant change in all the ages studied (Fig. 11). Slot blot analyses (Fig. 12A) and subsequent analyses of the intensity of the slot bands (Fig. 12B) corroborate the above findings. The glucocorticoid receptor concentrations in different tissues and at various postnatal ages are also summarized in Table I.

Scatchard analyses of glucocorticoid receptor

Scatchard analysis of the binding data was performed in the liver and kidney of chicken at the two age groups, where the changes in receptor concentration were maximum. Scatchard plot of the binding data in **liver** reveals no difference in the slopes of the linear-regressed plots obtained from 0- and 5-day old chicken (Fig. 13A). This shows that the dissociation constant (K_d), the value of which was found to be similar (2.84 nM for day 0 and 2.98 nM for day 5), indicating that affinity of hormone for the receptor remains unchanged at these two ages studied. However, the intercept of the plots on the x-axis showed that the value of specific binding sites in 5-day old chicken (435 fmol/mg protein) is significantly higher than that at day 0 (326 fmol/mg protein). In the **kidney,** Scatchard data from 0-and 5-day old chicken, similarly revealed no age-associated change in the dissociation constant values for both the age groups studied (Fig. 13B). However, the specific binding sites at day 0 (368 fmol/mg protein) is significantly higher than that at day 5 (313 fmol/mg protein). The data obtained from the Scatchard analyses in liver and kidney have also been outlined in Table II.

Studies on the activation of glucocorticoid receptor

Activation studies were performed in the liver and kidney of 0- and 30-day old chicken to monitor if there are any changes in the activation properties during these developmental

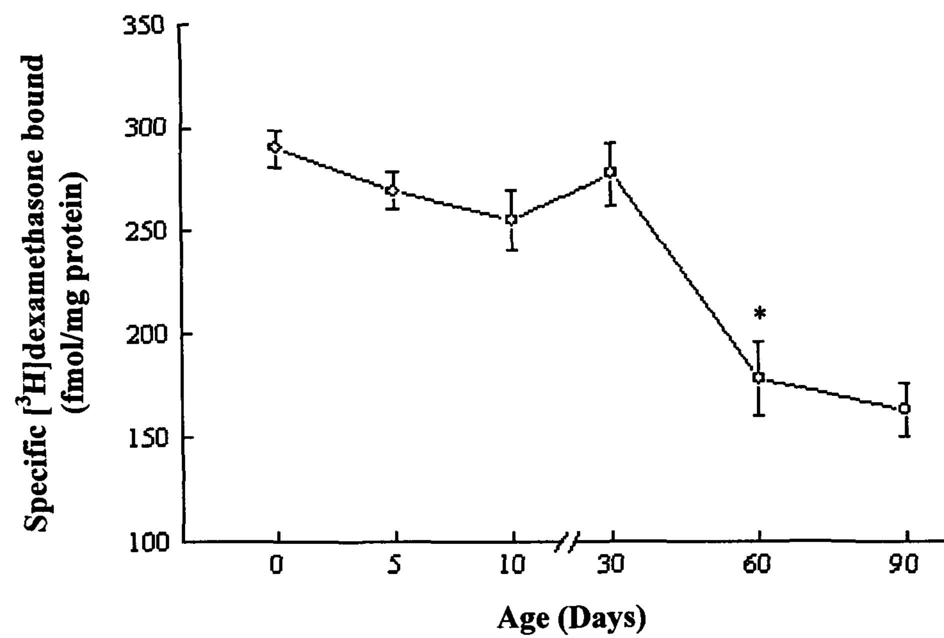


Fig. 7. Specific saturable binding of [^{3}H]dexamethasone in cardiac muscle of chicken at different postnatal ages. Tissues fractionation and receptor assay conditions are described in the Materials and Methods section. Values are mean ± standard deviation from 4-5 chicken of each age group. * statistically significant as compared to other each groups except day-90

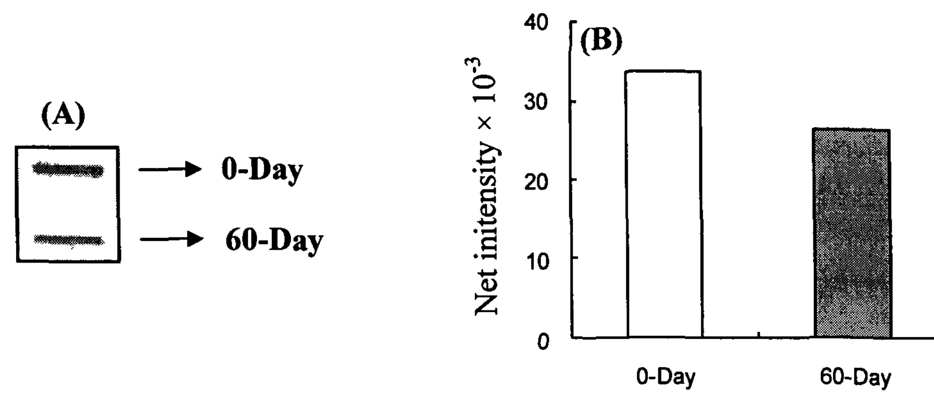


Fig. 8. Slot blot analysis of cardiac muscle glucocorticoid receptor from 0- and 60-day old chicken (A). Net intensity of the slot blot (B). The details of experimental conditions are described in Materials and Methods section. Equal amount of cardiac muscle cytosols containing GR from 0- and 60-day old chicken was applied to each slot and processed for *immunoblotting using anti-GR antibody and anti-rabbit IgG-alkaline phosphatase conjugate.* The net intensity of each band was ascertained using KDS-1D software (Kodak).

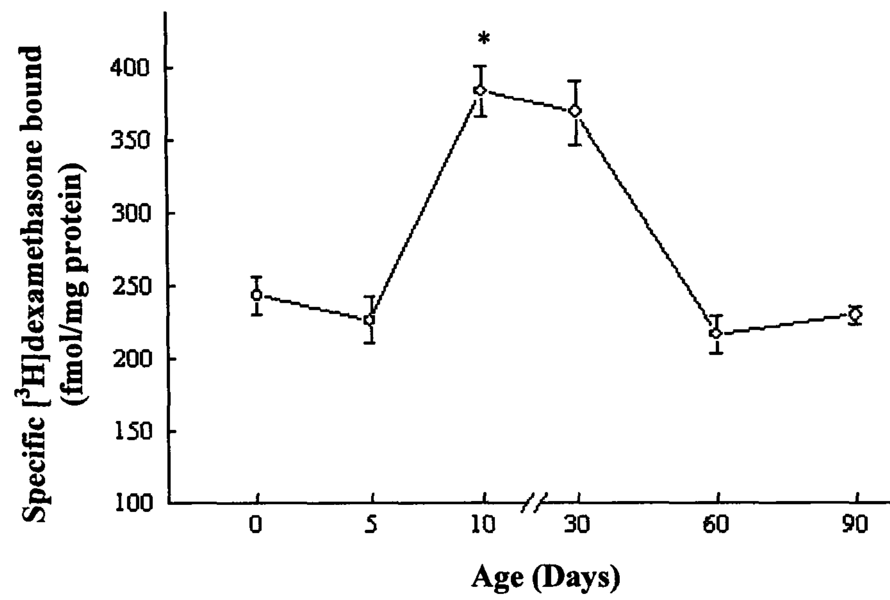


Fig. 9. Specific saturable binding of [^{3}H]dexamethasone in skeletal muscle of chicken at different postnatal ages. Tissues fractionation and receptor assay conditions are described in the Materials and Methods section. Values are mean ± standard deviation from 4-5 chicken of each age group. * statistically significant as compared to other each groups except day-30

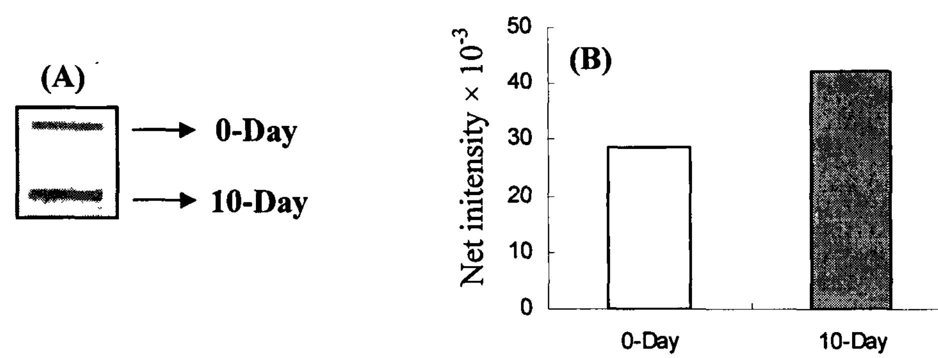


Fig. 10. Slot blot analysis of skeletal muscle glucocorticoid receptor from 0- and 10-day old chicken (A). Net intensity of the slot blot (B). The details of experimental conditions are described in Materials and Methods section. Equal amount of skeletal muscle cytosols containing GR from 0- and 10-day old chicken was applied to each slot and processed for immunoblotting using anti-GR antibody and anti-rabbit IgG-alkaline phosphatase conjugate. The net intensity of each band was ascertained using KDS-1D software (Kodak).

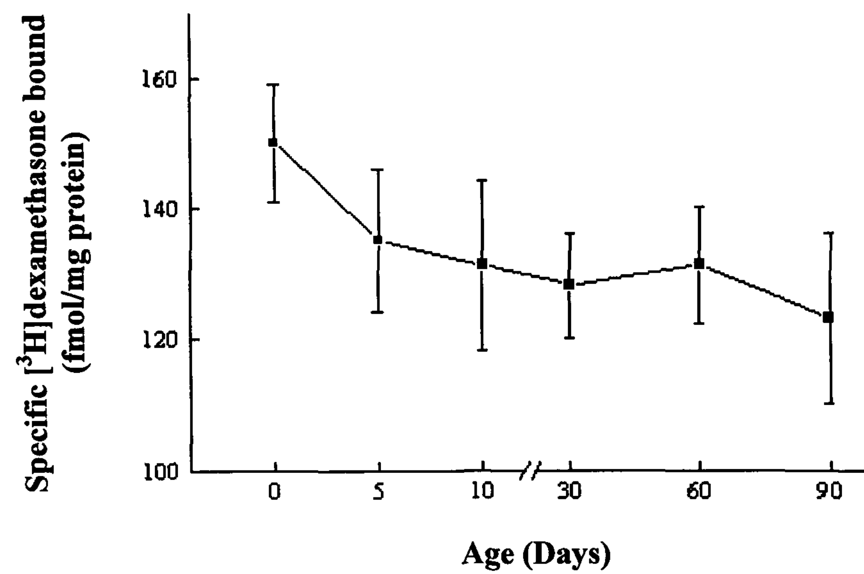


Fig. 11. Specific saturable binding of [^{3}H]dexamethasone in cerebral hemisphere of chicken at different postnatal ages. Tissues fractionation and receptor assay conditions are described in the Materials and Methods section. Values are mean ± standard deviation from 4-5 chicken of each age group

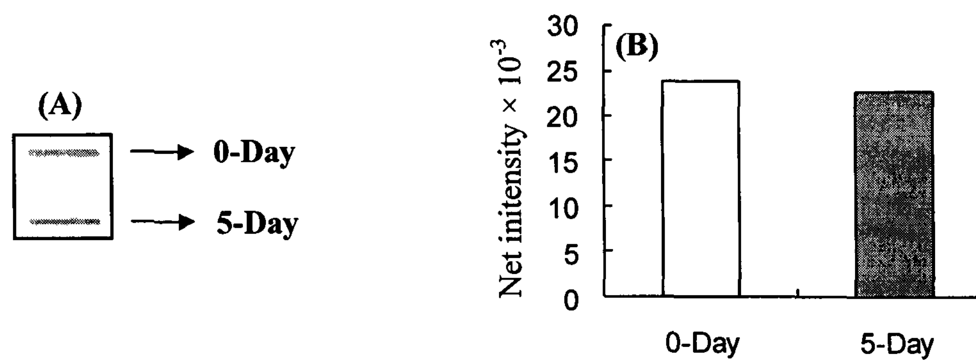


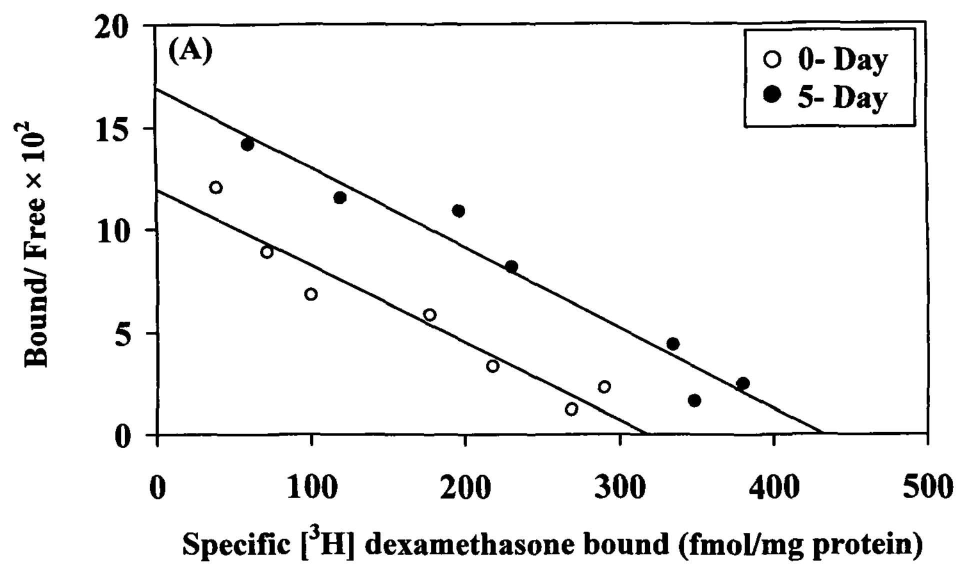
Fig. 12. Slot blot analysis of cerebral hemisphere glucocorticoid receptor from 0- and 5-day old chicken (A). Net intensity of the slot blot (B). The details of experimental conditions are described in Materials and Methods section. Equal amount of skeletal muscle cytosols containing GR from 0- and 5-day old chicken was applied to each slot and processed for immunoblotting using anti-GR antibody and anti-rabbit IgG-alkaline phosphatase conjugate. The net intensity of each band was ascertained using KDS-1D software (Kodak).

Table I

Level of glucocorticoid receptor at various postnatal ages of chicken						
	Age (Days)					
Tissue	**0**	**5**	**10**	**30**	**60**	**90**
Liver	332±15	472±13*	355±7	290±10	284±10	290±6
Kidney	374±12*	302±8	286±11	299±9	252±8	260±8
Cardiac muscles	290±9	269±9	255±14	277±15	178±18#	163±13
Skeletal muscle	243±13	226±16	383±18$^{\phi}$	368±22	216±13	229±6
Cerebral	150±9	135±11	131±13	128±8	131±9	123±13

Glucocorticoid receptor levels are expressed as fmol/mg protein; values are mean ± standard deviation from 5-6 animals of each age group.

* statistically significant ($p<0.05$) as compared to other age groups
# statistically significant ($p<0.05$) as compared to other age groups except day-90
ϕ statistically significant ($p<0.05$) as compared age groups except day-30



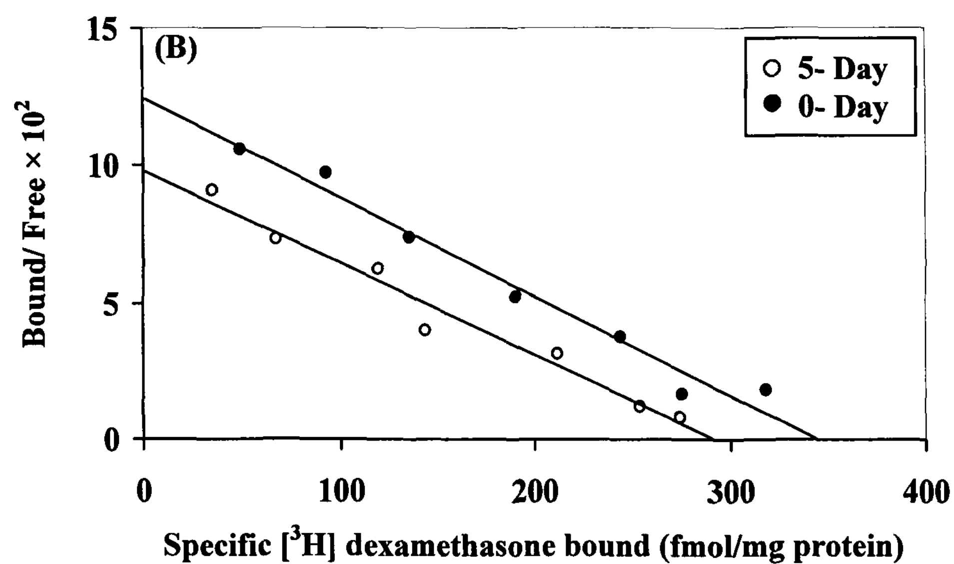


Fig. 13. Scatchard plot of [^{3}H]dexamethasone binding in the liver (A) and kidney (B) of chicken of day 0 and 5. Cytosols from liver and kidney were incubated with 2.5-120 nM [^{3}H]dexamethasone ± 500-fold excess non-radio active dexamethasone for 4 hr at 2° C. Specific binding at each concentration point was calculated as the difference between total binding and that in presence of excess non-radioactive hormone. The data obtained was analysed according to the method of Scatchard. The slope of the curve provided the dissociation constant (K_d) and the intercept on the x-axis gave the maximum receptor concentration value. Each data point is the mean of 3 experiments performed each time with tissues from 5-6 chicken of each age group.

Table II

Concentration and affinity of [^{3}H[dexamethasone receptors in the liver and kidney of chicken				
Parameters	**Liver**		**Kidney**	
	0-day	**5-day**	**0-day**	**5-day**
Number of specific binding sites (fmol/mg protein)	326±15	435±13*	368±8*	313±11
Dissociation constant, Kd (nM)	2.84±0.9	2.98±0.39	3.19±0.10	3.09±0.12

Data were collected from three sets of experiments with pooled tissues from 5-6 chicken of each age group and analysed using Scatchard plot as given in the Materials and Methods section. Values are mean ± standard deviation. *statistically significant ($p<0.05$) as compared to other age group.

phases. These ages were selected, as the level of GR was similar, hence any change in activation binding was not attributed to the GR level, but rather to its activation properties. The optimum temperature for maximum activation of GR complexes was found to be 25ºC (Fig. 14A) whereas, 25 mM $CaCl_2$ was the optimum concentration required for maximal binding of hormone-receptor complexes to the binding medium (Fig. 14B). The magnitude of thermal as well as salt (Ca^{2+}) dependent activation processes were judged using DNA-cellulose and purified nuclear binding assays.

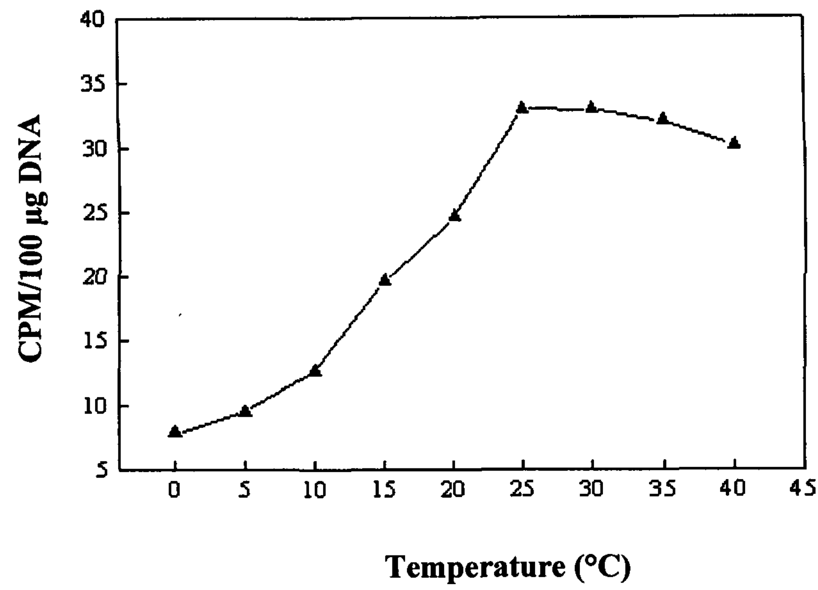
DNA-cellulose binding assay

DNA-cellulose binding assays in **liver** revealed that both the temperature and salt increase (2-2.5 fold) [^{3}H]dexamethasone-receptor complexes binding to DNA-cellulose as compared to the binding of unactivated receptor complexes incubated at 0ºC for 45 min. However, the degree of activation is similar for hepatic glucocorticoid receptors obtained from 0- and 30-day old chicken (Fig. 15A). The results indicate no postnatal difference in the *in vitro* activation of the hormone-receptor complexes under the conditions mentioned above.

In the **kidney** too, the thermal as well salt activation of [^{3}H]dexamethasone-receptor complexes were similar to that in liver since there was no difference in the magnitude of *in vitro* activation of the hormone-receptor complexes in the two ages studied. However, the fold of activation of [^{3}H]dexamethasone-receptor complexes both by temperature and salt is slightly lower (2-2.3 fold) compared to the liver (Fig. 15B).

Nuclear binding assay

Since DNA-cellulose by virtue of it being a non-specific assay system, could not implicate any age-related differences, purified nuclei were used to provide a more physiological assay system. The results indicate that the thermal as well as salt activation of the [^{3}H]dexamethasone-receptor complexes is similarly observed in both the age groups studied as seen using DNA-cellulose binding assays. However, in contrast to DNA-cellulose binding assay, nuclear binding of both thermal- and salt-activated glucocorticoid-receptor complexes is significantly higher in immature (0-day) compared to that of mature (30-day) chicken (Fig. 16A). Nuclear exchange (cross-mixing) assays, where in both the thermally and salt activated receptors of 30-day old were allowed to interact with the nuclei of 0-day and *vice versa*, showed significantly higher binding by the hepatic nuclei of 0-day old as compared to those from 30-day old chicken (Fig. 17A). These findings indicate higher binding capacity of immature nuclei to activated hormone-receptor complexes than those of mature ones.



