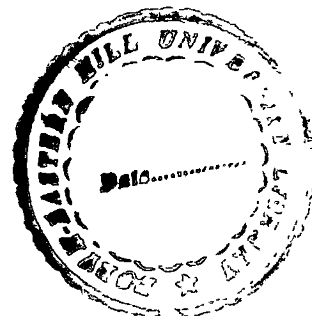
PHYSICOCHEMICAL CHANGES IN THE GLUCOCORTICOID RECEPTOR DURING DEVELOPMENT OF MICE



by

Md. Monsur Ahmed Borbhuiya

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.

North Eastern Hill University

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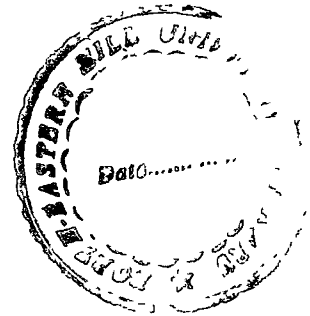

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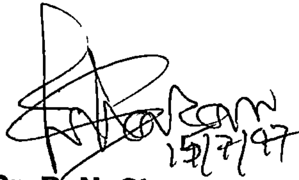
This is being submitted to the North Eastern Hill University for the degree of Doctor of Philosophy in Biochemistry.

(Md. Monsur Ahmed Borbhuiya)



Dr. R. N. Sharan
Head
Dept. of Biochemistry

Dr. R. Sharma
Supervisor
Dept. of Biochemistry

Head,
Deptt. Biochemistry
NEHU-Shillong 22

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Introduction

The period of time that an organism/species survives under a given environmental condition denotes the lifespan of that organism. In higher multicellular organisms, the onset of life begins with the fusion of the male and female gametes and ends with the death of the organism. This constitutes the whole lifespan that however, varies greatly among the different species. This makes it difficult to establish clear `chronological boundaries' of the different stages of lifespan. Some authors have divided lifespan into two major 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). Others have defined lifespan as a continuum with development at one end, followed by the reproductive phase; other end being the senescence or aging, each stage showing a characteristic set of sequential events, regulatory mechanisms, rate and duration (Kanungo, 1994).

Developmental phase

This phase encompasses all the events taking place during prenatal period and extends to a considerable length of the postnatal life too. It is characterised by striking changes in the morphological, physiological, biochemical and psychological features, which lead to suitable specialization of various cells, tissues and organ systems of the body. These changes confer reproductive ability upon the organism, at a definite stage of development, which in many cases continues even after the attainment of reproductive capacity (Timiras, 1988).

Reproductive phase

The transition from the developmental to reproductive phase is characterised by the appearance of specialized structures and functions that confer reproductive ability to the organism. This enables the organism to reproduce its own kind that not only aid in the perpetuation but also in the evolution of species. This period is characterised by great functional stability: it connotes the attainment of optimal and integrated function of all body systems. At the molecular level, several genes that play an important and specific role in the development and maintenance of reproductive ability are now expressed. The duration of this phase of lifespan is more or less defined, especially in females. There is a direct correlation between the time taken to

reach reproductive maturity and the maximum lifespan of an organism (Hayflick, 1987).

Senescent phase

This is characteristic of all multicellular organisms and is illustrated by an overall decline in the bodily functions. This decline is quite noticeable during the latter stages of the reproductive phase and affects most tissues, organs and the overall physiological competence. An important feature at this stage of lifespan is the appreciable loss in the reproductive ability of organisms. During this phase, the ability to adapt to both internal and external stresses decrease, leading to a decline in the homeostatic balance. Thus aging, whereby a time-dependent drift from the optimum bodily functions are seen, was postulated to be pleiotropic in nature (Cutler, 1984). This states that the aging process is due to

- normal by-products of the living processes.
- evolutionary non-selected endogenous properties.

Two classes of pleiotropic aging have been proposed, one liked to energy metabolism, called the continuously acting biosenescent process (CABPs) and the other associated with developmental processes, named as the developmentally linked biosenescent process (DLBPs). Questions exist as to which of the two processes play a decisive role in the aging phenomenon.