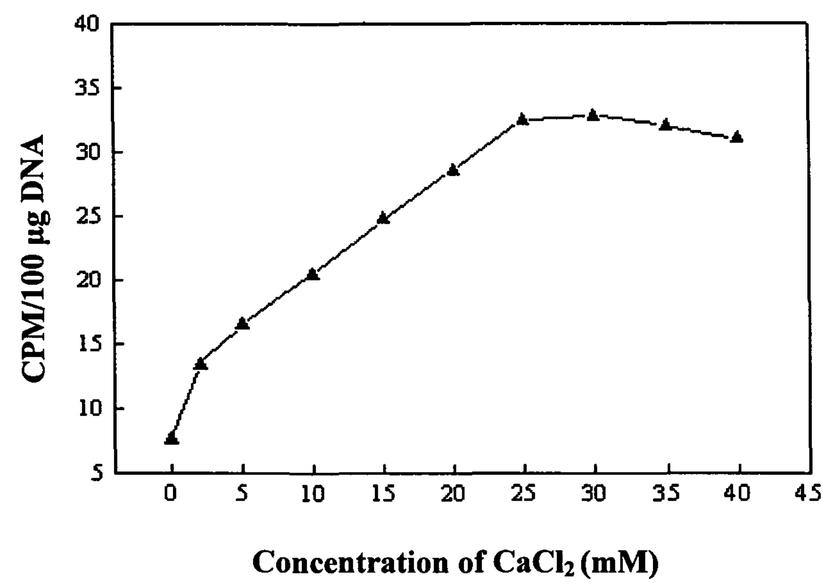


Fig. 14. Effect of temperature (A) and of $CaCl_2$ (B) on activation of [^{3}H]dexamethasone-receptor complexes. The hepatic hormone-receptor complexes were prepared and nuclear binding of the receptors activated by heat (A) and salt (B) were performed. Details of processing and determination of the bound complexes are as described in the Materials and Methods section. Each point represents the mean value of 4 separate set of experiments, each performed in duplicate.

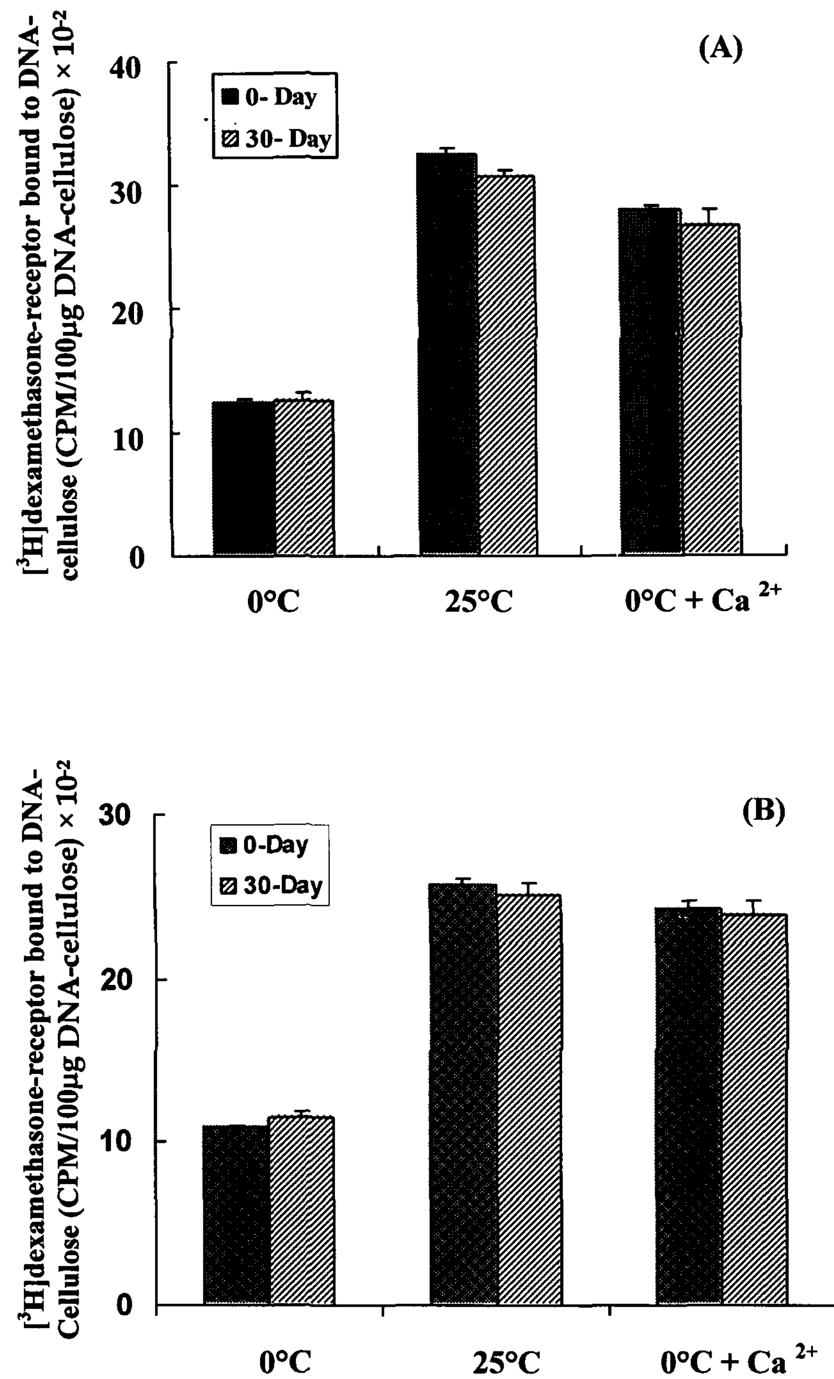


Fig. 15. Binding of [^{3}H]dexamethasone-receptor complexes to DNA-cellulose in the liver (A) and kidney (B) of chicken of day 0 and 30. Cytosols from liver and kidney were prepared in buffer B with 60 nM [^{3}H]dexamethasone for 4 hr at 2°C. Activation conditions, binding to DNA-cellulose and further processing of the pellets was done as described in the Materials and Methods section. The results are mean ± standard deviation of three experiments, each set done in triplicate with pooled tissues from 5-6 chicken of same age group. * *statistically significant as compared to other age*

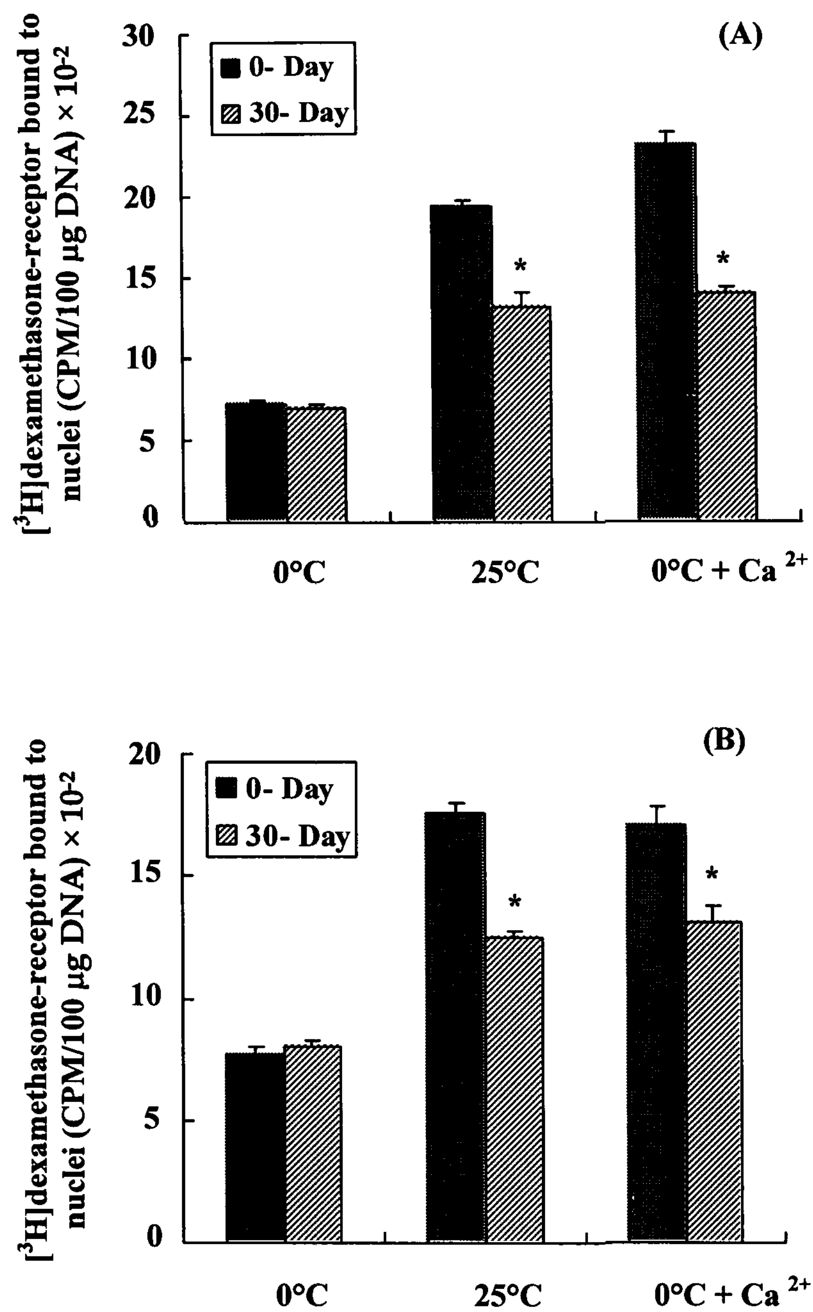


Fig. 16. Binding of [^{3}H]dexamethasone-receptor complexes to purified nuclei in the liver (A) and kidney (B) of chicken of day 0 and 30. Cytosols from liver and kidney were prepared in buffer B with 60 nM [^{3}H]dexamethasone for 4 hr at 2°C. Activation conditions, binding to purified nuclei and further processing of the nuclear pellets was done as described in the Materials and Methods section. The results are mean ± standard deviation of three experiments, each set done in triplicate with pooled tissues from 5-6 chicken of same age group. * statistically significant as compared to other each age

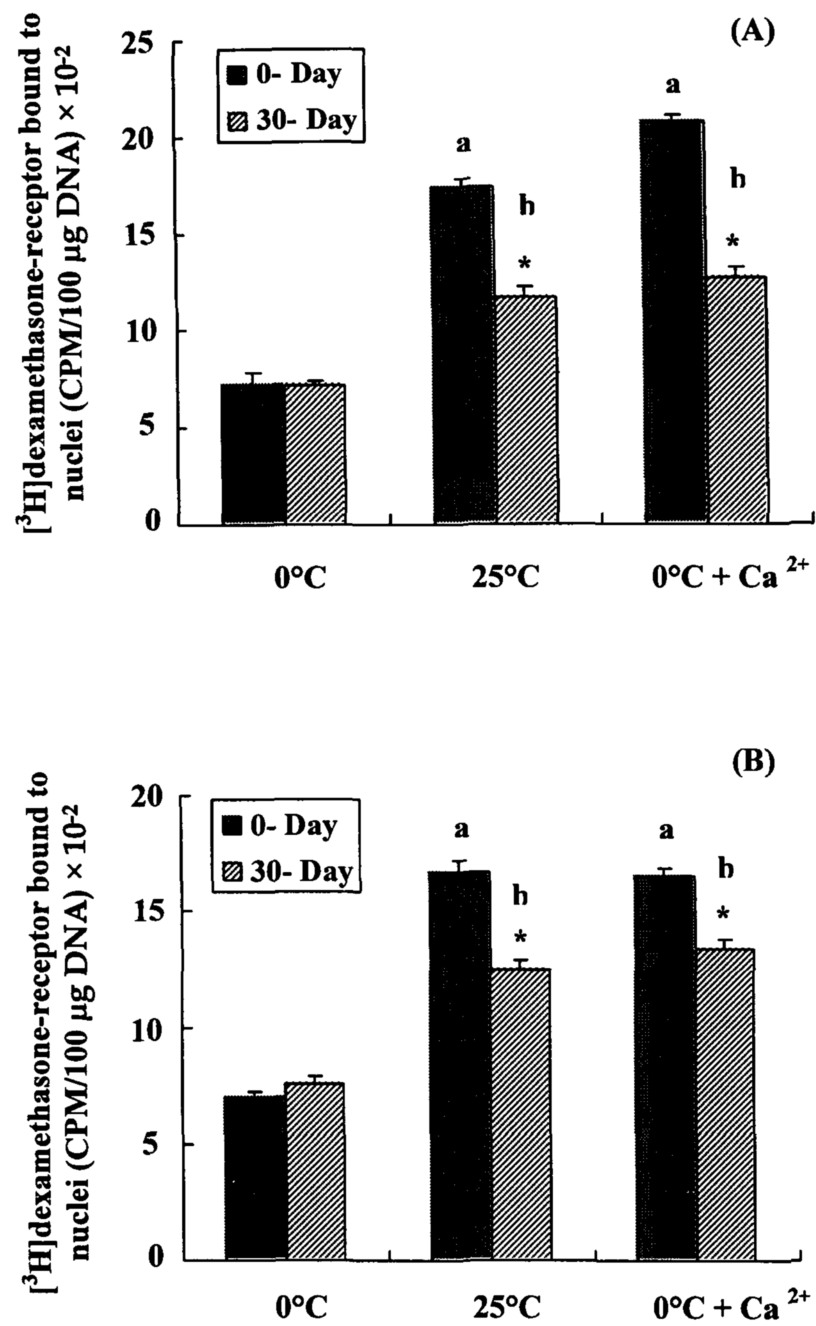


Fig. 17. Cross-mixing binding of [^{3}H]dexamethasone-receptor complexes to purified nuclei in the liver (A) and kidney (B) of chicken of day 0 and 30. Cytosols from liver and kidney were prepared in buffer B with 60 nM [^{3}H]dexamethasone for 4 hr at 2°C. Nuclear cross-mixing in which activated receptor complexes from 30-day old chicken were incubated with nuclei of 0-day old chicken (a) and vice versa (b). The results are mean ± standard deviation of three experiments, each set done in triplicate with pooled tissues from 5-6 chicken of same age group. * statistically significant as compared to other age

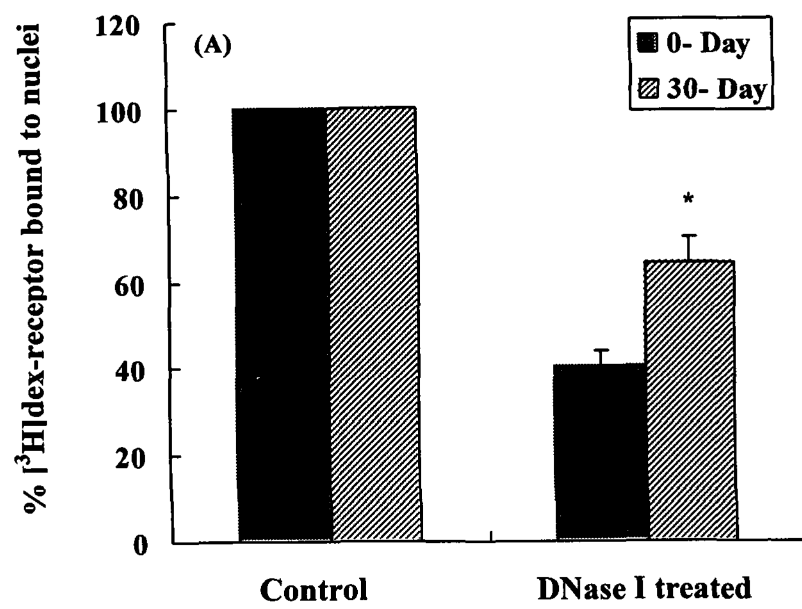
In **Kidney,** the pattern of nuclear binding by heat and salt activated receptor complexes was similar to that observed in liver (Fig. 16B). Nuclear exchange assay also revealed the same pattern of activation as seen in liver (Fig. 17B).

DNase I digestion studies

Nuclear exchange studies in the liver and kidney revealed a higher degree of binding of the thermally activated [^{3}H]dexamethasone-receptor complexes by the nuclei of 0-day as compared to 30 day nuclei. These findings indicated a change in nuclear chromatin property that may have contributed to the higher binding of hormone-receptor complexes in immature chicken. Therefore, DNase I digestion of the liver and kidney nuclei, from both the age groups were done to determine the extent of chromatin digested that will be estimated by the amount of bound hormone-receptor complexes from the nuclei. This will reflect the change in chromatin organization that might have been responsible for the observed changes in the nuclear binding at the two ages. DNase I digestion of the nuclear chromatin was able to extract a significantly higher number of nuclear bound, thermally activated [^{3}H]dexamethasone-receptor complexes from both the liver and kidney of 0-day with respect to 30-day old chicken. The degree of extraction of the nuclear bound receptors in the **liver** is 70% (0-day) and 44% (30-day) as compared to their respective controls taken as 100% (Fig. 18A). In the **Kidney** too, the percentage of extraction was observed to be 60% for 0-day and 46% for 30-day chicken (Fig. 18B).

Inhibition of activation studies

The inhibition of activation of GR by utilizing various exogenous and endogenous agents was studied to see their modulatory effects in the liver of 30-day old chicken. To examine the effect of molybdate (MO_4^{2-}) on receptor activation by heat, a concentration of 0-100 mM (MO_4^{2-}) was used. Result exhibited a dose-dependent inhibition (maximally 80 % at 60 mM) of heat activation of H-R complexes from the liver of chicken as assessed by binding to DNA-cellulose (Fig. 19A) and purified nuclei (Fig. 19B). Tungstate (WO_4^{2-}) was also utilized to see any modulatory effect on hepatic GR activation. Results indicated that tungstate (0-20 mM) appears to be a more effective inhibitory agent (80% at 15 mM) compared to molybdate, as assessed by binding to DNA-cellulose (Fig. 20A) and purified nuclei (Fig. 20B). Cadmium (0-6 mM) caused a maximal inhibition (60%) of hepatic GR heat activation at 2 mM by DNA-cellulose (Fig. 21A) and nuclear binding assays (Fig. 21B). Oleic acid, a polyunsaturated fatty acid (PUFA) has also been used to study its inhibitory role on *in vitro* receptor activation. Oleic acid (C 18:1) caused 45% maximal inhibition of heat activation at 40 μM as assessed by DNA-cellulose (Fig. 22A) and nuclear binding assays (Fig. 22B).



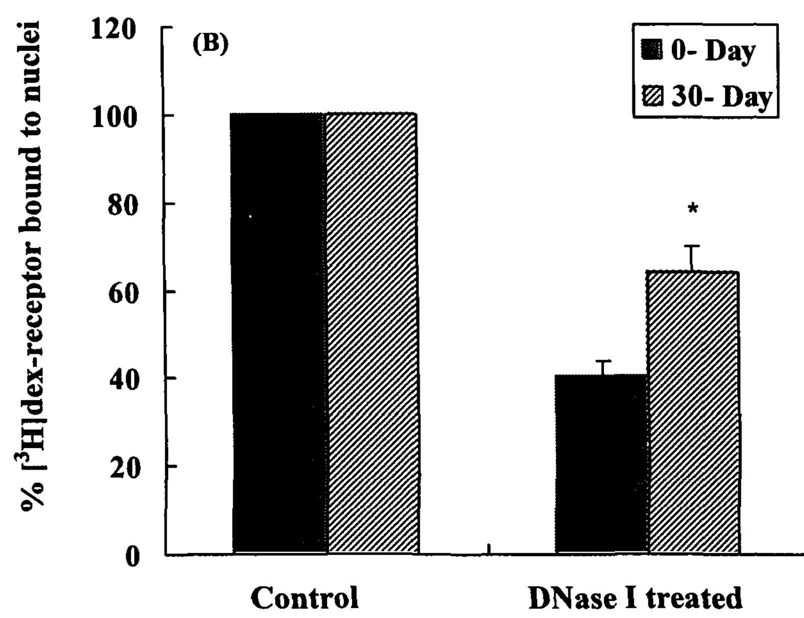
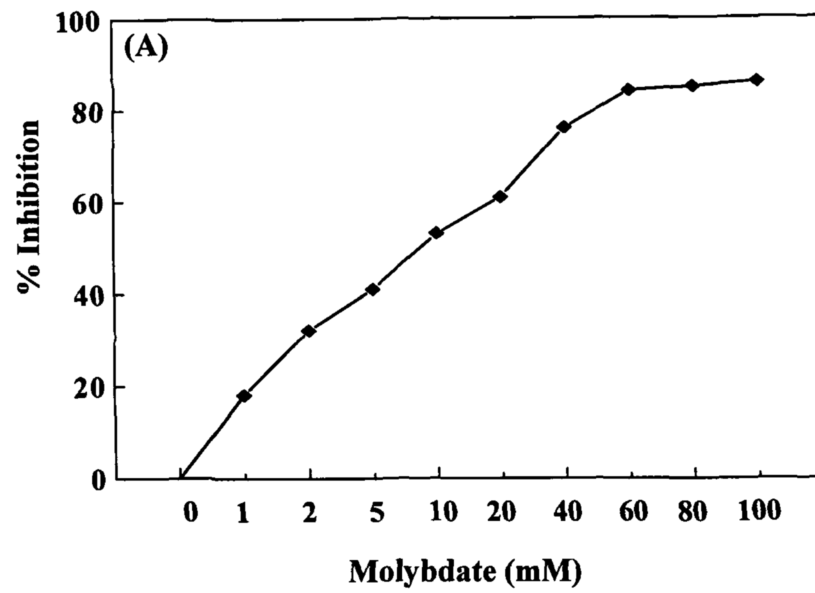


Fig. 18. Extraction of the nuclear bound [^{3}H]dexamethasone-receptor complexes by DNase I. Thermally activated, nuclear bound hormone-receptor complexes were extracted using DNase I (150 µg/ 100 µg DNA) in the liver (A) and kidney (B), for 45 min at 2°C. Details of the procedure and determination of the bound radioactivity are given in the text. The values are mean ± standard deviation of three experiments. * statistically significant with respect to other age groups



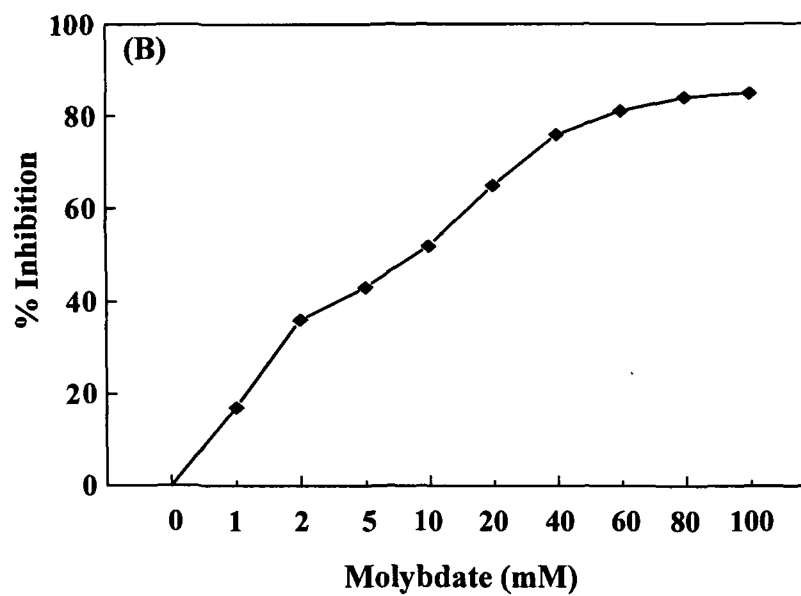


Fig. 19. Effect of molybdate (MO_4^{2-}) on the heat activation of hepatic [^{3}H]dexamethasone-receptor complexes as assessed by DNA-cellulose (A) and purified nuclear (B) binding assays. Cytosols from liver were prepared in buffer B and incubated with 60 nM [^{3}H]dexamethasone for 4 hr to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of molybdate. DNA-cellulose, nuclear binding and further processing were performed as indicated in the Materials and Methods section. Heat activation in the absence (control) of molybdate is taken as 0 % inhibition.