Since numerous variability exists in the rate as well as the time of incipience of senescence among various species, it becomes difficult to attribute any specific parameter or a single 'triggering event' as being responsible for the aging of the organism. Moreover, scientific works over the years appear to implicate the events occurring during the developmental phase, that not only induces but accelerates the process of senescence (Walford, 1987). Our comprehension of the basis of senescence may therefore, be reinforced by detailed information on the developmental period of lifespan.

As mentioned earlier, the developmental phase is characterised by striking changes. These include an increase in the number and size of cells, their differentiation to perform specialized functions and the formation of organ systems. At the molecular level, an intricate display of genes is seen as some are switched on while others, shut off. This gives rise to a whole array of proteins that not only support organogenesis but also provides the organism with catalytic power to lead an independent existence. The developmental phase is also characterised by the significant influence of environment, both internal and external on adaptation made by the organism. These also play a considerable role in regulation and maintenance of homeostatic balance necessary for proper development.

Many of the present efforts taken to comprehend the process of development is directed towards understanding the age-related changes that transpire at all levels of the organism. These changes can be organized into several broad headings; they encompass but, a few of the well-documented phenomena that contribute to our knowledge of the developmental and subsequent aging processes. Experimental evidence shows quantitative changes in the tRNAs and aminoacyl-tRNA synthesis, responsible for the translation process, during development (Ilan and Patel, 1970; Mays *et al.,* 1979). Also, an augmentation in the fidelity of transcription and translation processes during development increases the chances of having proteins and enzymes with altered structural and functional properties (Lamb, 1977). Other workers however, contradicted such reports (Sharma & Patnaik 1982, Fleming *et al.,* 1986). *The decline of immunologic competence and the concomitant increase in* autoantibody production may contribute, significantly to the aging process (Goidl *et al.*, 1983; Nandy and Bennett, 1983). Other changes include age-related differences in the protein turnover rates (Adelman and Dekker, 1985; Richardson, 1985) and in the enzymes responsible for DNA repair (Tice, 1978). More recently, the effect of dietary restriction and its influence on the various cellular activities have provided some interesting evidence on the role of diet in the development and aging processes (Weindruch, 1991; Timiras, 1994).

Inspite of the voluminous work being present no clear cut evidence has emerged, so far, as to the exact cause(s) that could be implicated for the aging phenomenon. However, it is generally believed to be due to a gradual and simultaneous deterioration of one or more processes mentioned above. This gradual loss in the functional ability and homeostatic balance as a whole lays the ground for onset of senescence or aging. It is important to emphasize here that the changes mentioned above constitute a few amongst the many well-studied processes. Some of these changes warrant a brief discussion to underline their importance in the development and aging process.

Changes in gene expression

The structure and functional capacity of an organism are directed by information that is encoded in the genes contained in DNA, the genetic material of a living system. These genes direct the production of biomolecules that ultimately dictate the form and function of the organism throughout its lifespan. Three major types of alterations in gene expression occur with age:

i) actively expressed genes may undergo a gradual decrease in expression due to a decline in regulatory factors or due to a structural/organizational change in the chromatin.

ii) an increase in expression of those genes which are already being expressed.

iii) genes that undergo alternate expression and depression.