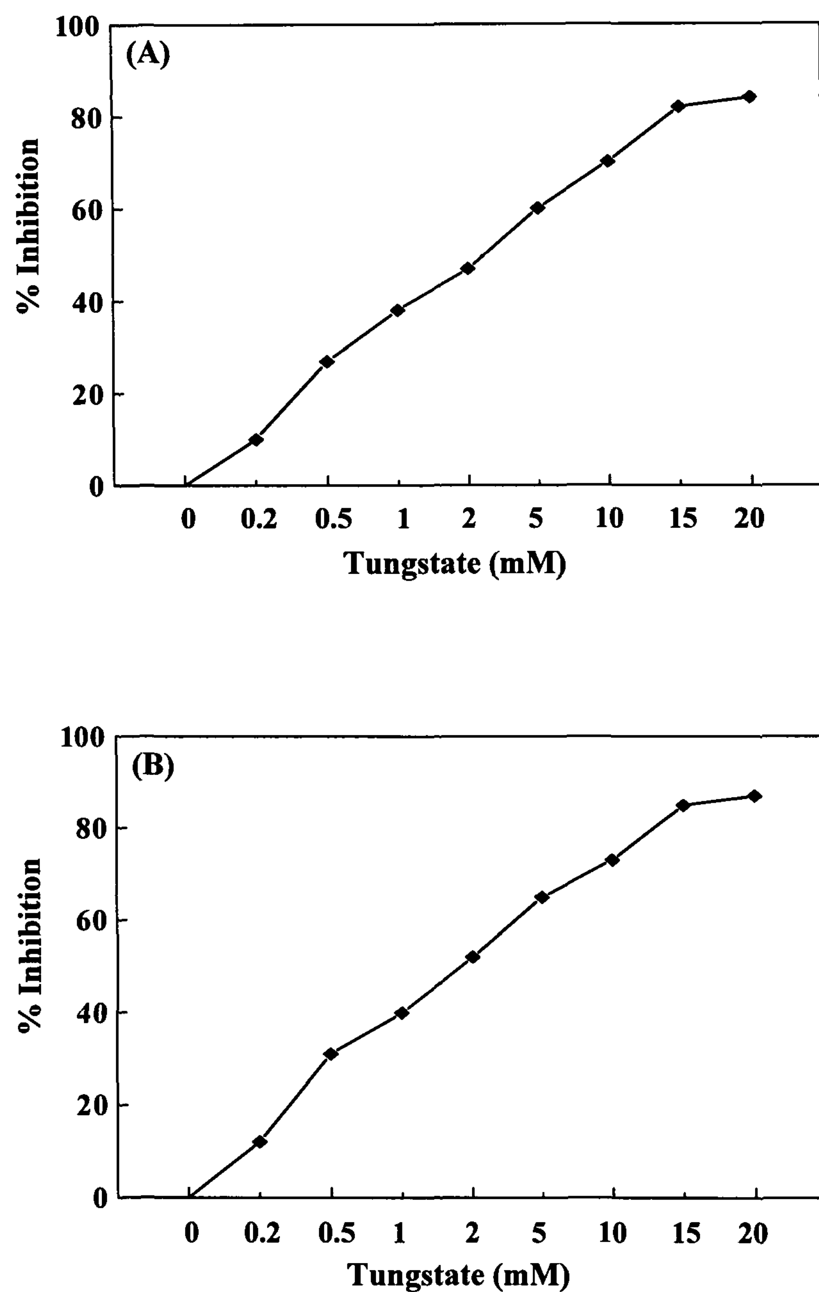
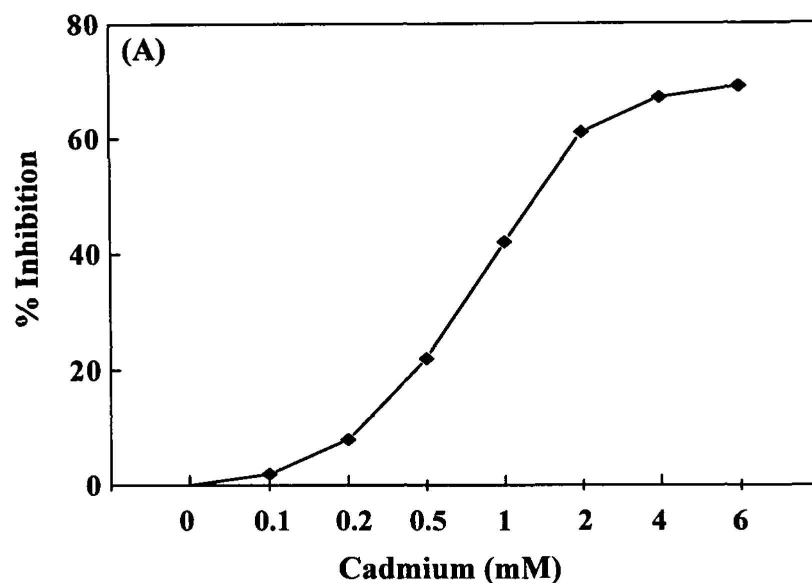


Fig. 20. Effect of tungstate (WO_4^{2-}) on the heat activation of hepatic [^{3}H]dexamethasone-receptor complexes as assessed by DNA-cellulose (A) and purified nuclear (B) binding assays. Cytosols from liver were prepared in buffer B and incubated with 60 nM [^{3}H]dexamethasone for 4 hr to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of tungstate. DNA-cellulose, nuclear binding and further processing were performed as indicated in the Materials and Methods section. Heat activation in the absence (control) of tungstate is taken as 0 % inhibition.



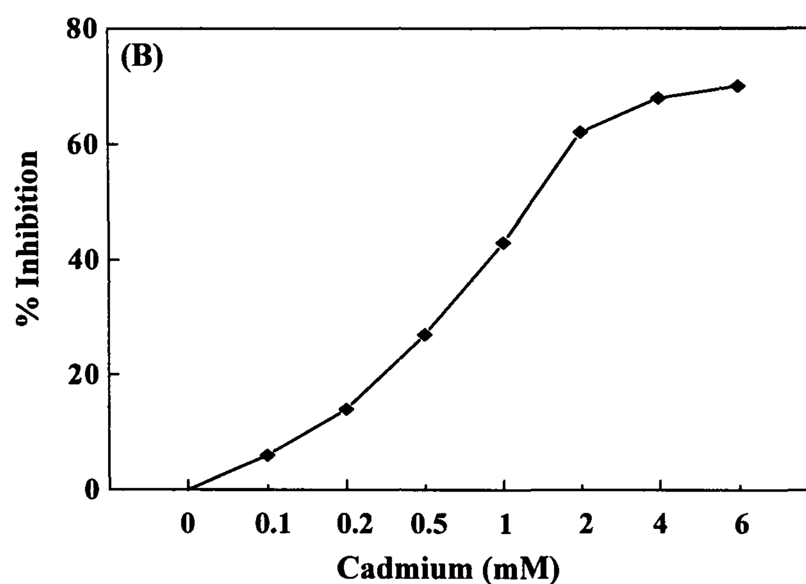


Fig. 21. Effect of cadmium (Cd^{2+}) on the heat activation of hepatic [^{3}H]dexamethasone-receptor complexes as assessed by DNA-cellulose (A) and purified nuclear (B) binding assays. Cytosols from liver were prepared in buffer B and incubated with 60 nM [^{3}H]dexamethasone for 4 hr to generate [^{3}Hdexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of cadmium. DNA-cellulose, nuclear binding and further processing were performed as indicated in the Materials and Methods section. Heat activation in the absence (control) of cadmium is taken as 0 % inhibition.

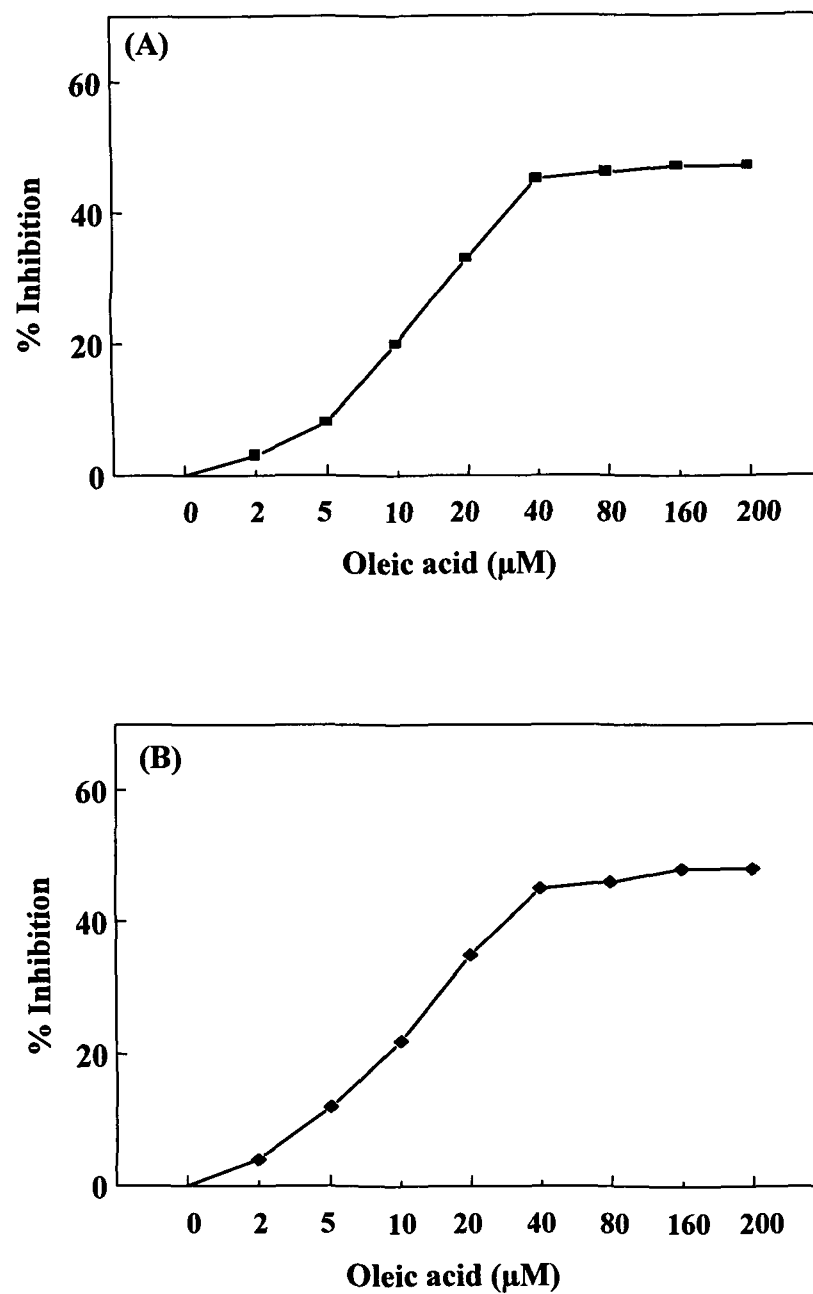
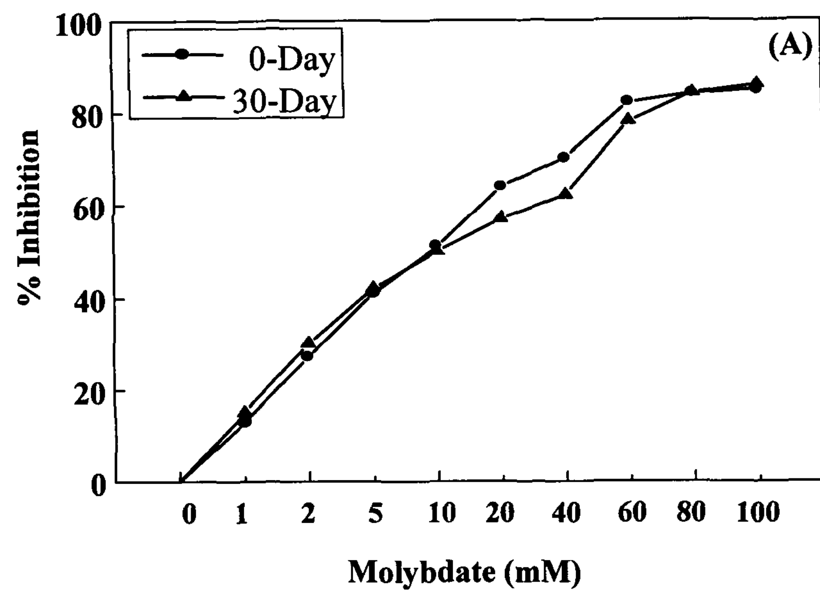


Fig. 22. Effect of oleic acid (C 18:1) on the heat activation of hepatic [^{3}H]dexamethasone-receptor complexes as assessed by DNA-cellulose (A) and purified nuclear (B) binding assays. Cytosols from liver were prepared in buffer B and incubated with 60 nM [^{3}H]dexamethasone for 4 hr to generate [^{3}H]dexamethasone-receptor complexes. Aliquots of cytosol containing [^{3}H]dexamethasone-receptor complexes were then subjected to heat activation at 25°C for 45 min in the presence or absence of different concentrations of oleic acid (prepared as stock in dimethyl sulfoxide, DMSO). DNA-cellulose, nuclear binding and further processing were performed as indicated in the Materials and Methods section. Control tubes received appropriate volume of DMSO instead of oleic acid. Heat activation in the absence (control) of oleic acid is taken as 0 % inhibition.

All the above inhibitors of activation were also used to study the modulation of heat and salt activation of liver and kidney GR from 0- and 30-day old chicken to see any tissue- and/or age-specific modulation. Molybdate was used at increasing concentration (0-100 mM) to see its inhibitory effect on GR heat and salt activation in the liver and kidney of 0- and 30-day old chicken. DNA-cellulose binding assay shows similar pattern of inhibition (~80% at 60 mM) of hepatic (Fig. 23A) and kidney (Fig. 23B) GR heat activation by molybdate in both the ages studied. Nuclear binding assay also revealed a similar pattern of inhibitory effect of molybdate in both the ages in the liver (Fig.24A) and kidney (Fig. 24B) of chicken. DNA-cellulose binding assay shows inhibition (75-80% at 60mM) of hepatic (Fig. 25A) and kidney (Fig. 25B) GR salt activation by molybdate in both the ages studied. Nuclear binding assay also revealed a similar pattern of inhibitory effect of molybdate in both the ages in the liver (Fig. 26A) and kidney (Fig. 26B) of chicken. Tungstate (0-20 mM) also inhibited GR heat activation maximally 80-85% at 15 mM in both the tissues and ages although, to a similar extent as assessed by DNA-cellulose (Fig. 27A&B) and nuclear (Fig. 28A&B) binding assays. Tungstate also inhibited GR salt activation in the same pattern as above in both tissues and ages as assessed by DNA-cellulose (Fig. 29A&B) and nuclear (Fig. 30A&B) binding assays. Cadmium (0-6 mM), caused a maximal inhibition (60% at 2 mM) of GR heat activation, but could not show any difference in % inhibition from the liver (Fig. 31A) and kidney (Fig. 31B) of 0- and 30-day chicken by DNA-cellulose binding assay, with a similar result in the case of nuclear binding assay (Fig. 32A&B). Similar observations were found where cadmium caused maximal inhibition (60% at 2 mM) of GR salt activation, but as above could not show any difference in % inhibition from the liver (Fig. 33A) and kidney (Fig. 33B) of 0- and 30-day chicken by DNA-cellulose binding assay, with a similar result in the case of nuclear binding assay (Fig. 34A&B). Oleic acid which is an endogenous PUFA was also employed to show if any difference in tissue- and age- specific inhibition of both heat and salt activation of GR. Oleic acid caused similar extent of inhibition (~42-47% at 40 µM) of heat activation of hepatic and renal GR in both the ages as observed by DNA-cellulose binding assay (Fig. 35A&B), with a similar extent in nuclear (Fig. 36A&B) binding assay. Oleic acid also caused a similar extent of inhibition of salt activation of hepatic and renal GR in both the ages as observed by DNA-cellulose binding assay (Fig. 37A&B), with a similar extent in nuclear binding assay (Fig. 38A&B).

Physicochemical characterization of glucocorticoid receptor

To study some of the physicochemical characteristics of the unactivated as well as activated glucocorticoid receptor from immature (0-day) and mature (30-day) chicken, gel filtration analyses and anion-exchange chromatography were performed only on the hepatic glucocorticoid receptors of chicken.



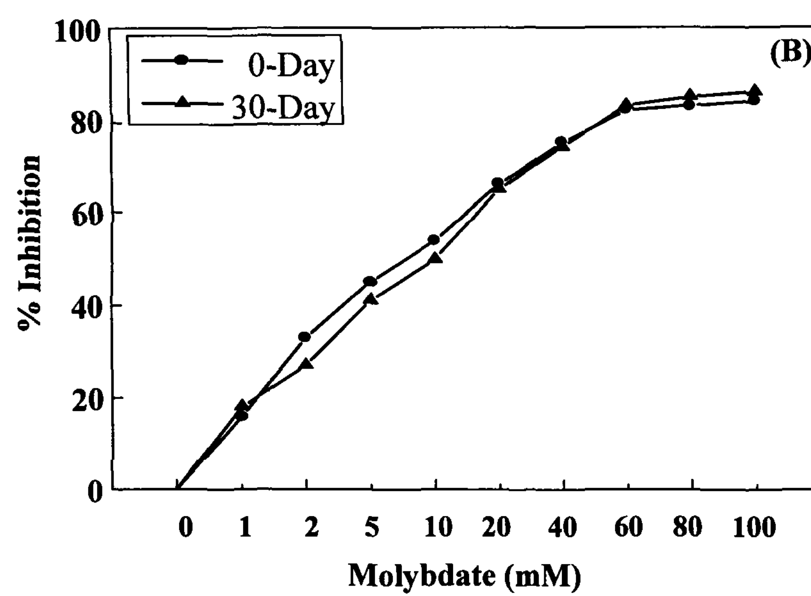
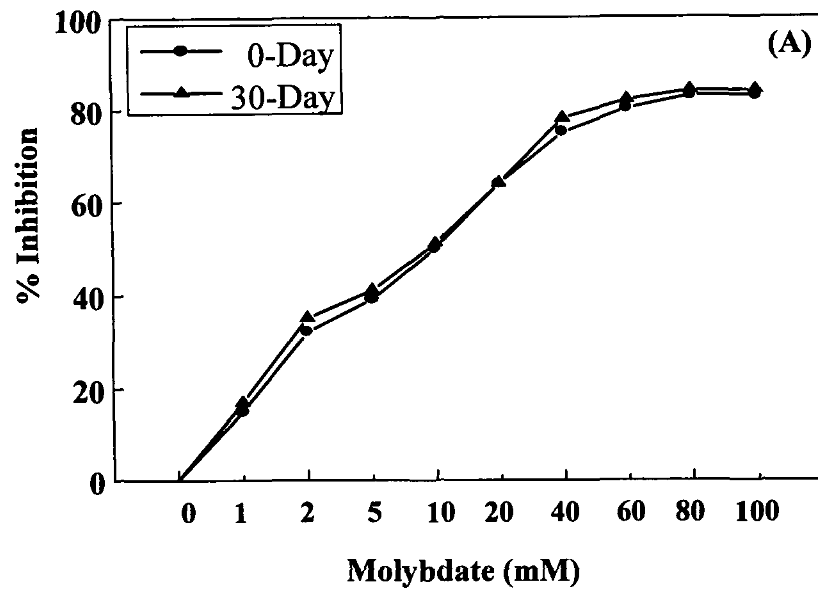


Fig. 23. Effect of molybdate (MO_4^{2-}) on the heat activation of hepatic (A) and kidney (B) [^3H]dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 19.



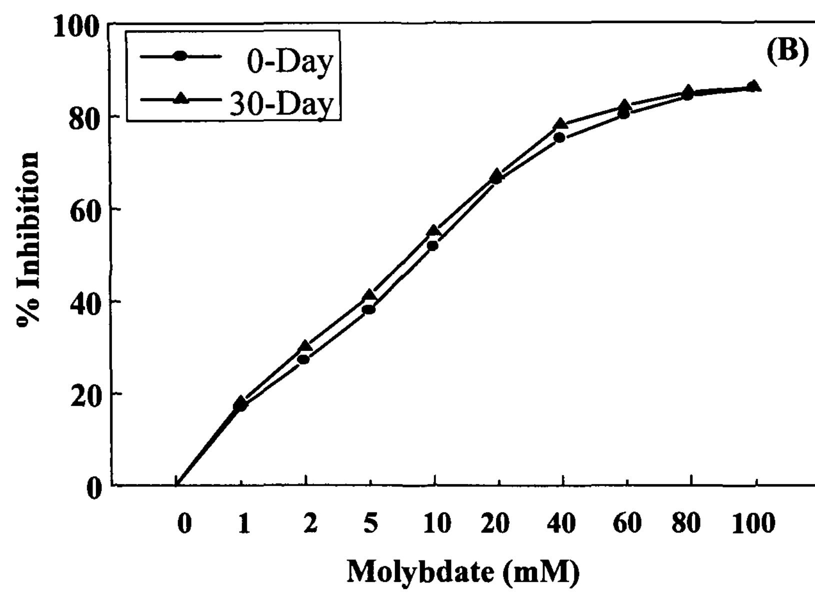
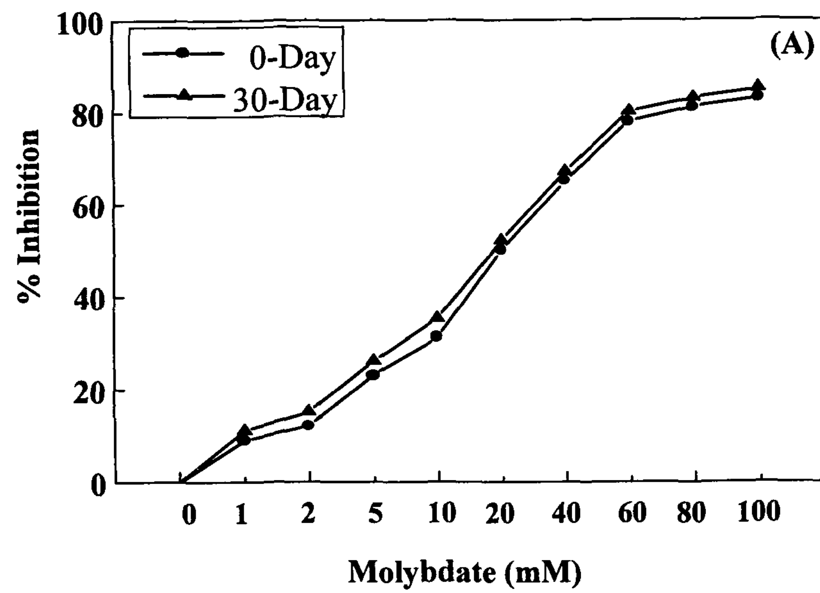


Fig. 24. Effect of molybdate (MO_4^{2-}) on the heat activation of hepatic (A) and kidney (B) [^{3}H] dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 19.



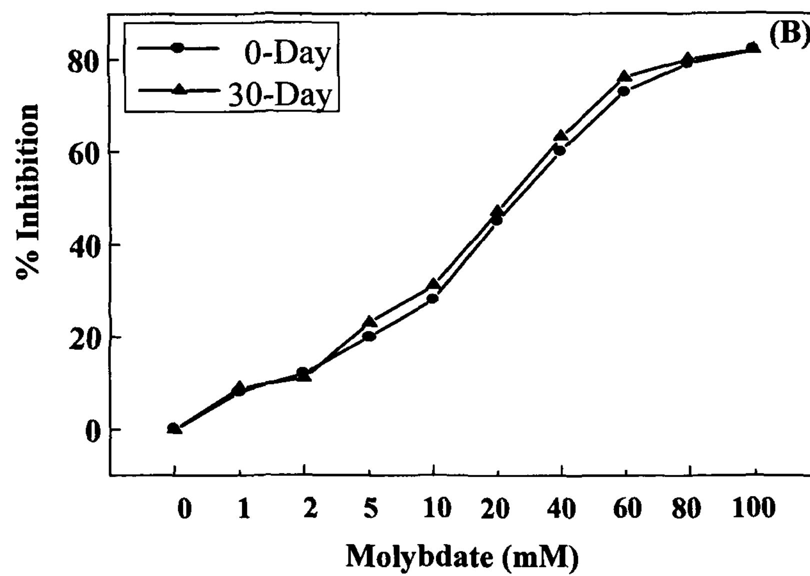
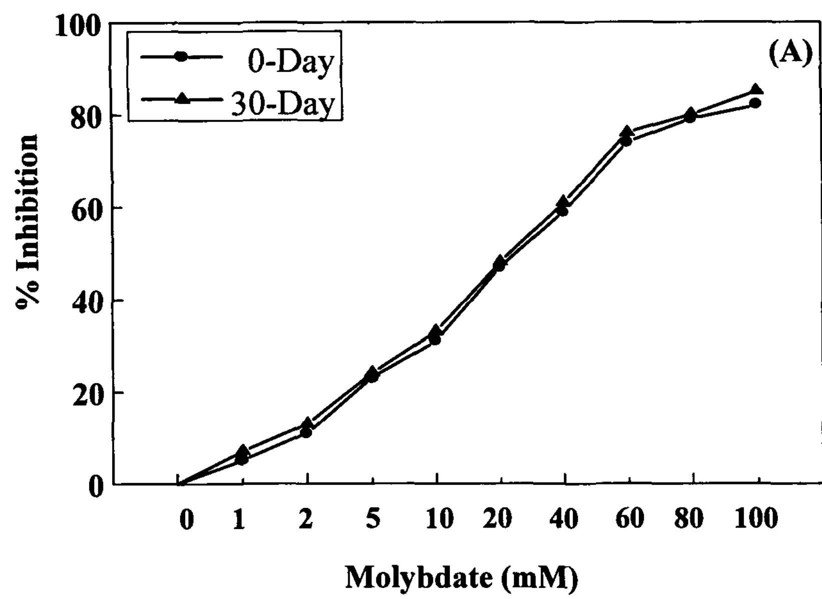


Fig. 25. Effect of molybdate (MO_4^{2-}) on the salt activation of hepatic (A) and kidney (B) [^{3}H] dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 19.



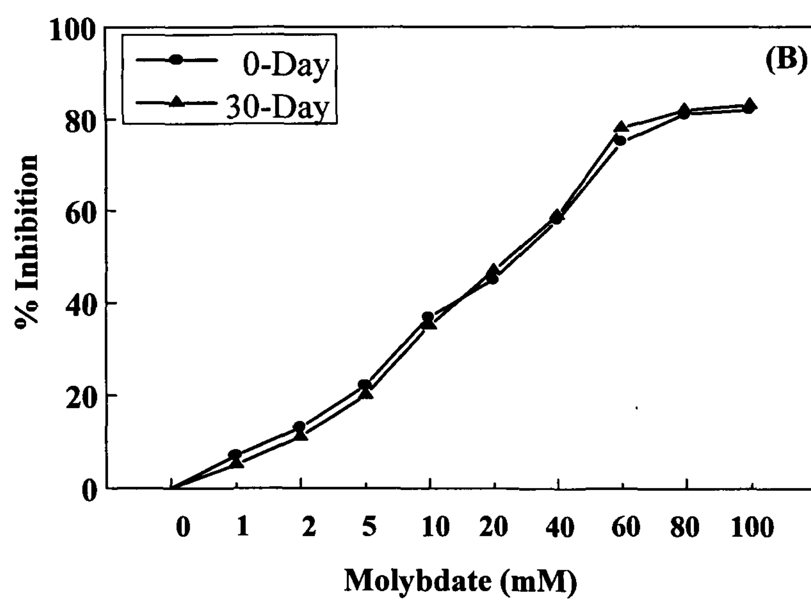
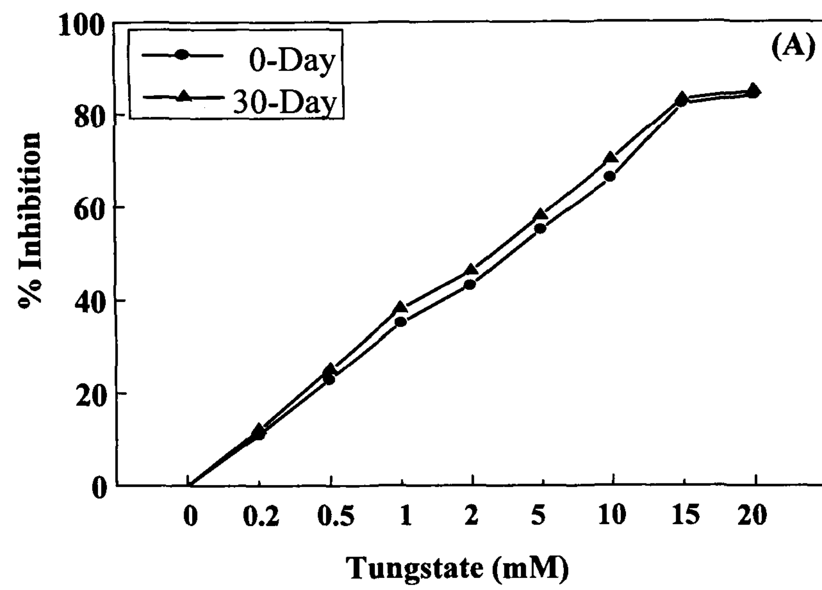


Fig. 26. Effect of molybdate (MO_4^{2-}) on the salt activation of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 19.



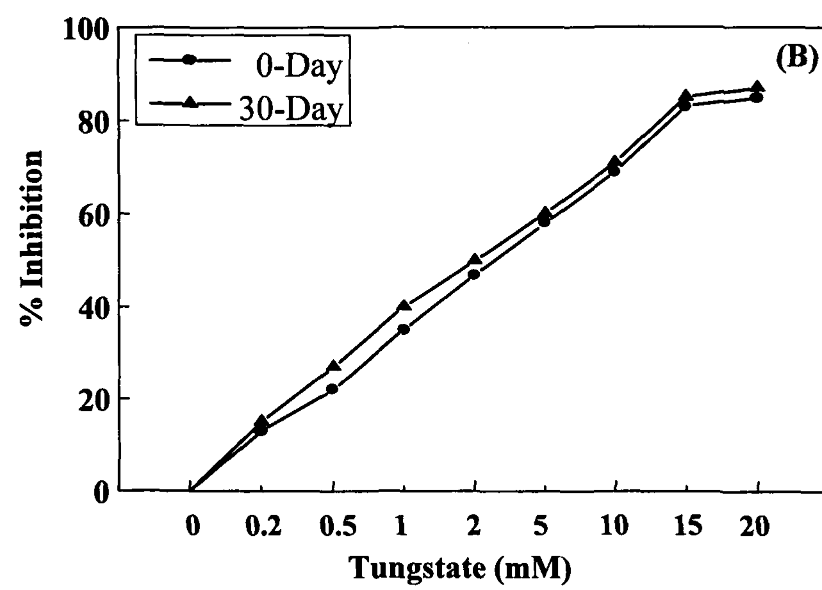
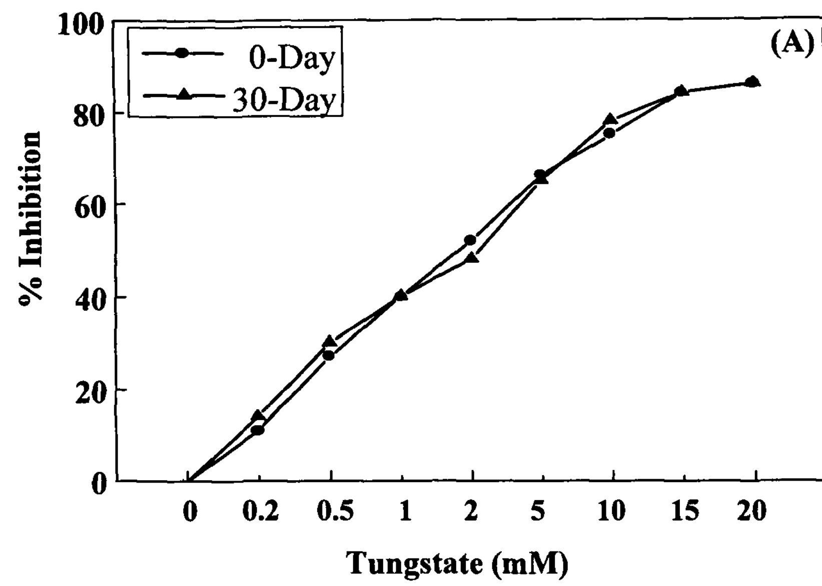


Fig. 27. Effect of tungstate (WO_4^{2-}) on the heat activation of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 20.



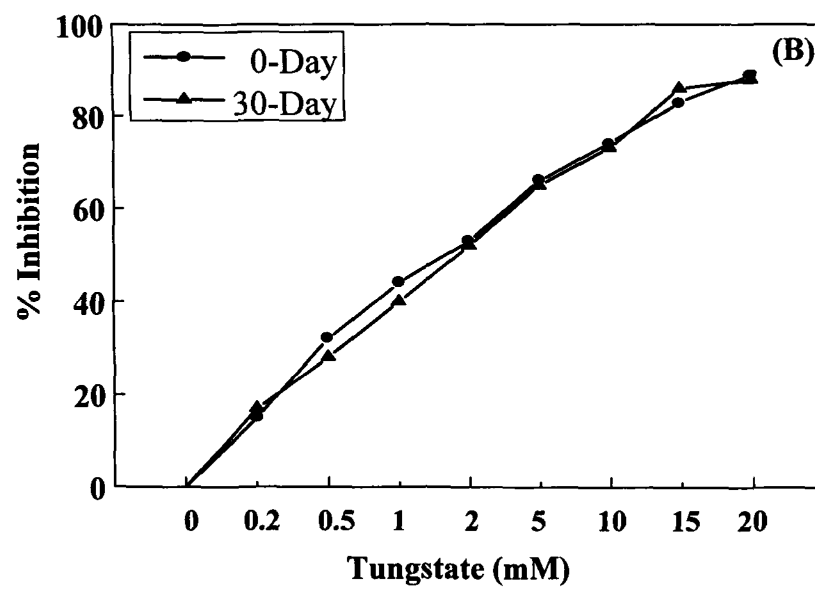
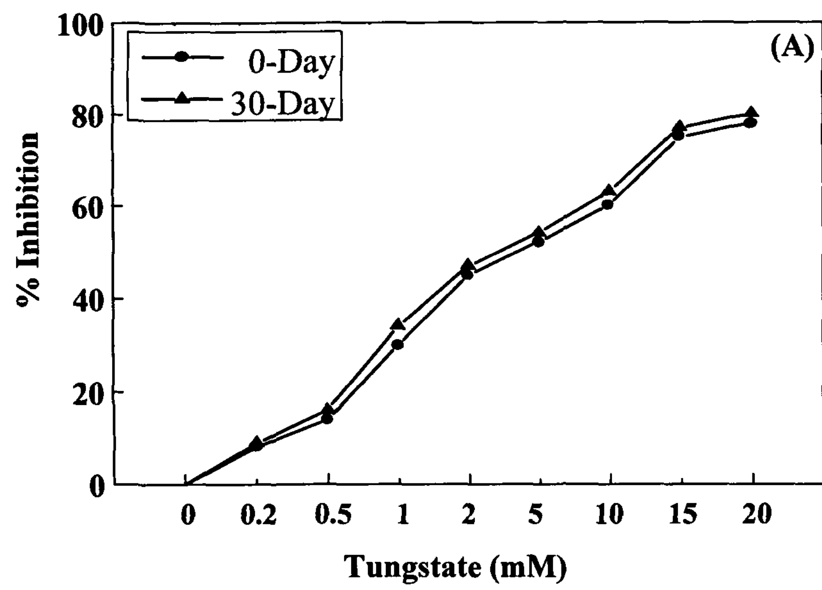


Fig. 28. Effect of tungstate (WO_4^{2-}) on the heat activation of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 20.



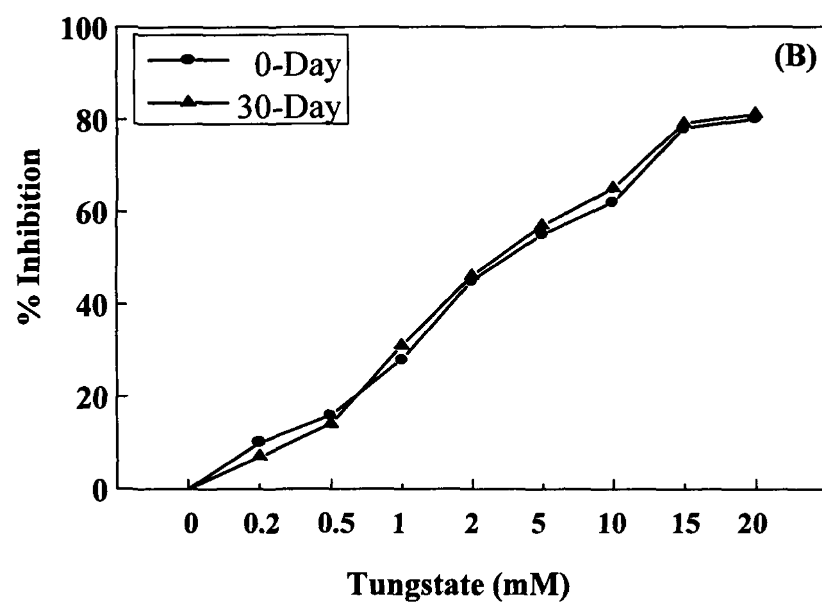
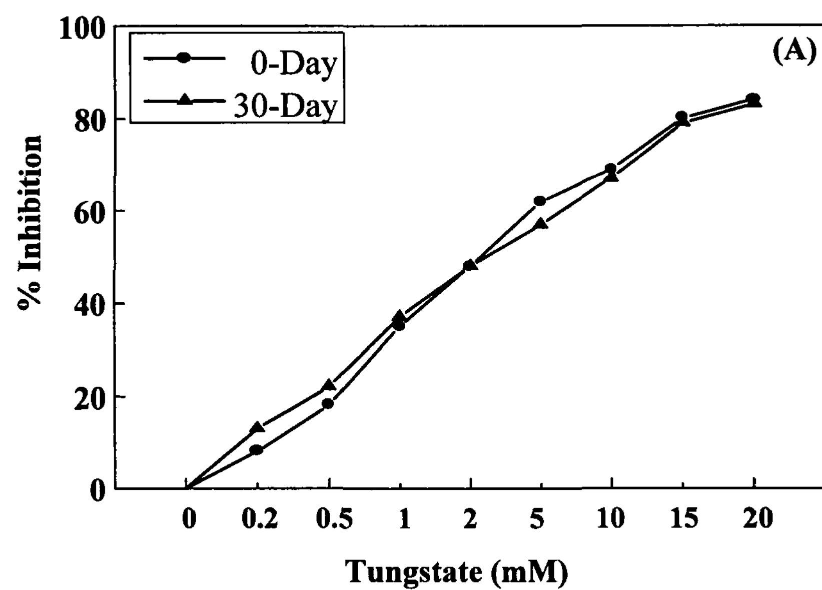


Fig. 29. Effect of tungstate (WO_4^{2-}) on the salt activation of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 20.



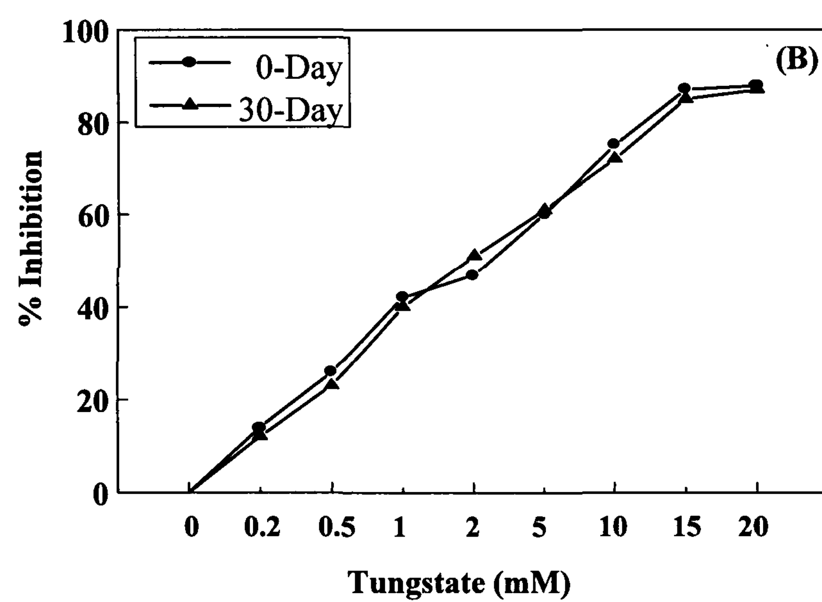
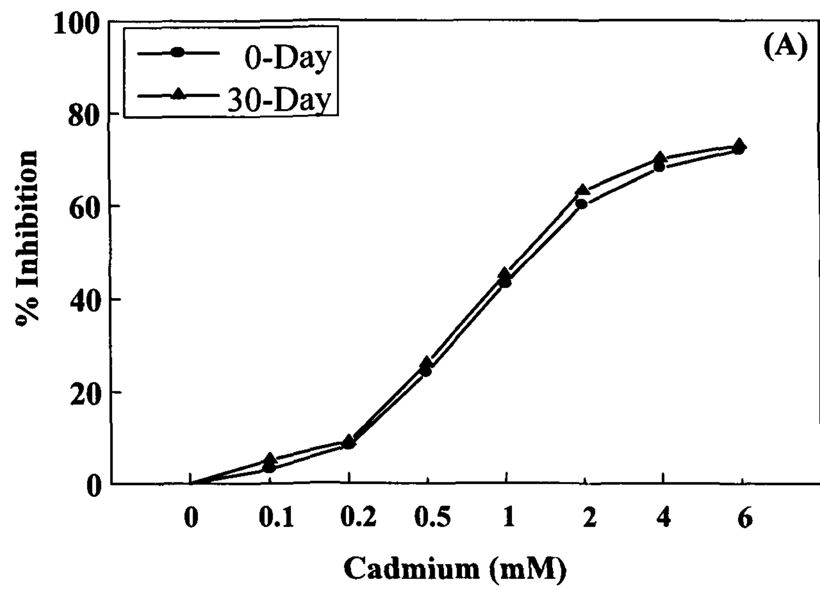


Fig. 30. Effect of tungstate (WO_4^{2-}) on the salt activation of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 20.