These points emphasize the control of gene expression at the transcriptional level. However, regulations at the processing, transport, stability and translation of mRNA as well as post-translational events are no less significant. Cellular signals due to cell-cell interactions in controlling gene expression are also important (Lewin, 1995). However, during developmental phase most of the changes in gene expression are seen as a result of sequential activation and repression of genes (Caplan and Ordahl, 1978; Wilkins, 1986). A classic example of this intricate control of gene expression is seen in the changing hemoglobin composition patterns during the early developmental period in humans (Zuckerkandl, 1965) The four globin chains- α, ε, γ, β, each encoded by a separate gene, constitute the tetrameric hemoglobin. The molecular composition is $\alpha_2\varepsilon_2$ in the 1-2 month old fetus that is replaced by $\alpha_2\gamma_2$ (fetal hemoglobin, HbF) at a later stage of the fetal life. In the newborn, the hemoglobin is of $\alpha_2\beta_2$ (adult hemoglobin, HbA) type. This clearly demonstrates the sequential activation and repression of different genes and that too for various durations, thus providing greater functional significance to hemoglobin at each stage of development. Changes in protein pattern of the larval tissues in developing *Drosophila. melanogaster* are attributed to sequential changes in the expression of genes (Arking, 1991). Work by Kanungo and his co-workers have shown such developmental changes in the isoenzymes of lactate dehydrogenase and of the subunit composition of alanine aminotransferase during aging of rats (Kanungo and Singh, 1965; Kanungo and Patnaik, 1975). Others have reported changes in the types and amount of RNA, with development in different tissues of mice (Cutler, 1982; Richardson, 1985). Of importance to the process of development and aging are genes that play a significant role in imparting sexual maturity and reproductive ability to an organism. Among these are genes for the production of sex-hormones as well as other hormones that not only help the organism attain sexual competence but also help in the maintenance of homeostatic balance. The loss of this balance, as reported by various workers, is a result of reproduction and this in turn is implicated to alter the expression of genes and usher in the process of senescence (Wodinsky, 1977; Diamond, 1982; Medvedev, 1990). Moreover, the direct and/or indirect action of other factors such as nutrition and stress in regulating gene expression cannot be ruled out.

It is of interest to know that many cis-acting elements in the promoter/enhancer regions as well as transcription and trans-acting factors have, over the past few years, been implicated to play an indispensable role in regulating gene expression. The work of Strähle *et al.* (1988) and others have not only shown the role of these

elements but also provided an insight into the regulatory differences between species expressing similar genes (Kelsey *et al.*, 1987; Koopman *et al.*, 1989; Cavener, 1992).

Thus, the onset and maintenance of the various stages of lifespan of an organism depend to a considerable extent upon the duration, rate and regulatory mechanisms involved in gene expression. The work on human genetic diseases- Progeria and Werner's syndrome helped in reinforcing the above observations (Kanungo, 1994).

Changes in chromatin structure and composition

In cells, the genetic material, DNA is complexed with histone and non-histone proteins to form an ordered, compact structure called chromatin. Changes in chromatin organization were reported as early as 1967, taking into account the variability in the types and amount of mRNA during development (Yaffe and Fuchs, 1967). Other parameters to suggest changes in the chromatin structure with age have also been reported, for example, elevation of melting temperature (T_m), the decrease in salt extractable proteins, increase in single strand breaks and sensitivity to nuclease S1. Additional changes include chemical modifications, as exemplified by decrease in phosphorylation, acetylation, methylation and poly-ADP ribosylation of both histone and non-histone proteins (Kanungo, 1994). The above mentioned changes have been credited to a conformational change which leads to condensation of the overall chromatin structure with development and aging. DNase I and MNase digestion of the chromatin as well as nick translation of DNA confirmed such a conformational change during development and aging (Chaturvedi and Kanungo, 1983). All these changes ultimately effect the efficacy and rate at which genes are transcribed and can provide mechanism(s) to control gene expression at various stages of lifespan.

Change in hormones/hormone receptors

Hormones are molecules, synthesized and secreted from specialized group of cells, which are capable of transducing intra-/inter-cellular messages that influence a wide variety of cellular and metabolic processes (Zubay *et al.*, 1995). Neural hormones (neuro-transmitters) and hormones secreted by the endocrine, paracrine and autocrine glands are necessary for the proper functioning of almost every cell in the body and are responsible for providing diverse functional abilities to the organism. An important aspect of hormone action is its modulation of certain enzymes in a tissue- and age-specific manner which appears crucial for the maintenance and adaptive response of the organism (Kanungo, 1980; Sharma, 1988). The role of hormones in maintaining the effectiveness of homeostatic balance and adaptation to internal as well as external stresses is immense for proper growth and development of an

organism. Reports indicate a decline on both these accounts during the latter stages of lifespan, suggestive of apparent changes in the complex signaling mechanism conferred by these molecules. Data over the years have shown that tissue responsiveness depends upon the level of hormones and their receptors and also on the post-receptor events (Roth, *1981; 1989). Hence, any change in these* parameters, may have profound influence on the process of development, growth, reproduction and aging.

Reports indicate significant changes in the level of certain hormones during early periods of development. A good example is that of glucocorticoids which appear in the fetal rats by the 19th. day of gestation and increase, significantly to reach adult levels by the 15th. day of postnatal age (Cohen, 1973; Martin *et al.,* 1977; Lu *et al.,* 1987). Similar observation was made in the avian system by Wise and Fyre (1973). After the attainment of adult values some hormones such as glucocorticoids, testosterone and serotonin do not show any further age-related changes. Others reported either an increase e.g., prolactin, GnRH, FSH, LH (Chakraborti *et al.,* 1976) or a decrease e.g., estrogen (Edman, 1983), ADH (Sladex *et al.,* 1981), dopamine and norepinephrine (Timiras *et al.,* 1985), aldosterone (Flood *et al.,* 1967) and DHEA (Orentreich *et al.,* 1984) with age.

The neuroendocrine system plays a notable role in the aging process and the importance of this system has over the years drawn considerable attention (Everitt and Walton, 1988; Timiras, 1991; Sharma, 1994). Changes in the neuroendocrine system is capable of altering various functional aspects of the organism. In this respect, the role of hormone receptors is central to the understanding of the control systems of the body and any age-related changes in the level or functional integrity of the receptors is likely to have diverse implications on the overall functioning of the organism.

It is now a well-documented fact that hormones exert their physiological effect either by way of receptors on the cell-surface or by intracellular receptors in target cells. For intracellular receptors, hormonal messages are transduced by the receptors themselves to the ultimate cellular centres. In the case of cell-surface receptors, the process of signal transmission extends to second and third messenger substances, which ultimately lead to an appropriate response. Furthermore, of late there is growing evidence of `cross-talk' between the various constituents of signal transduction pathways and this provides even more complexity to the whole system (Sharma, 1993). Moreover, a sizeable body of evidence suggests an alteration in the tissue responsiveness to hormones during development and aging. This being true, such changes can be attributed to either change (a) in the receptor concentration, (b)

in binding affinity of hormone to receptor or (c) in other receptor properties including changes in post-receptor events. Much evidence is present on the alteration in various hormone receptor levels and also in the post-receptor events (Roth, 1989). Examples of some of the representative members of each group and changes if any, in the receptor-related processes are discussed below.

Membrane bound receptors

Neurotransmitters which include amines (acetylcholine, cathecholamines, dopamine), amino acids (glutamate, aspartate), peptides (enkephalins, somatostatin) and even gases (nitric oxide, carbon monoxide) are synthesized and secreted by specialized neurons of the nervous system. They along with their cognate receptors and a variety of second messengers constitute an important vehicle for neuronal communication. One of the most studied aspects of age-related changes in nervous system involves alterations in the hormone-receptor levels at the synapse (Giacobini, 1982; Strong *et al.*, 1991)

Adrenergic receptors

Cathecholamines (epinephrine and norepinephrine) are the ligands for adrenergic receptors. Age-related studies on the beta-adrenergic receptors in various species have been reported. In cerebral cortex of rats, a decline with age was observed (Misra *et al.*, 1980; Enna and Strong, 1981). However, in the cerebellum of rats, an age-related increase in β-adrenergic receptors is reported by Pittman *et al.* (1980). Decline in the receptor concentration in rat erythrocytes (Bylund *et al.*, 1977), human lymphocytes (Schocker and Roth, 1977), submandibular glands of rats (Piantanelli *et al.*, 1980) is also reported. Similar trends have likewise been reported for alpha-adrenergic receptors in the cerebral cortex (Misra *et al.*, 1980) and heart (Partilla *et al.*, 1982) of rats. Analogous to changes in the receptor levels, loss in the adenylate cyclase activity (an amplifier enzyme necessary for second messenger production) has also been reported (Schmidt and Thornberry, 1978)

Cholinergic receptors

Acetylcholine is the principal ligand for this class of receptor. Reports suggest an age-associated decline in acetylcholine receptors in the cerebral cortex of rat (Lippa *et al.*, 1981) and human (Perry, 1980). Observations for other tissues e.g., rat hippocampus (Nordberg and Winblad, 1981) and anterior pituitary (Avissar *et al.*, 1981) show similar trend. Contrary to these findings, reports by Davies and Verth (1978) and Strong *et al.* (1980) show no such age-related changes.