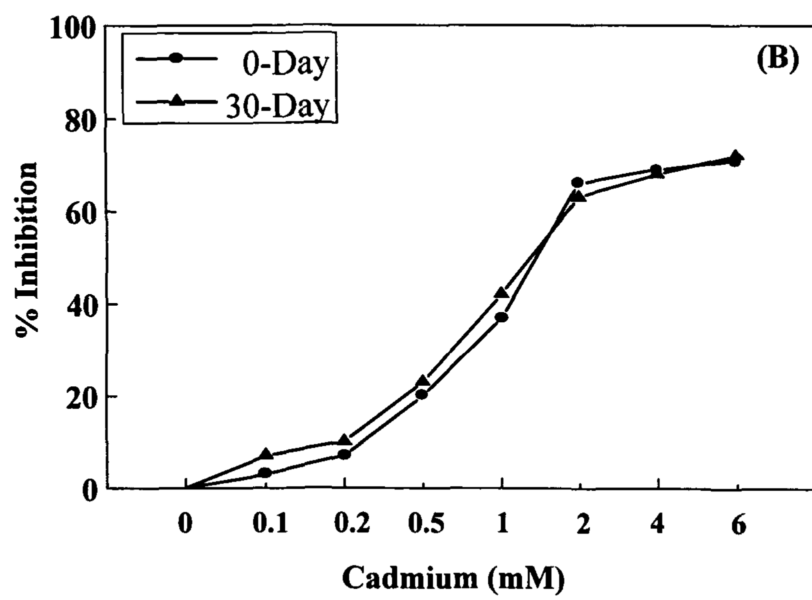
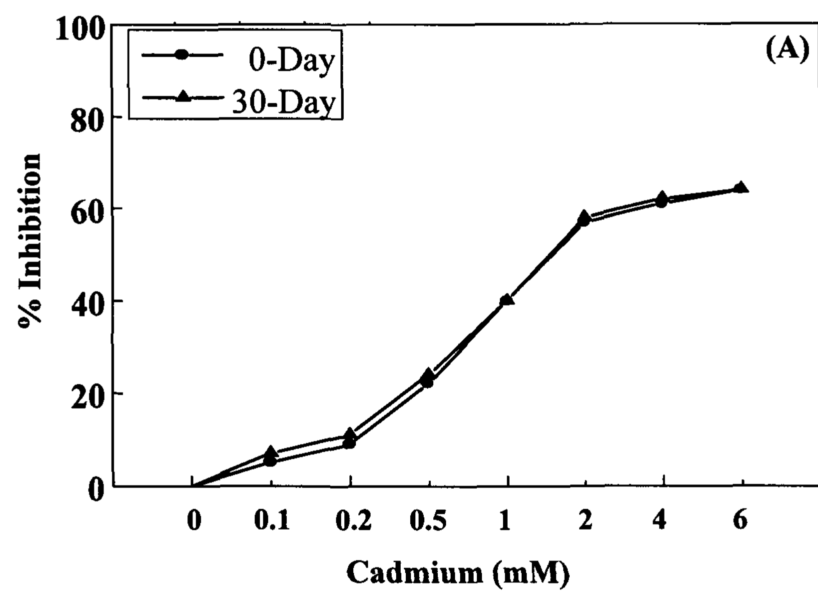


Fig. 31. Effect of cadmium (Cd^{2+}) on the heat activation of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 21.



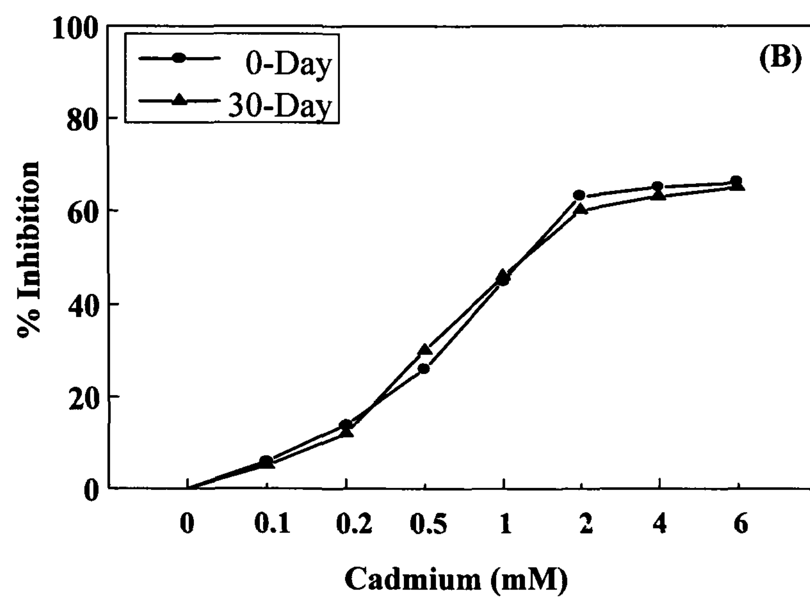
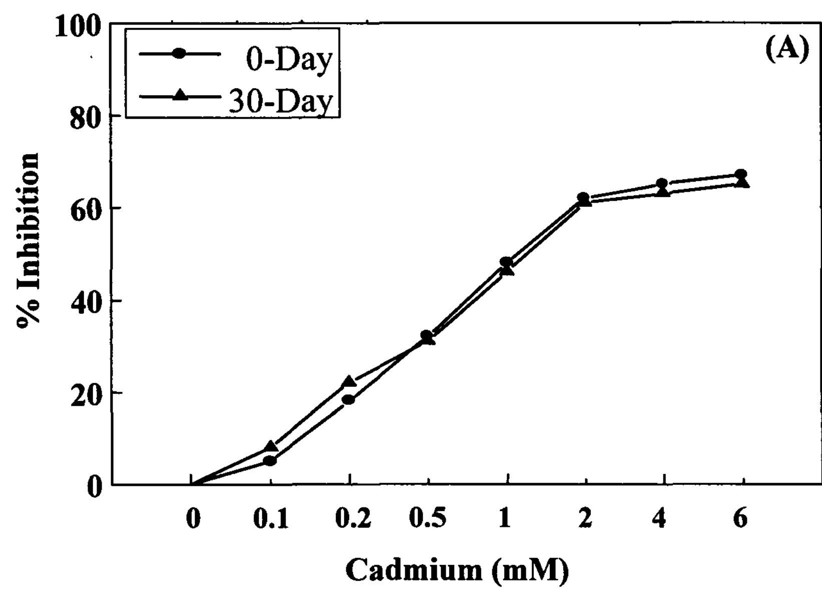


Fig. 32. Effect of cadmium (Cd^{2+}) on the heat activation of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 21.



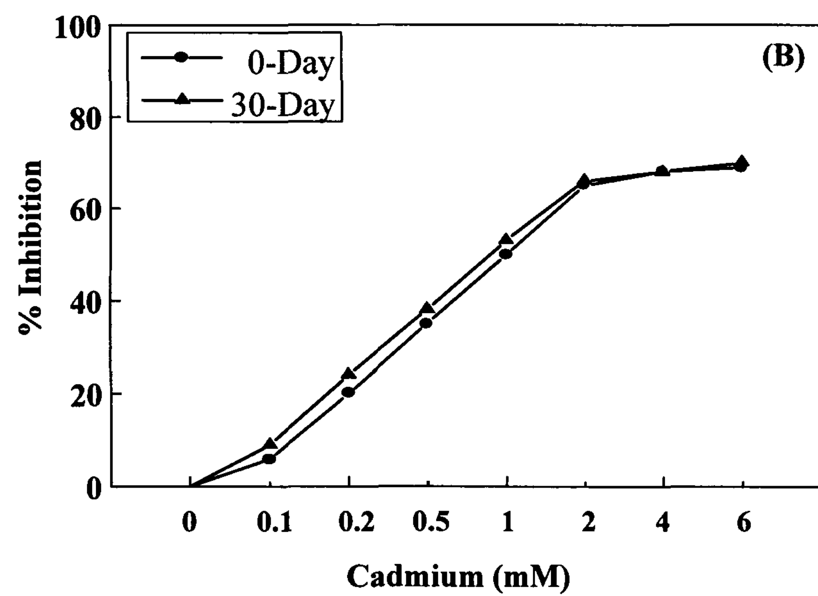
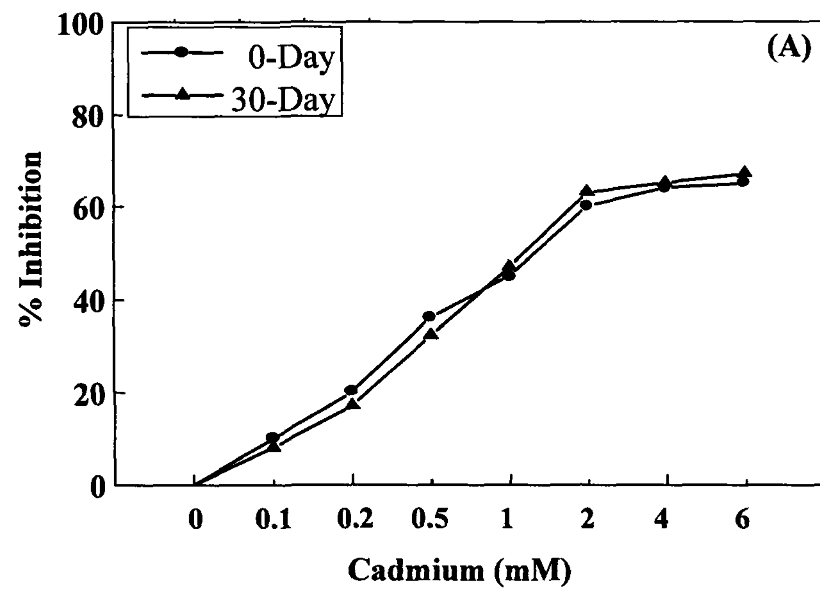


Fig. 33. Effect of cadmium (Cd^{2+}) on the salt activation of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 21.



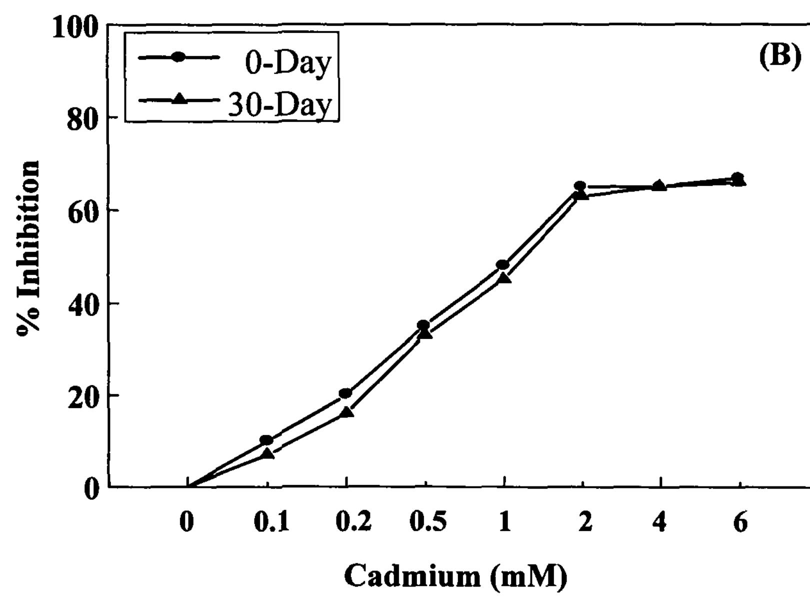
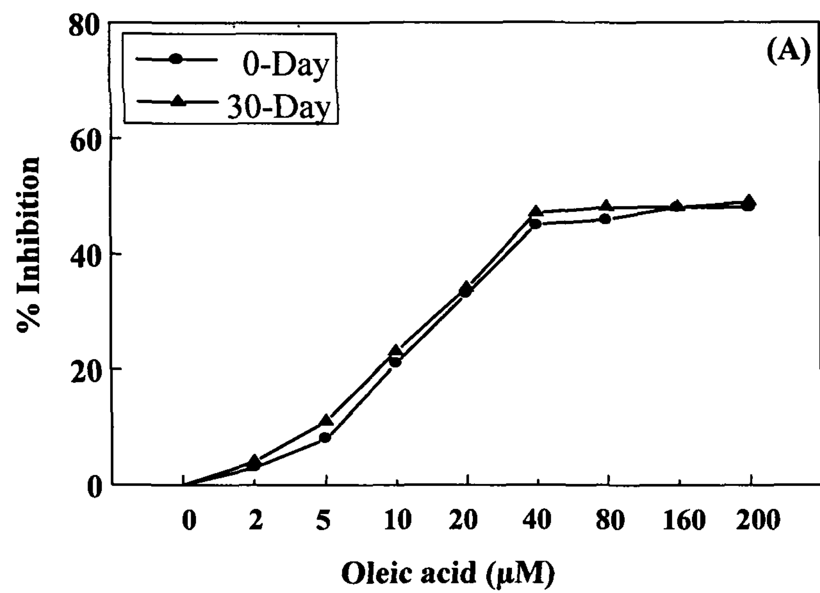


Fig. 34. Effect of cadmium (Cd^{2+}) on the salt activation of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 21.



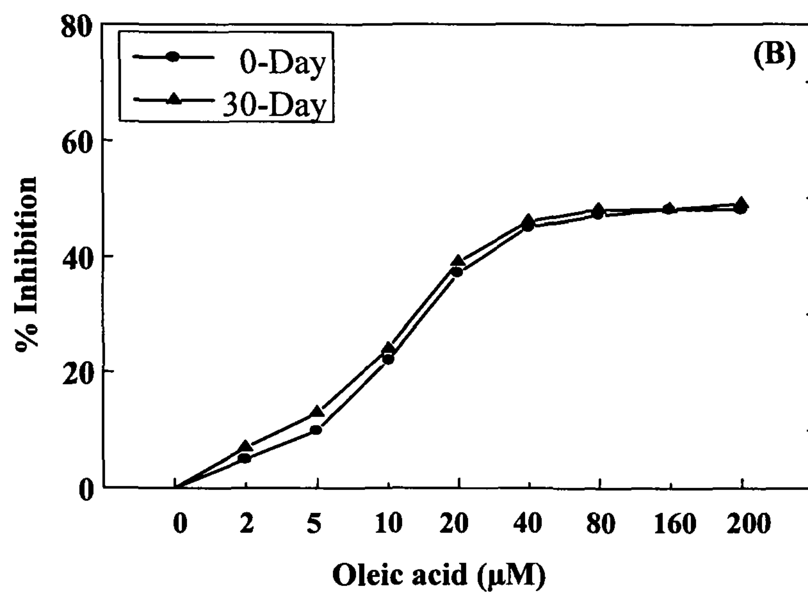
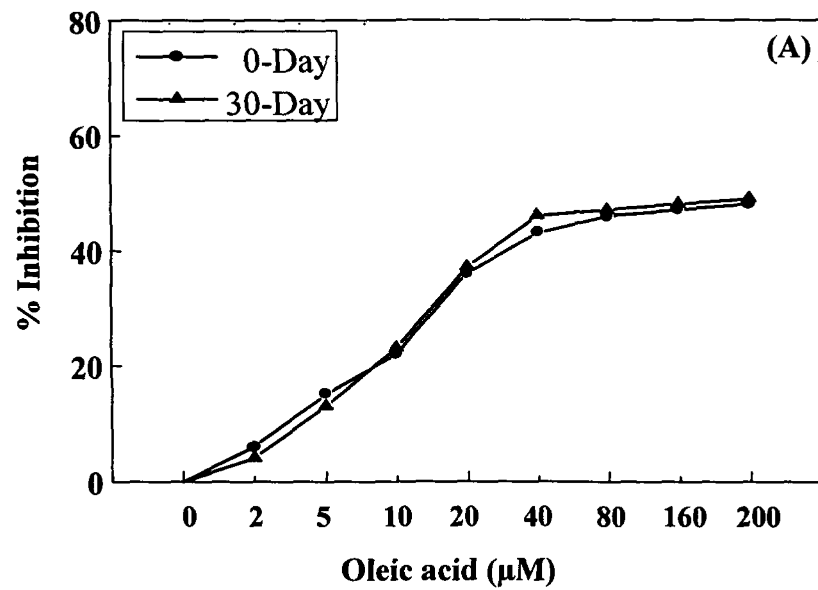


Fig. 35. Effect of oleic acid (C 18:1) on the heat activation of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 22.



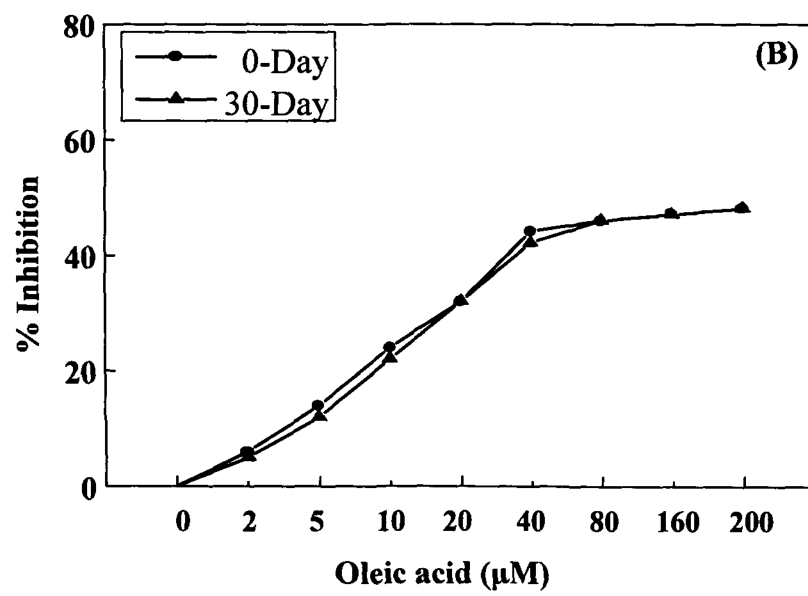
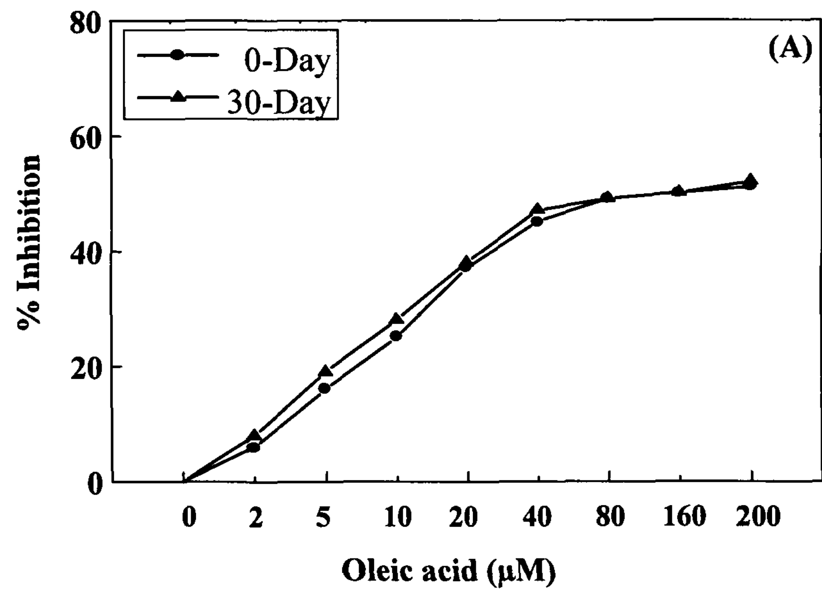


Fig. 36. Effect of oleic acid (C 18:1) on the heat activation of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 22.



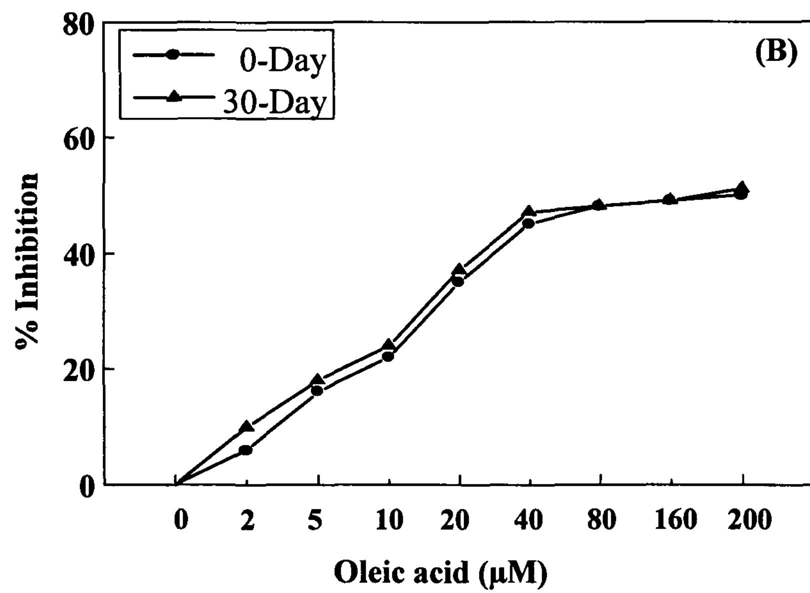
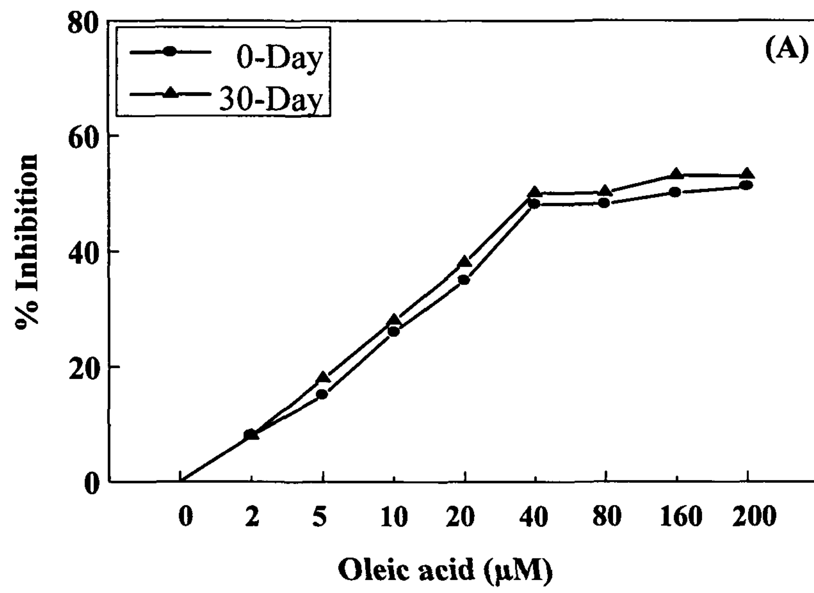


Fig. 37. Effect of oleic acid (C 18:1) on the salt activation of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes from 0- and 30-day old chicken by DNA-cellulose binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 22.



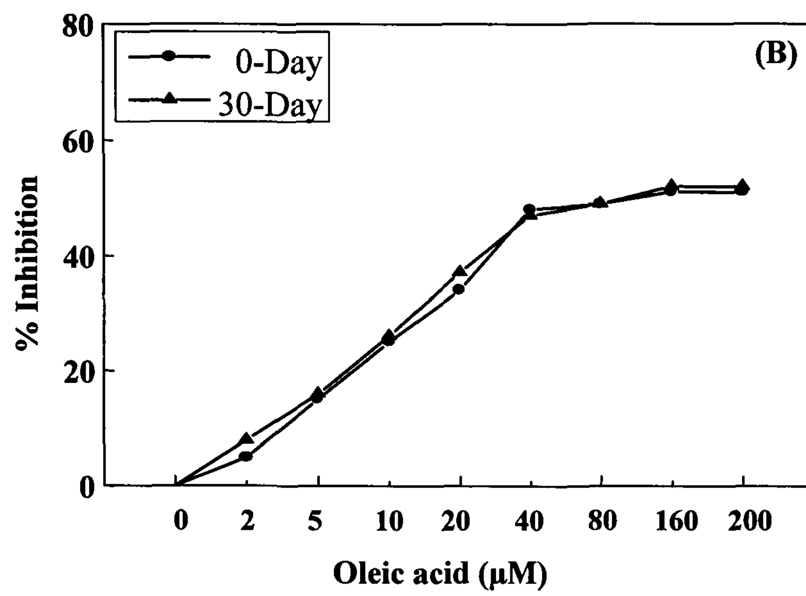


Fig. 38. Effect of oleic acid (C 18:1) on the salt activation of hepatic (A) and kidney (B) [^{3}H]dexamethasone-receptor complexes from 0- and 30-day old chicken by nuclear binding assay. Receptor preparation, activation conditions, DNA-cellulose binding and further processing of pellets are same as described in fig. 22.

Gel filtration analyses

Fig. 39 depicts the elution profile of the unactivated [^{3}H]dexamethasone-receptor complexes analysed on a sephadex G-200 column. The receptor from both the ages (0-and 30-day) eluted as a single peak at the same elution volume between the standard molecular weight markers, ferritin and β-amylase. The data generated from the above studies were plotted and linear regressed curves obtained, from which molecular weight and the stokes radii (R_s) were calculated. The plot of log M *vs.* V_e/V_o gave molecular weight of 255 kDa and 256 kDa for 0- and 30-day receptor, respectively (Fig. 40). The stokes radii were calculated from a plot of -(log $K_{av})^{1/3}$ *vs.* R_s and calculated to be 5.65 and 5.68 nm for the receptors of 0- and 30-day, respectively (Fig. 41).

The thermally activated [^{3}H]dexamethasone-receptor complexes were analysed on a sephadex G-100 gel column. The elution profile of the activated receptors showed that the receptors from both the ages eluted between the molecular markers, alcohol dehydrogenase and bovine serum albumin as a single peak and at the same elution volume (Fig. 42). A small peak of radioactivity, which eluted in the void volume, is probably due to the fraction of receptors remaining in the unactivated state. From the plot of log M *vs.* V_e/V_o, the molecular weight was calculated to be 86 kDa (0-day) and 87 kDa (30-day) (Fig. 43). The stokes radii, calculated from the plot of -(log $K_{av})^{1/3}$ *vs.* R_s gave the values as 3.28 nm (0-day) and 3.31 nm (30-day) (Fig. 44).

Ion-exchange analyses

To study the charge content of the unactivated as well as activated [^{3}H]dexamethasone-receptor complexes from liver of 0- and 30-day chicken, ion-exchange analyses were performed. The elution profile of the unactivated receptors (Fig. 45) does not reveal any age-related difference in the concentration of salt required to elute the receptors of the two ages. The hormone bound receptors from both the age groups eluted as a single peak at ~248 mM of KCl. Upon thermal activation, the elution of receptors from DE-52, reveals two radioactivity associated peaks, one eluting at ~100 mM KCl and the other at ~247 mM of KCl (Fig. 46). The peak which eluted at ~100 mM KCl represents the fraction of glucocorticoid receptors that have undergone thermal activation, while the peak at higher salt concentration is contributed by the fraction of receptors that remained unactivated. The data obtained from the above studies are also presented in a tabular form in Table III. However, these data did not reveal any differences in the charge content of the GR from the the two ages of chicken.

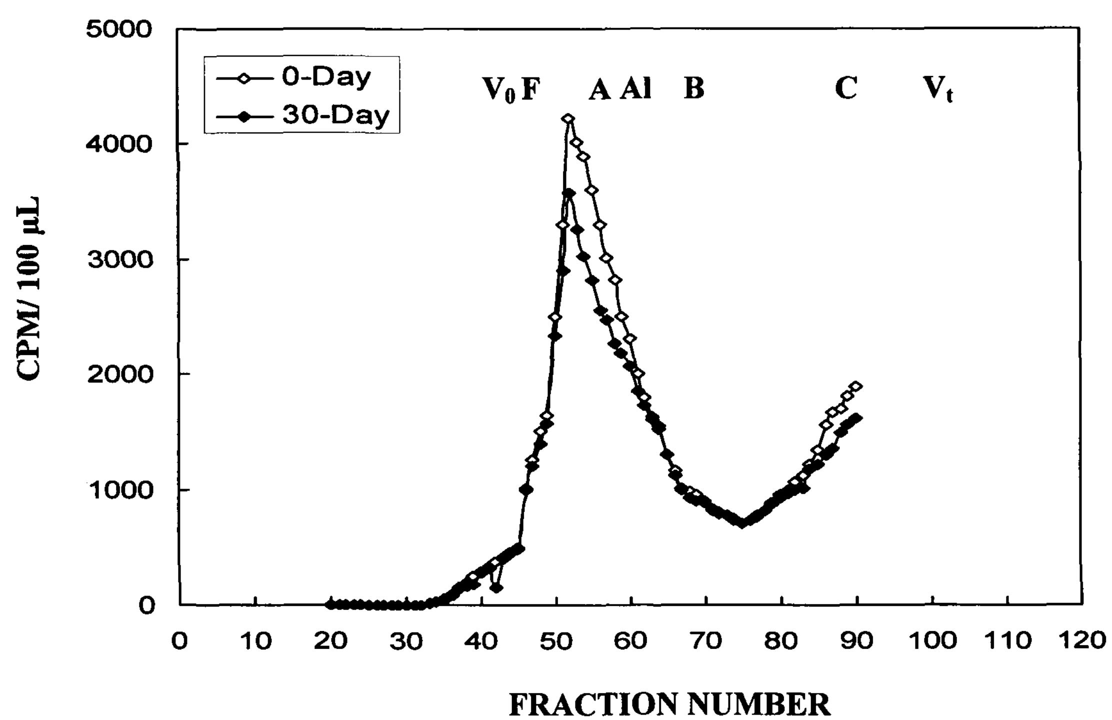


Fig. 39. Gel permeation column chromatography of hepatic unactivated glucocorticoid receptors. A column (1.8 x 90 cm) of sephadex G-200, equilibrated with buffer C (i) at 2-4°C was used. Cytosol prepared in buffer A was incubated with 60 mM [^{3}H]dexamethasone for 4 h at 0°C. After DCC treatment, 2 ml of the cytosol was loaded onto the column and eluted with buffer C (i). Fractions of 2 ml each were collected and 100 µl from each fraction was counted for bound radioactivity. Standard protein markers used were F, ferritin (443 kDa); A, β-amylase (200 kDa); Al, alcohol dehydrogenase (150 kDa); B, bovine serum albumin (66 kDa) and C, cytochrome C (12.4 kDa). V_o and V_t represent the elution volume of blue dextran and [^{3}H]dexamethasone, respectively. Each point in the elution volume represents the mean value of 4 experiments.

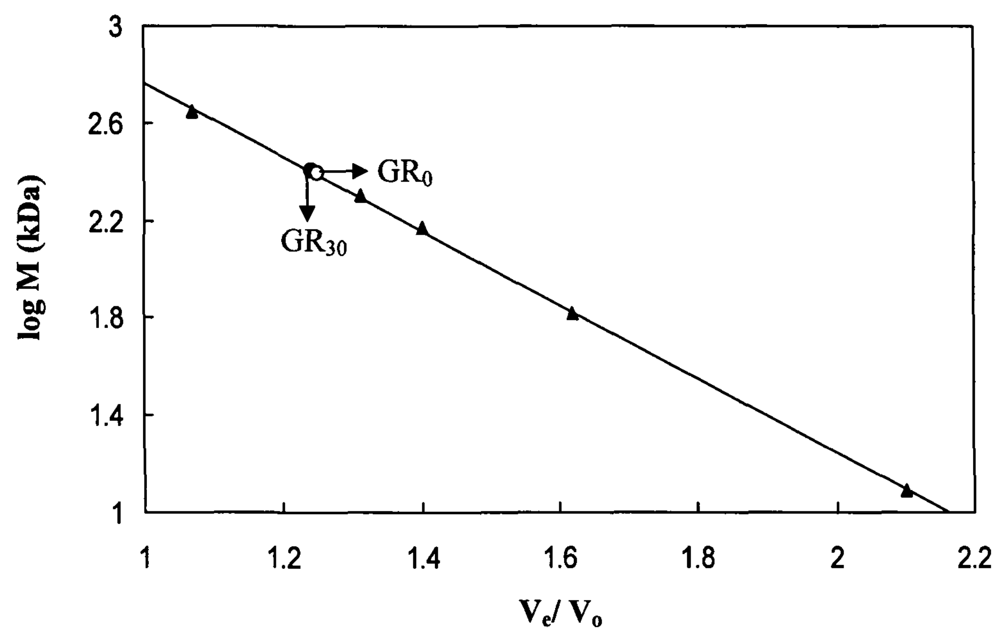


Fig. 40. Plot of log M *vs.* V_e/V_o for the determination of molecular weight of unactivated glucocorticoid receptors. The data from sephadex G-200 gel chromatography were plotted to obtain a linear-regressed curve. The standard protein markers used were ferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (156 kDa), BSA (66 kDa) and cytochrome C (12.4 kDa). GR_0 and GR_{30} represent the positions of unactivated hepatic glucocorticoid receptors from 0- and 30-day old chicken, respectively. The data is the mean of 4 separate experiments.

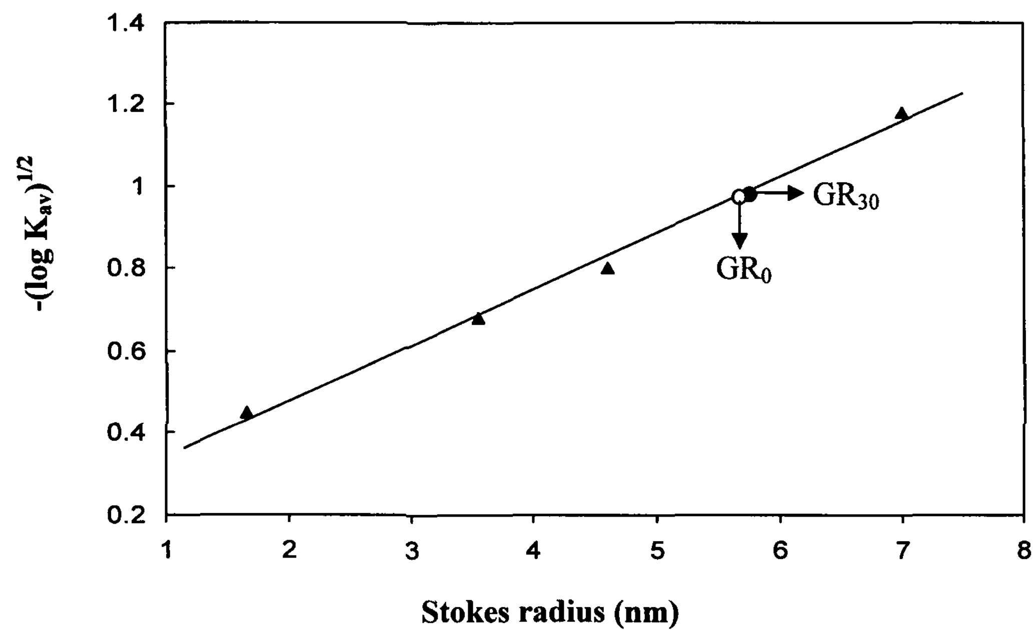


Fig. 41. Plot of -(log Kav)$^{1/2}$ *vs.* Rs for the determination of stokes radii of unactivated liver glucocorticoid receptors. The plot was generated using data from G-200 gel chromatography. The protein markers of known stokes radius were ferritin (7.0 nm), alcohol dehydrogenase (4.6 nm), bovine serum albumin (3.55 nm) and cytochrome c (1.64 nm). The positions of the receptor from the two age groups are indicated as GR_0 and GR_{30}, respectively. The values are mean of 4 experiments.

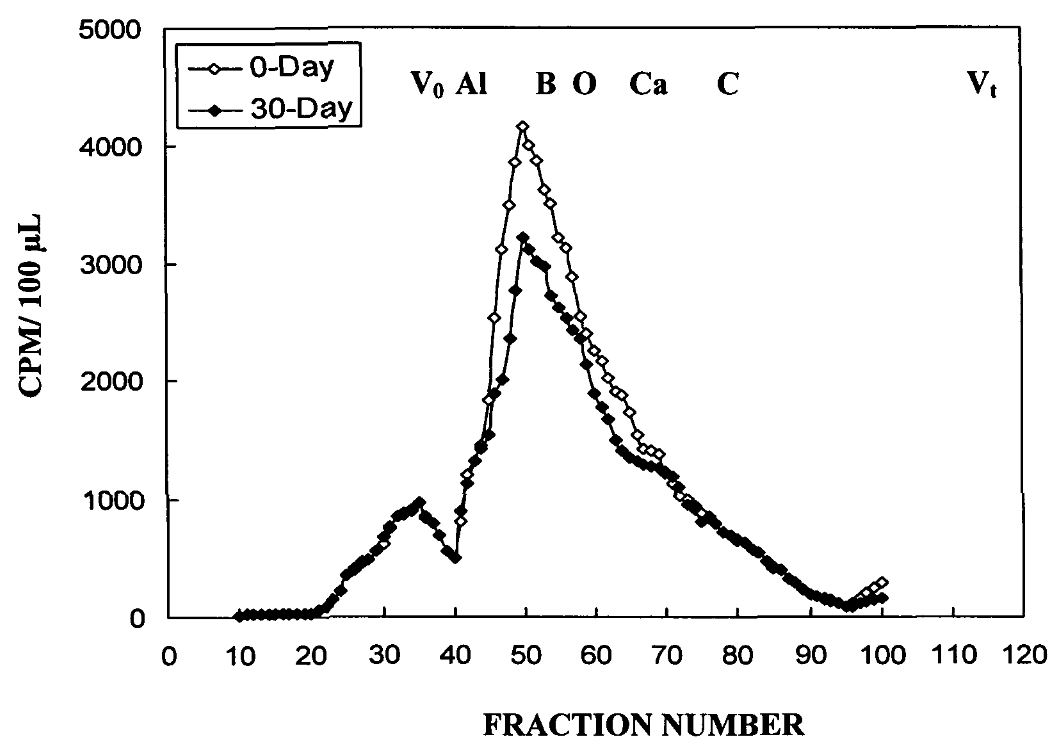


Fig. 42. Gel permeation column chromatography of hepatic activated glucocorticoid receptors. A column (3 x 40 cm) of sephadex G-100, equilibrated with buffer C (ii) at 2-4°C was used. After DCC treatment, the hormone-receptor complexes were activated at 25°C for 45 min and 2 ml of it loaded onto the column and eluted with buffer C (ii). Fractions of 2 ml each were collected and 100 µl from each counted for bound radioactivity. Standard protein markers used were A, alcohol dehydrogenase (150 kDa); B, bovine serum albumin (66 kDa); O, ovalbumin (45 kDa); Ca, Carbonic anhydrase (29 kDa) and and C, cytochrome C (12.4 kDa). V_o and V_t represent the elution volume of blue dextran and [^{3}H]dexamethasone, respectively. Each point in the elution volume represents the mean value of 4 experiments.

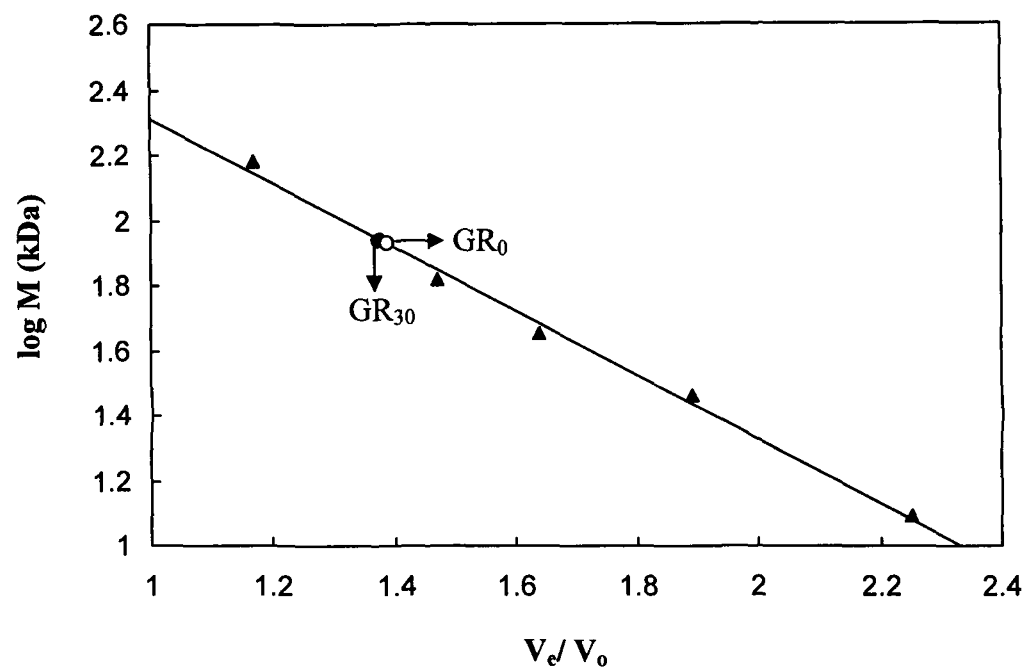


Fig. 43. Plot of log M *vs.* V_e/V_0 for the determination of molecular weight of activated glucocorticoid receptors. The data from sephadex G-100 gel chromatography were plotted to obtain a linear-regressed curve. The standard protein markers used were alcohol dehydrogenase (150 kDa), BSA (66 kDa), ovalbumin (45 kDa) and cytochrome C (12.4 kDa). GR_0 and GR_{30} represent the positions of the activated glucocorticoid receptors from 0- and 30-day old chicken, respectively. The data presented is the mean of 4 separate experiments.

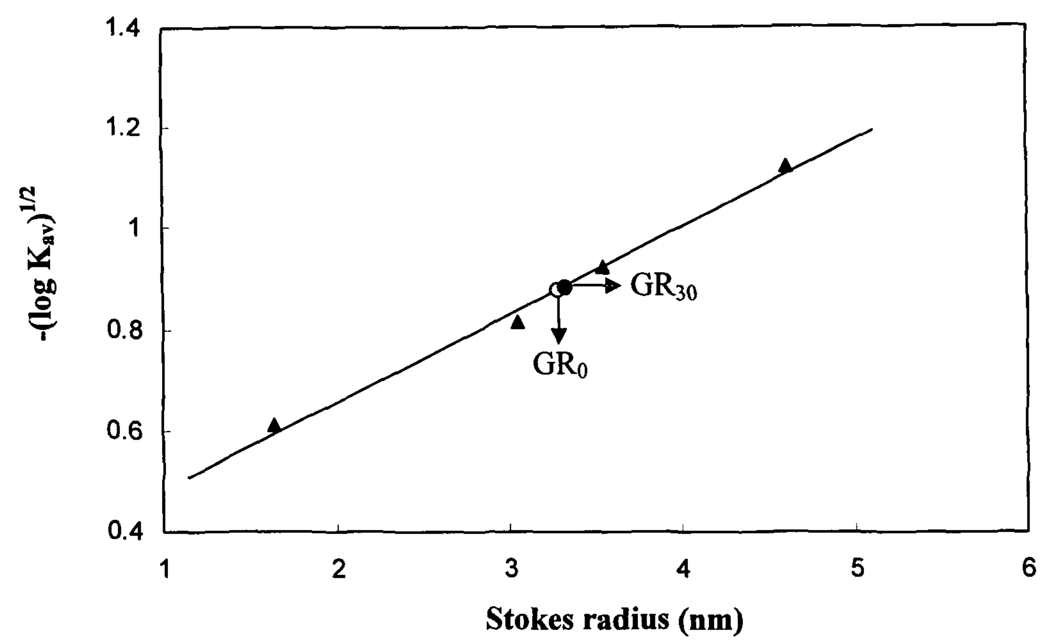


Fig. 44. Plot of -(log Kav)$^{1/2}$ *vs.* R_s for the determination of stokes radii of activated liver glucocorticoid receptors. The plot was generated using the data from sephadex G-100 gel chromatography. The molecular markers used were alcohol dehydrogenase (4.6 nm), bovine serum albumin (3.55 nm), ovalbumin (3.05 nm) and cytochrome C (16.4 nm). The positions of the receptor from the two ages are indicated by GR_0 and GR_{30}, respectively. The values are mean of 4 experiments.

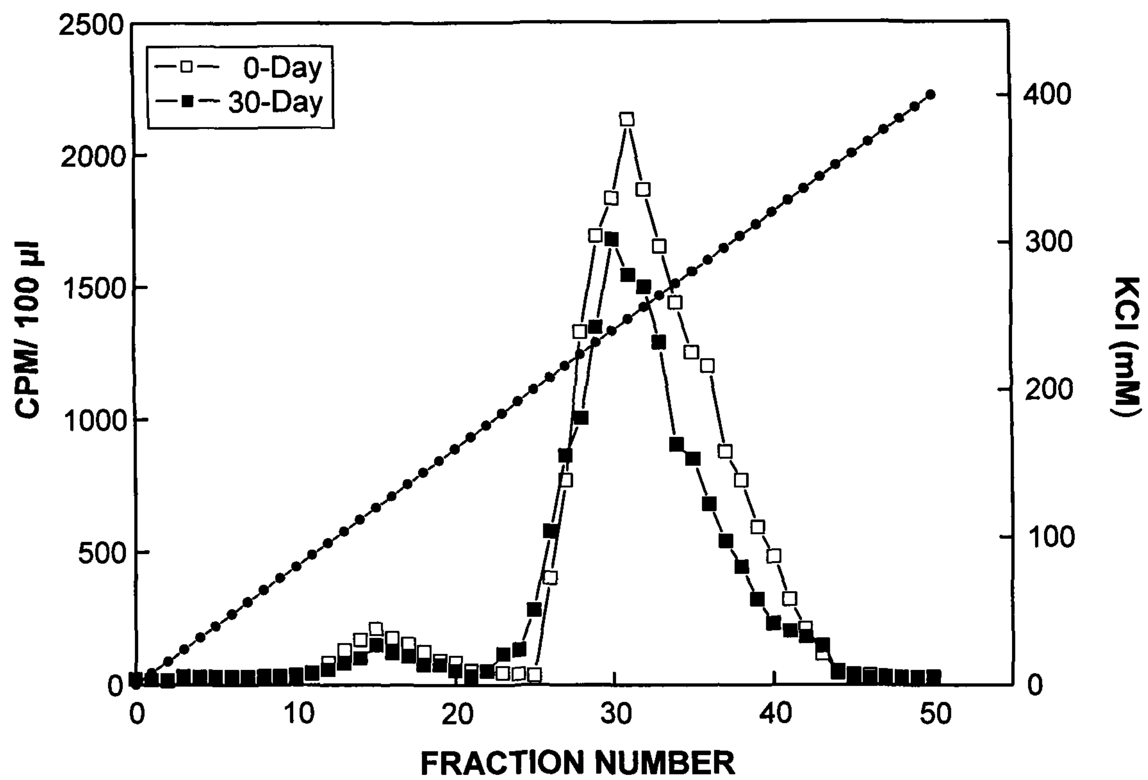


Fig. 45. Elution profile of the unactivated glucocorticoid receptors from anion-exchanger DE-52. A 5 ml glass syringe containing 3 cm high gel-bed layered over a 2 mm thick dextran coated charcoal layer was used. The gel was equilibrated at 2-4°C with buffer D. Unactivated [^{3}H]dexamethasone receptor complexes from the liver were prepared as given in the Materials and Methods section and 2 ml loaded onto the column. After washing off the unbound proteins, the bound proteins were eluted with a linear salt gradient (0-400 mM KCl in the above buffer). One ml fractions were collected and 100 µl from each fraction counted for radioactivity. Each point in the profile represents the mean of 4 experiments.