Dopaminergic receptors

Age-related decrease in dopamine receptors have been reported in the corpus striatum of rabbits (Thal *et al.*, 1980), mice (Marquis *et al.*, 1981) and rats (Roth, 1982). However, an increase in dopamine receptors with age, in the retina of rats is reported by Riccardi *et al.* (1981). Consequently, dopamine induced adenylate cyclase activity has been observed to decrease with age (Puri and Volicer, 1977).

Since neurotransmitters convey chemical information among neurons, any change in the signal transmission pathway via receptor-mediated processes will be directly reflected in altered physiological functions with age. Decrease in peripheral motor system, loss of memory and sensory functions as seen in Parkinson's and Alzheimer's diseases are manifestations often associated with the aging process (*Timiras, 1994*).

Insulin receptors

Insulin is one of the important hormones responsible for the regulation of carbohydrate metabolism, maintenance of glucose level and other metabolic processes involving gluconeogenesis, lipogenesis, protein synthesis and general growth in mammalian system (Norman and Litwack, 1987). Receptors for insulin are known to be present in a variety of tissues. Reports suggest a tissue- and species-specific alteration in the receptor concentration with age. Rosenbloom *et al.* (1976) reported an increase in the receptor number in human skin fibroblasts. However, most reports suggest a decrease in insulin receptor number, e.g., in rat liver (Pagano *et al.*, 1981), adipose tissues (Olefsky, 1975) and in human erythrocytes (Dons *et al.*, 1981). Goldfine (1987), however, suggests that the number and affinity of insulin receptors does not change, but the possibility of defects in the cascade of post-receptor reactions is being actively investigated upon (Caro, 1987; Fink *et al.*, 1983). Pathophysiological conditions e.g., increased incidence of diabetes and its associated disorders may reflect changes in receptor and/or post-receptor events with age (Bolinder *et al.*, 1983; Green, 1986)

Gonadotropin receptors

Gonadotropins (FSH and LH), secreted by the anterior pituitary control the sex steroid hormone production and reproductive function in both sexes. No change in the receptor concentration has been reported in the interstitial cells and ovaries in rats (Steger and Huang, 1983). On the other hand, Pirke *et al.* (1978) have observed a decline in the gonadotropin receptors in rat testes and leydig cells, respectively.

Intracellular hormone receptors

Thyroid hormone receptors

Thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), play an indispensable role in affecting a variety of biochemical reactions, in controlling the basal metabolic activity and also in regulating the growth and development. Receptors for thyroid hormone, primarily for T_3, is present in many tissues- liver, brain, lung, kidney and anterior pituitary, the receptor being present predominantly in the nucleus and also non-specifically, in the cytoplasm, mitochondria and plasma membrane (Eberhardt *et al.*, 1978). The biological actions of the hormone occur primarily through nuclear binding and stimulation of protein synthesis. Reports on age-related changes in this receptor are few. A decrease with age in the brain and liver of rats have also been reported (Margeuity *et al.*, 1985). However, no such change in the rat brain and liver was reported by Cutler (1981). The level of thyroid hormones, conversion of T_4 to T_3 and the rate of secretion of T_4 from the thyroid gland also affect the receptor concentration with age.

Androgen, Estrogen and Progesterone receptors

Androgens (testosterone and 5α-dihydrotestosterone) are primarily responsible for the differentiation, growth, maturation and maintenance of male reproductive organs. They are also responsible for the development of secondary sex characteristics and behavioral manifestations related to muscularity. The androgen receptor is present in a number of tissues- testes, prostrate, seminal vesicles, epididymis, kidney, liver, uterus, brain and pituitary. Studies on most of the tissues show a decline in the receptor numbers with age, e.g., in rat cerebral cortex and testes, ventral and lateral prostrate (Haji *et al.*, 1981), and in liver (Roy *et al.*, 1974).