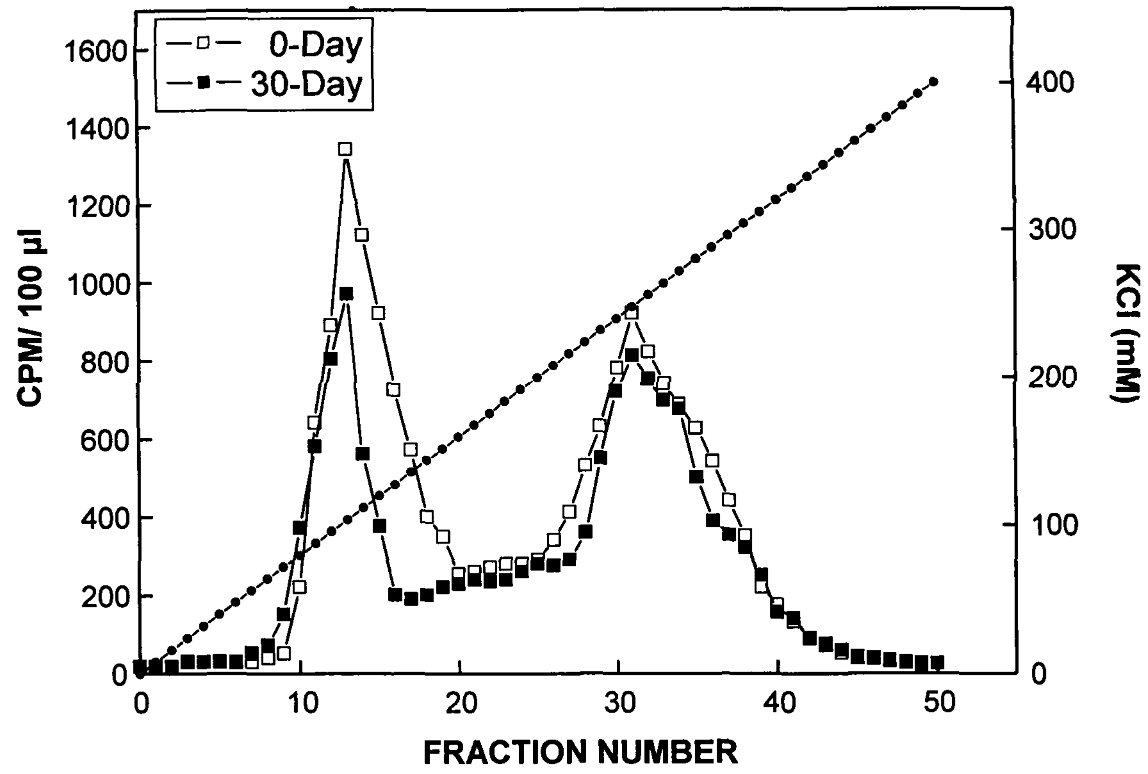


Fig. 46. Elution profile of the activated glucocorticoid receptors from anion-exchanger DE-52. A 5 ml glass syringe containing 3 cm high gel-bed layered over a 2 mm thick dextran coated charcoal layer was used. The gel was equilibrated at 2-4°C with buffer D. Heat activated [^{3}H]dexamethasone receptor complexes from the liver were prepared as given in the Materials and Methods section and 2 ml loaded onto the column. After washing off the unbound proteins, the bound proteins were eluted with a linear salt gradient (0-400 mM KCl in the above buffer). One ml fractions were collected and 100 μl from each fraction counted for radioactivity. Each point in the profile represents the mean of 4 experiments.

Table III

Physicochemical properties of hepatic GR from 0- and 30-day old male chicken		
Parameters	**0-Day**	**30-Day**
Unactivated receptors		
Molecular weight (kDa)	255.50 ± 2.50	256.20 ± 2.80
Stokes radius (nm)	5.65 ± 0.06	5.68 ± 0.14
Elution from DE 52	248 ± 4.00	241 ± 5.00
Activated receptors		
Molecular weight (kDa)	86.60 ± 4.00	87.30 ± 2.00
Stoke radius (nm)	3.28 ± 0.20	3.31 ± 0.07
Elution from DE 52 by KCI (mM)	100 ± 2.00	101 ± 3.00

DISCUSSION

An animal starts its life as a single cell- a fertilized egg. During development, this cell divides repeatedly to produce many different cells in a final pattern of spectacular complexity and precision. The genome is normally identical in every cell; the cells differ not because they contain different genetic information, but because they express different sets of genes. This selective gene expression controls the four essential processes by which the embryo is constructed: a) cell proliferation, producing many cells from one, b) cell specialization, creating cells with different characteristics at different positions, c) cell interactions, coordinating the behaviour of one cell with that of its neighbours, and d) cell movement, rearranging the cells to form structured tissues and organs. In a developing embryo, all these processes are happening at once, in a variety of different ways and in different parts of the organism (Alberts *et al.*, 2002). All changes in the animal form and functions during development are due to changes in the gene expression. Such changes determine which proteins are made, and where and when, during development. The selective gene expressions are important during development since it is the expressed proteins that determine how cells behave.

There are a number of ways by which control of gene expression is achieved *in vivo*. One of the important means by which such a control is brought about is through hormones which act as chemical messengers. Hormones coordinate cellular interactions which are of central importance during development of different tissues or organs. A cohort of hormones has long been recognized as players in the control of growth and development and glucocorticoids are no exception. Glucocorticoids (GCs) control several metabolic processes. They are the key regulators of homeostasis, stress responses and adaptation in animals (Baxter and Forsham, 1972; Sapolsky *et al.*, 2000). GCs play pivotal role in immune responses, inflammatory reactions, development and aging processes (Kalimi, 1984; Sharma, 1988). They are also required for normal maturation of the lung and the central nervous system and play a critical role in the control of postnatal growth (Buckingham, 2006). Many of these actions are 'activational' i.e. they are reverse in the absence of steroid. However, some of the actions of GCs are irreversible or 'organisational' and effectively programme adult physiology (Buckingham, 2006). Disturbances in the glucocorticoid milieu at critical stage of development may thus have long-term and potentially harmful effects on physiology, thereby influencing disease susceptibility. Evidence that adverse events may increase susceptibility in adulthood first emerged from the work of Barker's group which noted the correlation between low birth weight and increased risk of cardiovascular and metabolic disorders in later life (Barker *et al.*, 1989). Therefore, glucocorticoid levels are normally maintained within very low levels during development (Buckingham, 2006). Avian

growth and development, like in mammals, is under intricate hormonal controls, which involve endocrine, paracrine and autocrine signaling. The synthesis and secretion of adrenal hormones are initiated during embryogenesis and have been purportedly involved in avian development (Freeman and Vince, 1974). Corticosterone, the principal glucocorticoid, increases during the early stages of chicken development and induces several enzymes of carbohydrate metabolism (Cohen *et al.*, 1972; Wise and Frye, 1973). In addition, GCs also have differentiative effects in many eukaryotic embryonic tissues (Koike and Shiojiri, 1996). They also induce growth hormone (GH) producing cell differentiation in rats and chicken (Bossis *et al.*, 2004). Corticosterone promotes changes in behaviour and metabolism that help birds to adjust stressful situations (Cockrem *et al.*, 2004).

The actions of GCs are mediated by ubiquitous intracellular protein, the GR, which functions as a hormone-activated transcription factor for many target genes. The circadian and stress-induced secretion of the glucocorticoid is governed by the hypothalamo-pituitary-adrenal axis. Glucocorticoids exert their actions principally via intracellular receptors which belong to the nuclear receptor superfamily that includes receptors for other steroid hormones, thyroid hormone, retinoic acid, vitamin D, fatty acids and eicosanoids. Unbound glucocorticoid receptor is associated within the cytoplasm in an inactive oligomeric complex with other regulatory proteins such as the heat shock protein 90 (hsp90) which bind as dimer, hsp70, the p59 immunophilin, FKBP52 and the small p23 phosphoprotein. The interaction of GR with hsp90 is required to maintain the C-terminal domain in a favourable conformation for ligand binding (Davies *et al.*, 2005). Binding of hormone releases GR from its interaction with the inhibitory complex, thus inducing a conformational change which results in the unmasking of the receptor nuclear localization signal. Upon activation, GR thereby translocates to the nucleus and binds as a dimer to DNA through its central domain, which is structurally characterized by DNA binding motif (Pelaia *et al.*, 2003). GR either interacts with DNA via GRE or nGRE which results either in the activation or repression of genes, respectively. Activity of GR is not simply gene transactivation, but considerable cross-talk also occurs between the GR and a cohort of molecules like CBP (CREB-binding protein), SRC1 (steroid receptor coactivator-1) to mediate their function as transcriptional regulators (Pelaia *et al.*, 2003; Grenier *et al.*, 2004). Alternatively, GR can also modulate the expression of genes through a GRE-independent mechanism, which is mediated in part through protein-protein interactions of GR with other sequence-specific DNA-binding factors or coactivators (Stellato, 2004).

Earlier, the age-related changes in GR concentration, activation/transformation and their binding to nuclear chromatin in different mammalian tissues during postnatal development studies have been reported (Sharma and Timiras, 1987; Kalimi *et al.*, 1988; Borbhuiya and Sharma, 1995a & b). However, studies in the level and in the

physicochemical properties of GR during avian development are lacking. The work embodied in this thesis was planned to study the changes in the level of GR, as well as the magnitude of activation of the hormone-receptor complexes in order to ascertain the GR regulation during postnatal development. Conformational changes in the chromatin organization during postnatal ages, which may be involved in glucocorticoid regulation of gene expression in developing chicken, have also been studied.

Studies on the binding of [^{3}H]dexamethasone to glucocorticoid receptor

Responsiveness to GCs requires a critical threshold of transcriptionally active GR molecules in the cells (Okret *et al.*, 1991). When the receptor level is below that threshold or when some steps essential for receptor function, such as activation, is inhibited, transcription of target genes will not occur. Timed changes in the activity of transcription factors, including the GR, are required for orchestrated expression of gene networks during development (Ben-Dror *et al.*, 1993). Modulation of their activity is an important mechanism for control of gene function. Such modulation may involve changes in synthesis of a particular transcription factor, translational modifications and/or induction of antagonist that specifically repress the factor's transcriptional activity. Development and aging may partly be characterized by changes in the responsiveness of tissue and cells to certain hormonal modulators (Singh and Sharma, 1995). The magnitude of cell response to GCs depends on the concentration of receptor, as well as in the efficiency of GR-mediated signal transduction (Bamberger *et al.*, 1996). In many cases, changes in the receptor function appear to be closely associated with altered cell responsiveness to hormone during lifespan (Kalimi *et al.*, 1988). The occurrence of quantitative changes in receptor molecules are demonstrated during postnatal development and aging of mouse (Borbhuiya and Sharma, 1995b; Dutta and Sharma, 2004). The magnitude of action of GCs primarily depends on the level of its receptors and also on the post-receptor events (Borbhuiya and Sharma, 1995a & b). Cell responsiveness to GCs depends not only on the presence of glucocorticoid receptors but also on their concentration, which are known to fluctuate, for example, during development, during the cell cycle and following disturbances in endocrine status (De Kloet *et al.*, 1998).

Our findings, using [^{3}H]dexamethasone binding studies, demonstrate changes in the glucocorticoid receptor concentration in tissue- and age-specific manner during postnatal development of chicken. In the liver, the level of GR is maximum at day 5 of postnatal age, and this may possibly be associated with changing dietary and metabolic adjustments at this phase of the lifespan (Nongbri and Sharma, 2007). The yolk sac absorbed during hatching can supplement the nutritional needs for a limited period only, so by day 5 of postnatal age, there is total dependency on external diet. Earlier studies showed that hepatic GR

concentration is higher in weaning rats as compared to mature (Sharma and Timiras, 1987). Thus, our studies show similar correlations between dietary requirements and GR concentration.

In the kidney, the receptor level is maximum in the early postnatal age (day 0) and thereafter the GR level shows a steady decline until day 60 of postnatal age. In studies of mice, however, the receptor level is minimal in early postnatal ages (Borbhuiya and Sharma, 1995b). In kidney, glucocorticoids are known to influence glomerular filtration rate, ion-transport and other metabolic functions (Fanestill and Park, 1981; Sharma and Timiras, 1988).

The observed increase in receptor concentration at day 5 in liver and day 0 in kidney could be due to the increase in the receptor *per se* or due to the increase in receptor affinity for the hormones. Scatchard analyses of the binding data were, therefore performed to see whether increase in receptor concentration or increase in affinity of receptor for hormones or both that contributed to our observations. The data obtained confirmed our findings of increase in receptor concentration but not the affinity of the receptor for the hormone. The maximum specific binding sites (fmol/mg protein), obtained from the Scatchard plot are also similar to that observed from binding studies. However, there is no age-associated alteration in the binding affinity, as given by the equilibrium dissociation constant (K_d) values in both the tissues. It was also reported earlier that the affinity of GR for hormone does not change as function of age in different tissues of animals (Sharma and Timiras, 1987; Kalimi *et al.*, 1988; Ranhotra and Sharma, 2001; Dutta and Sharma, 2003; Nongbri and Sharma, 2007). Slot blot analyses also confirmed the higher concentration of GR at day 5 and day 0 in liver and kidney, respectively.

In the cardiac muscle, the receptor concentration is maximum during the early postnatal ages but unlike the kidney the receptor concentration is constant up to day 30 and by day 60 of postnatal age the receptor level declines. Slot blot analyses of the receptor preparation and the intensity of the slot bands confirm a decline in receptor protein level at day 60 compared to day 0 of postnatal age. The concentration of cardiac glucocorticoid receptors was much lower than that of liver receptors which agrees the report by Boer and Oddos (1979). Several observations suggest that GCs have an important effect upon the heart: patients with Addison's disease and adrenalectomized animals show hypotrophy of the heart and also the contractile force of the heart is impaired (Brown and Remington, 1955). Glucocorticoids effectively restore the normal circulatory parameters in animals that are in adrenal crisis (Swingle *et al.*, 1957) and potentiate the circulatory and myocardial effects of catecholamines (Baxter and Forsham, 1972).

In muscles, GCs have catabolic actions and result in decreased synthesis and increased degradation of protein and RNA (Baxter and Forsham, 1972). In skeletal muscle, the receptor concentration is highest at day 10 and then declines by day 60 of postnatal age. It was reaffirmed by slot blot analysis that the level of GR protein was higher at day 10 in the skeletal muscle of chicken. Such an expression pattern in GR level may reflect the metabolic differences in relation to the role of glucocorticoids in the skeletal muscle of developing chicks.

In the cerebral hemisphere, the receptor concentration is low compared to the other tissues studied. Furthermore, unlike the other tissues, the receptor level does not elicit any significant changes in all the ages studied. Within the central nervous system (CNS), GCs target both the neurons and the glial cells. During development these actions underpin important organizational events in the brain, while in adulthood, they contribute to neuronal plasticity and are implicated in the process of neurodegenaration (Sapolsky and Meaney, 1986).

Studies on the activation of [^{3}H]dexamethasone-receptor complexes

In cell-free systems, unoccupied glucocorticoid receptors are found in the cytosolic fraction where they can be incubated with hormones to form complexes. These complexes exhibit very little affinity for nuclear sites (Milgrom *et al.*, 1973). Activation or transformation is a process which involves a conformational change in the glucocorticoid-receptor complex resulting in the exposure of positively charged amino acid residues on the surface of the protein (Milgrom *et al.*, 1973; DiSorbo *et al.*, 1980). Although *in vitro* activation of glucocorticoid receptor complexes can be achieved by a variety of manipulations including elevated temperature, increased ionic strength, elevated pH or gel filtration (Milgrom *et al.*, 1973; Goidl *et al.* 1977), the underlying biochemical mechanism(s) is not very clear. Activated GR acquires an increased affinity for isolated nuclei and polyanions such as phospho-cellulose, ATP-sepharose and DNA-cellulose. The activation of glucocorticoid-receptor complexes has also been described in intact cells under physiological conditions (Munck and Foley, 1979; Holbrook *et al.*, 1983).

Activation studies were performed in the liver and kidney of chicken of two-age groups (0- and 30-day), to determine the physicochemical changes in the receptor molecules in the activation and nuclear binding stages. These ages were selected initially in liver keeping in mind that the GR concentrations were similar, hence, any alterations in the activation/nuclear binding properties could be attributed to reasons other than change in receptor number. The degree of activation of the [^{3}H]dexamethasone-receptor complexes by heat (25ºC for 45 min) and salt (25 mM $CaCl_2$ at 0ºC for 45 min) were judged using DNA-cellulose and purified nuclei. In chicken, the optimum concentration of $CaCl_2$ for maximum

activation of glucocorticoid-receptor complex was higher than that required for murine system. DNA-cellulose served as a non-specific binding media (open DNA-binding sites), whereas a more physiological *in vitro* binding medium was provided by the purified nuclei.

DNA-cellulose binding assays in the liver show that the magnitude of activation of [^{3}H]dexamethasone-receptor complexes both by heat (25ºC for 45 min) and salt (0ºC + 25 mM $CaCl_2$ for 45 min) are significantly higher (~2.5 fold) as compared to the unactivated receptor complexes which were incubated at 0ºC for 45 min. However, the degree of activation is similar for hepatic glucocorticoid receptors obtained from 0- and 30-day old chicken. Similar observations were seen in kidney where the degrees of thermal- as well as salt-mediated activation processes were significantly higher (~2 fold) as compared to the unactivated hormone-receptor complexes.

Using purified nuclei as a binding medium showed that the binding of both heat- and salt-activated hormone-receptor complexes is significantly lower in 30-day old chicken as compared to day 0. The decreased nuclear binding of both the heat and salt activated [^{3}H]dexamethasone-receptor complexes in 30-day old chicken tissues could be due to the decline in the affinity of the receptor (receptor property) for nuclear components or due to the changes in the DNA/chromatin (nuclear property), that allowed lesser binding of the activated hormone-receptors in 30-day old chicken. To determine which of these two properties contributed to the observed differences in nuclear binding, cross-mixing experiments were performed, wherein both thermally- and salt-activated receptor complexes of 30 day-old chicken were allowed to interact with the nuclei of 0-day chicken and *vice versa*. Our findings of a similar pattern of binding by the mixed as well as the non-mixed groups indicated a change in the nuclear property as a probable reason for the decreased nuclear binding in 30-day old chicken (Borbhuiya and Sharma, 1995a & b; Nongbri and Sharma, 2007).

The supercoiling of DNA double helix around histone core confers the specificity of digestion of DNA in chromatin by pancreatic DNase I (McGhee and Felsenfeld, 1980). It makes single stranded cuts in the double stranded DNA at 10-bp intervals and its multiples and it is widely used to identify chromatin organization in animals (Chaurasia and Thakur, 1997). DNase I cut the DNA where it is maximally exposed and thus depends on the degree of chromatin organization. Our data both on liver and kidney reveal higher digestion of chromatin in day 0 reflects a less condensed chromatin organization at this stage of postnatal development. This probably allows a higher *in vitro* binding of activated hormone-receptor complexes. However, at day 30, where extraction of bound complexes is less, chromatin might have acquired a more compact organization and hence a lower binding of activated hormone-receptor complexes. Our findings corroborate with earlier reports that digestibility of chromatin by DNase I decreases as development proceeds in older group of animals (Chaturvedi and Kanungo,1983; Kanungo, 1994; Borbhuiya and Sharma, 1995b).

Inhibition of activation studies

In order to gain a better understanding of the process of glucocorticoid receptor activation, a variety of approaches have been applied. One of these approaches has been to study the interaction of GR with isolated nuclei, or DNA-cellulose. Consequently, various compounds have been identified which inhibit the activation of GR *in vitro*. Extensive analysis of avian progesterone receptor with inhibitors of activation has been reported (Nishigori and Toft, 1980; Moudgil, 1983). We have utilized some of these agents for our study to monitor their inhibitory effects on the activation process. Study was also extended to find out tissue- and age-specific inhibition of activation process of GR by these inhibitors. Molybdate is a transition metal ion whose inhibitory effect on GR activation was reported (Moudgil *et al.*, 1984; Nishigori and Toft, 1980). Molybdate is known inhibitor of phosphatase activity, and the observations that phosphatase inhibitors like molybdate and tungstate stabilize steroid receptors and block transformation of cytosolic receptor suggested that these compounds may act indirectly by blocking the action of phosphatases (Leach *et al.*, 1979). It was also reported that the rate of activation of glucocorticoid-receptor complex is stimulated by incubation with alkaline phosphatase (Barnett *et al.*, 1980). Therefore, it was proposed that activation of glucocorticoid receptors involves a dephosphorylation of receptor itself or of some related regulatory component(s) of cytosol (Leach *et al.*, 1979; Barnett *et al.*, 1980). Modarress *et al.* (1994) reported that the metal oxyanions, like molybdate, tungstate and vanadate interaction with the GR or an associated protein(s) is independent of thiols. Studies by Murakami and Moudgil (1981) on rat liver GR indicate that molybdate blocks the *in vitro* activation of glucocorticoid receptor and also inhibits the binding of activated glucocorticoid-receptor complexes to acceptors such as purified nuclei, DNA-cellulose and ATP-sepharose. Therefore, both non-activated and activated forms of GR are sensitive to molybdate action. Moreover, activated glucocorticoid-receptor complexes bound to acceptors could be extracted by molybdate, thus suggesting an interaction between GR and molybdate (Moudgil *et al.*, 1984). Heat activation of glucocorticoid-receptor complex can be blocked by treatment of cytosol with sodium tungstate (Moudgil *et al.*, 1984). Tungstate, unlike molybdate results in irreversible change in the properties of glucocorticoid-receptor complex leading to a loss of its DNA-binding capacity (Murakami *et al.*, 1982; Moudgil *et al.*, 1984). Our findings on the inhibition of molybdate and tungstate on the heat and salt activation of GR from liver and kidney of chicken are consistent with the earlier reports (Kalimi *et al.*,1983; Moudgil *et al.*, 1984; Zakula and Moudgil, 1991). However, our data suggest that tungstate to be more effective agent than molybdate for blocking receptor activation and this finding is consistent with that of Moudgil *et al.* (1984).