The estrogens and progesterone are responsible for the development and maintenance of female reproductive system. Gessel and Roth (1981), and Kaur and Thakur (1991) observed a decrease in rat uterine receptors with age. Also, an age-related decrease in estrogen receptor concentration in cerebral cortex and amygdala of rats and in mouse brain cortex has been reported (Kanungo *et al.*, 1975; Roselli *et al.*, 1993; Asaithambi *et al.*, 1997). However, progesterone receptors show no such age-related decline in rat (Saiuddin and Zassenhaur, 1979).

Glucocorticoid receptors

Glucocorticoids partake in a number of crucial metabolic reactions, especially those occurring in the liver, muscle, adipocytes and brain. These processes are important during the developmental period and more so during periods of stress (Sapolsky *et al.*, 1986). Glucocorticoid receptors are ubiquitous in their distribution and studies reveal an early developmental increase in the receptor number upto about 15-20

days of postnatal age in rats (Henning, 1978). Thereafter a gradual decrease is seen in most tissues, e.g., in rat liver (Kalimi, 1984), kidney (Sharma and Timiras, 1988), brain (Kitkari *et al.*, 1984; Pfeiffer *et al.*, 1991), pituitary (Pfeiffer *et al.*, 1991), skeletal and cardiac muscles (Mayer *et al.*, 1981; Sharma and Timiras, 1987). Keeping in mind our interest in this receptor, a brief discussion on the recent advances in the study of receptor structure, mechanism of action and also the tissue- and age-specific changes, associated with receptor during development, is given below.

Inspite of the voluminous work being present, no clear cut consensus have emerged which could possibly explain the alterations in various aspects of hormone action during development and aging. Moreover, recent data adds to the overall complexity by suggesting age-dependent alterations that may occur at every step of the signal transduction process.

Structure and function of glucocorticoid receptor

Glucocorticoids are synthesized and secreted by the adrenal cortex under the control of hypothalamus-pituitary axis. At cellular level, most known effects of glucocorticoids are mediated by a ~94 kDa intracellular protein, the glucocorticoid receptor (GR) (Evans, 1988). Till the mid-sixties the concept of receptor for hormones was unclear. However, the availability of radioactive hormones (Jensen *et al.*, 1966), capable of selective binding to receptors provided a method of studying these proteins and their mechanism of action in greater detail. Glucocorticoid receptor was first detected in the thymus (Munck and Brinck-Johnsen, 1968) and since then its presence in almost all the tissues of mammals has been confirmed. During the last three decades, use of classical techniques and more recently the use of powerful molecular genetic tools have provided much information on the receptor structure and other details of its action mechanism. Glucocorticoid receptor belongs to a phylogenetically conserved superfamily of nuclear hormone receptors, that also include other steroid receptors, thyroid hormone receptors, oncogene products and the recently discovered `orphan receptors´, whose ligand requirement has not yet been identified (Laudet *et al.*, 1991; Mangelsdorf *et al.*, 1995). This family constitutes the largest known group of eukaryotic transcription factors and it is of interest to know that glucocorticoid receptor is the first transcription factor to be isolated and studied in detail.

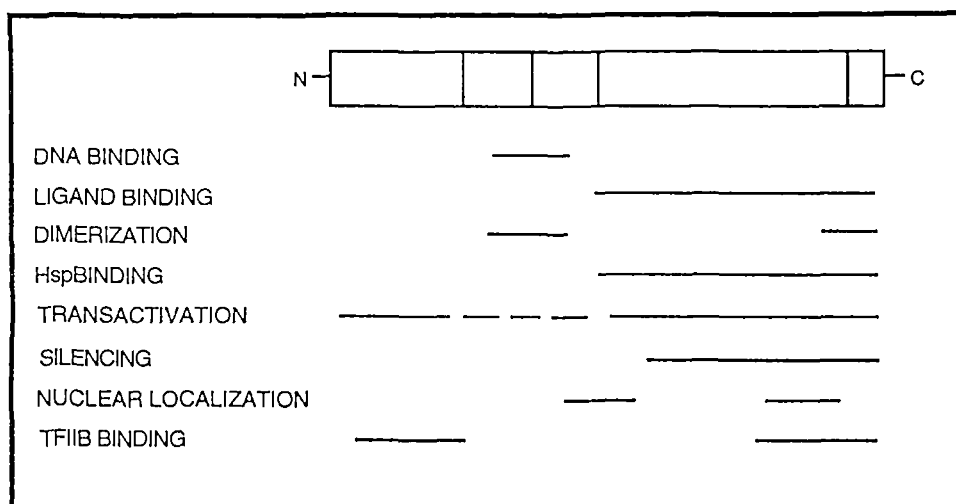
GR gene structure

The ability to clone genomic genes transcribing for GR in mouse and in humans has revealed a complex organization of the GR gene. The gene is about ~60 kb in size and the translated portion is found to be distributed among 8 exons for mouse GR. A