Thiols are the most reactive nucleophiles commonly found in biological systems. There are about 20 thiols in the glucocorticoid receptor, and thiols have been found to be involved in steroid binding (Simons *et al.*, 1987; Carlstedt-Duke *et al.*, 1988; Miller and Simons, 1988), DNA binding (Bodwell *et al.*, 1984; Tienrungroj *et al.*, 1987; Miller and Simons, 1988), and possibly activation (Tienrungroj *et al.*, 1987; Blicq *et al.*, 1988). Divalent cadmium (Cd^{2+}) has been shown to be an efficient inhibitor of enzymes containing dithiols (Joshi and Hughes, 1981). For our study, we used Cd^{2+} as a chemical probe to determine the importance of vicinal dithiols in the activation of glucocorticoid-receptor complex. Findings revealed that Cd^{2+} inhibits the *in vitro* heat and salt activation of hepatic and renal glucocorticoid-receptor complexes in a dose-dependent manner, attaining a maximal inhibition (~60%) at 2 mM. However, the precise role of Cd^{2+} on the inhibition of activation of GR is not known. The observed inhibition of actvation by Cd^{2+} may probably be mediated by reaction with vicinal dithiols present in the GR.

Glucocorticoids facilitate lipolysis in adipocytes and the released fatty acids are metabolized in many tissues, including liver and kidney. Polyunsaturated fatty acids (PUFAs) have been attributed to play important roles in growth and development of mammals, where GCs also act as a regulator of such processes (Simopoulous, 1991). PUFAs have also been shown to reduce hormone binding to GR (Sumida *et al.*, 1993; Haourigui *et al.*, 1994). Ranhotra and Sharma (2004) demonstrated the inhibitory effects of PUFAs on the *in vitro* activation of hepatic glucocorticoid receptor activation. To ascertain whether PUFAs have similar inhibitory actions on the *in vitro* activation of avian GR we used oleic acid and found that it is a potent inhibitor of both heat and salt activation of hepatic and renal GR complexes in a dose dependent manner. Earlier experiments performed by Ranhotra and Sharma (2004) indicate a possible involvement of degree of unsaturation and fatty acid chain length in inhibiting the activation of GR. The exact mechanism(s) of these inhibitory effects are unclear yet. Probably, PUFAs induce a conformational change in the receptor molecule through the involvement of their unsaturated moieties, and thereby may inhibit the release of bound heat shock proteins that keep the receptor in an unactivated form.

Both the thermal as well as salt-mediated activation processes were inhibited to a similar magnitude in the liver and kidney of chicken. Moreover, no age-related differences in the degree of inhibition are seen, suggesting no tissue- and age-specific differences in sensitivity of the receptor to these chemical agents. This agrees well with the observation of Kalimi *et al.* (1983), who reported identical sensitivity of liver glucocorticoid receptor from adult and aged rats to inhibitors of thermal activation. The above observations support the opinion that alterations in chemical properties of receptors do not occur with age.

Studies on the physicochemical characterization of glucocorticoid receptors

Studies on the physicochemical properties of the glucocorticoid receptors did not reveal any change in hepatic receptor from 0- and 30-day old chicken. Gel filtration analyses of both unactivated and thermally activated liver glucocorticoid receptors showed similar values for both 0- and 30-day old chicken. However, the low molecular weight of the activated receptors from both the age groups is indicative of monomeric form upon activation as compared to the oligomeric form of the unactivated receptor complexes where the molecular weight is higher because of presence of other associated proteins. A similar observation was seen while looking at stokes radii for both unactivated as well as activated receptors from 0- and 30-day old chicken. Similarly, the elution pattern from DE-52 did not reveal any charge differences in the hepatic glucocorticoid receptors in the two ages studied. The above findings correlate with the findings of Borbhuiya and Sharma, (1999) who reported no change in the physicochemical properties of hepatic GR from pre- and post-weaned mice. Our findings on chicken reveal that the molecular mass of chicken GR was slightly lower than that reported earlier in mice and rats (Kalimi *et al.*, 1983; Grandics *et al.*, 1984; Borbhuiya and Sharma, 1999). Cloning of the full-length cDNA for chicken kidney GR revealed that it encodes 772 amino acids which is slightly smaller than the rat GR having 794 amino acids (Kwok *et al.*, 2007). The high affinity of anti-rat GR antibody to the chicken GR, reflects a high degree of structural homology as far as the immunogenic domain is concerned and this support the observation that there is ~ 55% cross-reactivity between the anti-rat GR and the chicken GR. Taken together, these findings did not convey any fundamental differences in the physical and chemical properties of the GR during development of chicken.

In conclusion, it is emphasized that the glucocorticoid receptor concentration and not the hormone binding affinity alters during postnatal development of chicken. The above findings also reveal a decreased nuclear binding of the heat and salt activated receptors in developed animals. This is probably due to the change in the chromatin organization to a higher ordered, more compact conformation thereby limiting the availability of nuclear binding sites in mature animals. Moreover, there is no age related difference in the inhibition of activation by various modulators and also in the physicochemical properties of chicken glucocorticoid receptors. Such changes in the level of glucocorticoid receptors and their nuclear binding may contribute towards functional changes in glucocorticoid responsiveness in developing animals.

Summary

The lifespan of an animal may be broadly divided into three phases: developmental, reproductive, and senescence. All changes in the animal form and functions during development depend on unique set of genes, which are sequentially activated and repressed. Such changes determine which proteins are made, and where and when, during development. The duration of this phase may vary within certain limits and may be influenced by intrinsic factors such as hormones and other effectors, and extrinsic factors such as nutrition and stresses. Hormones coordinate cellular interactions which are of central importance during development of different tissues and organs.

Glucocorticoids, an essential class of stress-induced endogenous steroid hormones under control of the hypothalamic-pituitary-adrenal axis, affect virtually all tissues and regulate various biologically important functions, from metabolism, behaviour, immune response to growth and development in cell-specific manner. Most of the physiological actions of glucocorticoids are mediated by binding to a specific intracellular protein called glucocorticoid receptor (GR), which is ubiquitously distributed and acts as ligand-dependent transcription factor belonging to the superfamily of nuclear receptors. Nuclear receptors are still being found, and more than 300 sequences have been reported. Many are important transcriptional regulators involved in widely different physiological functions such as the control of embryonic development, cell differentiation, and metabolic homeostasis.

As lipophilic molecules, glucocorticoids cross the cell membrane readily to interact with GR which resides in the cytoplasm in association with heat shock proteins and other chaperonic and immunophilic proteins. Upon hormone binding, the protein complex dissociates due to conformational change in the GR, a process termed 'activation' or 'transformation'. Activation of GR facilitates nuclear import where they dimerize and then bind specifically to glucocorticoid response elements (GREs), enhancing transcription or suppress transcription when it binds to negative GREs (nGREs). GR also modulates gene transcription via cross-talk with other transcription factors such as activator protein-1, nuclear factor-κB, members from the signal transduction and activator of transcription family.

Numerous studies have been made on the age-related changes in GR concentration, activation/transformation and their binding to nuclear chromatin in different mammalian tissues during postnatal development. It has also been demonstrated that changes in physicochemical properties of hepatic GR occur as a function of age. However, information on the changes in the level and in the physicochemical properties of GR during avian development is scarce. The present study describes the changes in the level of GR and also in the physicochemical properties of GR during postnatal development. Conformational

changes in the chromatin organization during postnatal ages which may be involved in glucocorticoid regulation of gene expression in developing chicken are also examined.

Studies on the binding of [^{3}H]dexamethasone to glucocorticoid receptor

Using [^{3}H]dexamethasone binding studies, our data show a change in the glucocorticoid receptor concentration in a tissue- and age-specific manner during the postnatal development of chicken. In the liver, the receptor level reaches a peak value by day 5 of postnatal age, which is significantly higher than the values observed in other age groups. In the kidney, the receptor concentration is maximum in the early postnatal age (0-day), and thereafter shows a gradual decline. The observed increase in receptor concentration at day 5 in liver and day 0 in kidney could be due to the increase in the receptor concentration *per se* or an increase in the receptor affinity for the hormone. To ascertain the above possibilities, slot blot analyses were performed and results indicate the increase in GR concentration at day 5 and day 0 in liver and kidney, respectively. Scatchard analysis of the binding data also confirmed the above findings. Moreover, there is no age-associated alteration in the dissociation constant (K_d) values in both the tissues. The glucocorticoid receptor concentration in the cardiac muscle (heart) is constant in the early postnatal ages up to day 30. By day 60, the receptor level declines and the level at day 90 is similar to that at day 60 of postnatal age. Slot blot analyses of the receptor preparation and the intensity of the slot bands confirm a decline in receptor protein level at day 60 compared to day 0 of postnatal age. In skeletal muscle, the glucocorticoid receptor level reaches the peak value by day 10 of postnatal age, which is significantly higher than the value observed at day 5 of postnatal age. The receptor level then declines at day 60 of postnatal age and the receptor level at day 90 is similar to that at day 60 of postnatal age. Slot blot analyses and the intensity of the slot bands show that the receptor protein level is higher at day 10 compared to day 0 of postnatal age. In the cerebral hemisphere, the receptor levels are quite low compared to the other tissues studied, however, the receptor level does not elicit any significant change in all the ages studied. Slot blot analyses and subsequent analyses of the intensity of the slot bands corroborate the above findings.

Studies on the activation of [^{3}H]dexamethasone-receptor complexes

DNA-cellulose binding assays both in liver and kidney show that the activation of [^{3}H]dexamethasone-receptor complexes both by heat and salt are significantly higher as compared to the unactivated receptor complexes. However, the degree of activation is

similar for hepatic glucocorticoid receptors obtained from 0- and 30-day old chicken. The results indicate no postnatal difference in the *in vitro* activation of the hormone-receptor complexes under the conditions mentioned above. In the kidney too, the thermal as well salt activation of [^{3}H]dexamethasone-receptor complexes were similar to that in liver since there was no difference in the magnitude of *in vitro* activation of the hormone-receptor complexes in the two ages studied. However, the fold of activation of [^{3}H]dexamethasone-receptor complexes both by temperature and salt is slightly lower (2-2.3 fold) compared to the liver. Nuclear binding assays in both the liver and kidney indicate that the thermal as well as salt activation of [^{3}H]dexamethasone-receptor complexes is similarly observed in both the age groups studied as seen using DNA-cellulose binding assay. However, in contrast to DNA-cellulose binding assay, nuclear binding of both the thermally- and salt-activated glucocorticoid-receptor complexes is significantly higher in immature (0-day) compared to that of mature (30-day) chicken. Nuclear exchange (cross-mixing) assays in both liver and kidney revealed that the higher magnitude of activation in 0-day old chicken is not due to the [^{3}H]dexamethasone-receptor of the two age groups studied but because of the differences of the nuclear properties. The sensitivity of both liver and kidney nuclear chromatin digestion by DNase I shows that 0-day old chromatin reflects a less condensed chromatin as compared to that of 30-day old, hence allowing greater binding to [^{3}H]dexamethasone-receptor complexes at this age of lifespan.

Studies on inhibition of activation of [^{3}H]dexamethasone-receptor complexes

Various exogenous and endogenous agents were used to inhibit the receptor activation process by heat and salt. Molybdate was found to be a more potent inhibitor compared to tungstate. Also, cadmium and oleic acid were found to be potent inhibitors of both heat and salt activation of GR. However, the magnitude of activation inhibition by these inhibitors remains the same at the two ages studied, indicating that the mechanism(s) of activation inhibition does not get altered during these ages of chicken.

Studies on the physicochemical properties of glucocorticoid receptors

Studies on determine the physicochemical properties of glucocorticoid receptors did not reveal any difference in the hepatic receptor from 0- and 30-day old chicken. Gel filtration analyses indicated the molecular weight and stoke radii values of ~255 kDa and ~5.65 nm, respectively for the unactivated glucocorticoid receptor complexes. For the thermally activated glucocorticoid receptors the values were ~86 kDa and 3.28 nm, respectively. It is

also evident from our study that the elution pattern from DE-52 did not reveal any charge differences in the glucocorticoid receptors in the two ages studied. The unactivated glucocorticoid receptor complexes from both the age-groups eluted at ~248 mM KCl, whereas the activated receptors eluted at a salt strength of ~100 mM KCl. These findings reveal no differences in the physical and chemical properties of the glucocorticoid receptors during postnatal development of chicken.

From these studies, it is concluded that the glucocorticoid receptor concentration and not the hormone binding affinity alters during postnatal development. In addition, an increased nuclear binding of both the heat- and salt-activated receptors is due to the more relaxed chromatin organization in immature animals as compared to mature ones, where chromatin is more compact. Molybdate, tungstate, cadmium and oleic acid were found to be inhibitors of heat and salt activation of GR in liver and kidney. However, the magnitude of activation inhibition by these inhibitors remains the same at the two ages studied, indicating that the mechanism(s) of activation inhibition does not get altered during these ages of chicken. Physicochemical properties studied remain unaltered during postnatal development. The observed changes in the level and activation binding of GR as well as chromatin organization may contribute to functional alterations in glucocorticoid actions which may provide a better adaptation to the changing demands made upon animals during development and also be responsible for tissue's responsiveness to glucocorticoids during such phase of animal's lifespan.

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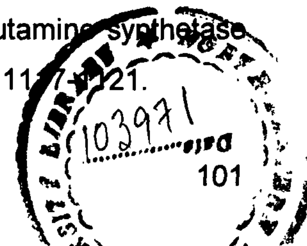
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APPENDIX I

Total protein concentration determination by the dye-binding method of Bradford

The dye-binding method of **Bradford** (1976) was used to determine the protein concentrations of samples.

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml of absolute ethanol, and to this further 100 ml of orthophosphoric acid (85% w/v) was added. This stock solution was mixed and stored under refrigeration.

Just before use, 15 ml of the stock solution was made up to 100 ml by adding water and filtered through Whatman No.1 qualitative filter paper to give the **working solution**.

Bovine serum albumin (BSA) was used as a standard protein. Stock solution of BSA was dissolved in distilled water (1 mg/ml). Then, aliquots containing 0.01-0.1 mg protein were pipetted out into the test tubes in duplicate, and further distilled water was added to make the final volume to 100 μl. To this, 5 ml of working solution was added and mixed. The test tubes were then incubated for 10 min at room temperature for color development. Finally, absorbance of each test solutions was determined at a visible wavelength of 595 nm, against a reagent blank. Next, a plot of absorbance against protein concentrations used was obtained.

20-30 μl of the cytosol, from which total protein concentration to be estimated was pipetted out in duplicate, into the test tubes and the final volume was made to 100 μl with distilled water. Furthermore, 5 ml of working solution was added and the absorbance determined as indicated above. Total cytosolic protein concentration was then determined from the standard plot.

APPENDIX II

Use of conversion factor to convert CPM into concentration (mmol)

2 μl of [^{3}H]dexamethasone (specific activity, 83 Ci/mmol) was diluted to 20 μl with the homogenization buffer. Then, 5 μl of this was pipetted into scintillation vials in triplicate and further 4 ml of Scintillation cocktail-T was added, and mixed throughly. The radioactivity (CPM) was obtained using liquid scintillation counter. The average CPM (X) was converted to concentration unit (mmol), using

$$\frac{\text{Average CPM (X)}}{\text{Vol. Of [}^3\text{H]dex (ml)}} \times \frac{1}{\substack{\text{Radioactive concentration} \\ \text{(Ci/ml)}}} \times \substack{\text{Specific activity of [}^3\text{H]dex} \\ \text{(Ci/mmol)}} \times \text{dilution factor}$$

Using the above equation, a value of X CPM/mmol was obtained. It was inverted to give1/X mmol/CPM and then used as a factor to convert CPM into concentration of receptor in mmol. This was further converted to fmol by multiplying with 10^{12}.

APPENDIX III

DNA concentration determination by diphenylamine method.

The method of Burton (1968) which is a modified version of Burton (1956) procedure was used to determine DNA content in purified nuclear suspension.

Preparation of diphenylamine reagent

1.5 g of diphenylamine was dissolved in 100 ml of glacial acetic acid. To this, 1.5 ml of concentrated sulfuric acid was added and the solution stored in amber bottle at room temperature. Prior to use, 0.5 ml of an aqueous solution of acetaldehyde from a stock (1.6 %) was added.

Preparation of standard DNA solution

2 mg of calf thymus DNA was added to 5 ml of 5 mM sodium hydroxide solution and incubated overnight at room temperature to allow the DNA to dissolve. Furthermore, to this, 5 ml of 1 N perchloric acid was added and the solution incubated at 70°C, in a water bath for 15 min. The solution was then brought to room temperature and the final volume was made to 10 ml by adding 1 N perchloric acid. This resulted in a standard DNA solution of concentration 200 μg/ml.

For standard plot, 0.1-1 ml (20-200 μg DNA) of standard DNA solution was pipetted out in duplicate into the test tubes and the volume made to 1 ml by adding 0.5 N perchloric acid. Next, to each tube 2 ml of diphenylamine reagent was added and the solution mixed properly by vortex machine. The tubes were then incubated at 30°C for 17 hr in a water bath. The tubes were then brought to room temperature and the absorbance obtained at a wavelength of 600 nm against a reagent blank.

DNA estimation in nuclear suspension

50 μl of purified nuclear suspension was pipetted out into the test tubes and the volume made to 1 ml with 0.5 N perchloric acid and the solution incubated at 70°C for 15 min. The solution was then brought to room temperature and further 2 ml of diphenylamine reagent was added, followed by incubation for 17 hr at 30°C in a water bath. The absorbance was determined as mentioned above. DNA concentration in the samples was then obtained from the standard plot.

BIO-DATA

Name: **DANIEL NONGBRI**

Present & Permanent address	: Upper-Lumparing, Lumdorbar, : P.O. Laban, Shillong-793004
Date of birth	: 17th December, 1979
Educational qualifications	
M.Sc$_{NEHU}$ (Biochemistry) (2003)	: 1st Class. 68.55% (Rank 5th)
B.Sc$_{NEHU}$ (Biotechnology) (2001)	: 1st Class. 70% (Rank 9th)
Research experience	: Five years
Teaching experience	: 2 years, as Lecturer in Biochemistry, : Lady Keane College, Shillong-1
Research field	: Steriod receptor biology, Signal : Transduction, Development and Aiging
Fellowship awarded	: CSIR Junior Research Fellowship, 2002.

Experience in Biochemical techniques and methods

Radioreceptor assay, Liquid scintillation counting, Centrifugation, Spectrophotometry, SDS-PAGE, Gel filtration chromatography, Ion-exchange chromatography, Agarose gel electrophoresis, Radioimmuno assay (RIA), ELISA, genomic DNA isolation and purification, working knowledge of HPLC, PCR, Gas chromatography, Western and Slot blotting, Pharmacia Phast system.

.

Experience in computer

Windows XP and Vista, Microsoft Word XP & 2007, MS Excel XP & 2007, MS paint, MS photoeditor, Internet explorer, Origin 7.

Research publications

D. Nongbri and R. Sharma (2007) Postnatal regulation of glucocorticoid receptors in the liver of chicken. ***Indian Journal Of Biochem. & Biophys***. 44, 7-13.

Conferences/symposia/seminars

(i) D. Nongbri and R. Sharma (2004) Postnatal changes in the level of glucocorticoid receptors in liver and kidney of chicken. 73rd Annual Meeting of Society Biological Chemist (India), G.B. Pant University of Agriculture & Technology, Pantnagar-263145, Uttaranchal, Nov 21-24, 2004.

(ii) D. Nongbri and R. Sharma (2005) Postnatal changes in the level of glucocorticoid receptors in different tissues of chicken. Silver Jubilee Conference of Dept. of Biochemistry on advances in Biochemical Education and Research, North Eastern Hill University (NEHU), Shillong-793 022, Meghalaya, Feb. 25-26, 2005.

(iii) D. Nongbri and R. Sharma (2006) Postnatal regulation of glucocorticoid receptors in the liver of chicken. Seminar on Trends in Biochemical Research, Department of Biochemistry, North Eastern Hill University, Shillong, March 31, 2006.

(iv) D. Nongbri and R. Sharma (2006) Renal glucocorticoid receptor changes during postnatal development of chicken. 75th Annual Meeting of Society of Biological Chemist (India), Jawaharlal Nehru University, New Delhi, India, Dec. 8-11, 2006.

(v) D. Nongbri and R. Sharma (2007) Effect of modulators on activation of hepatic glucocorticoid receptor of chicken. National Seminar on Adaptation Biochemistry, Department of Biochemistry, North Eastern Hill University, Shillong, March 22-23, 2007, under auspices of UGC-SAP (DRS).

(vi) D. Nongbri and R. Sharma (2007) Inhibition of *in vitro* activation of renal glucocorticoid receptors by various modulators. 76th Annual Meeting of Society of Biological Chemist (India), Sri Venkateswara University, Tirupati, India, Nov.25-27, 2007.

(vii) D. Nongbri and R. Sharma (2008) *In vitro* activation of hepatic glucocorticoid receptor of chicken and its inhibition by various modulators during postnatal. National Seminar on Adaptation Biochemistry, Department of Biochemistry, North Eastern Hill University, Shillong, March 13-14, 2008, under auspices of UGC-SAP (DRS).

(viii) D. Nongbri (2008) Ageing in India with special reference to North East India. ICSSR, North Eastern Regional Centre, Shillong, October 1-2, 2008, sponsored by the Ministry of Social Justice & Empowerment, Govt. of India, New Delhi & North Eastern Council.

Workshop attended:

(i) A one-day workshop on 'Science and technology for the better quality of life' organized by Study Forum for Advanced Technology, SFAT (NE) India, Feb. 25, 2006, Sponsored by Deptt. Of Atomic Energy, Govt. of India.

(ii) Training course on 'Application of Bioinformatics', conducted by the Bio-informatics Centre, NEHU, Shillong, 13-15th April, 2007 funded by DBT, Ministry of Science & Technology, Govt. of India.

(iii) Two-day workshop on 'Faculty Development and Administration', organized by Lady Keane College, Shillong, May 20-30, 2009.

National tests cleared National Eligibility Test (NET), 2002.

Scholarship/fellowship NEHU Post Graduate Scholarship (2001-2003), CSIR Junior Research Fellowship (2003).

Member of Scientific bodies

Society of Biological Chemists (SBC), Bangalore, India.

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