single exon of about 1230 nucleotides codes for the N-terminal end of the receptor protein whereas the DNA- and steroid-binding domains are encoded by more than one separate exons. It has a promoter region similar to that of a housekeeping gene with multiple transcription initiation sites (Encio and Detera-Wadleigh, 1991; Cole TJ *et al.,* 1993).

Domain structure of GR

The use of classical proteolytic analysis of intact GR as well as the recent advances in amino acid sequencing, mutation/deletion studies, and expression of chimeric GR have provided much information about the structural and functional organization of the GR protein (Vedeckis, 1983; Rusconi and Yamamoto, 1987; Evans, 1988; Muller and Renkawitz, 1991). GR has a characteristic three domain structure similar to that of other members of the steroid receptor superfamily A schematic diagram of the GR domains and their functional attributes is given in the figure below.



Functional Domains of Glucocorticoid Receptor

Hormone binding domain (HBD)

The first step in the glucocorticoid action mechanism, is its binding to high affinity intracellular receptors present in the cytoplasm of target cells. The portion of GR responsible for ensuring proper binding of the ligand is located at the carboxyl terminus, encompassing about one third of the receptor molecule and is referred to as the hormone binding domain (HBD) (Godowski *et al.*, 1987)). Proteolytic analysis revealed a 16 kDa fragment (Thr^{537} to Arg^{673}) which binds steroid but, with a much lowered affinity than the intact molecule. The HBD must be properly folded to have a high affinity binding site and this is helped by the presence of other regions in the GR itself and through the association of GR with the components of the protein

folding system (Xu *et al.*, 1996). This system , termed `foldosome´ consists of two molecules of 90 kDa heat shock proteins (hsp90), a molecule of hsp70, hsp56 (an immunophillin) and a 23 kDa acidic protein (Pratt , 1993; Stancato *et al*, 1996). The HBD region is rich in amino acids, cysteine and methionine and several of them, e. g. $cys^{638, 665}$ in human GR, met^{622} and $cys^{640, 656, 661, 674}$ in rat GR and the associated intramolecular S-S linkages are involved in high affinity steroid binding (Yu *et al.*, 1995; Simons and Pratt, 1995). These studies revealed the three dimensional structure of HBD to consist of a conformationally flexible pocket where the binding of ligand occurs with maximum hydrophobic interaction. Recent studies also attribute the role for HBD as a repressor of transactivation. Deletion of the HBD yields a molecule whose ability to undergo transactivation equals almost that of an intact GR and that too in the absence of the hormone ligand. Transactivation of chimeric construct with rat GR HBD and E1a is repressed in the absence of hormone. Other fusion proteins with GR-HBD have their activity controlled by steroid binding (Hollenberg *et al.*, 1987; Picard *et al.*, 1988). Moreover, in addition to repression of DNA-binding activity, the HBD may also have a role in controlling the nuclear localization and transcriptional regulation activity of GR (Picard and Yamamoto, 1987; Lanz and Rusconi, 1994). Major receptor dimerization function is also ascribed to the C-terminal end of this domain, which contains leucine-rich sequences. These sequences are thought to form a hydrophobic dimerization interface with an α-helix, in a structure similar to leucine zipper dimerization motif present in other transcription factors (Dahlman-Wright *et al.*, 1992).

DNA-binding domain (DBD)

Cloning of cDNA coding for both the intact GR or only the DBD allowed elucidation of the tertiary structure, initially by NMR which was later refined and confirmed by X-ray crystallographic studies (Härd *et al.*, 1990; Luisi *et al.*, 1991). The central portion of the GR, rich in basic residues constitutes the DBD, which is highly conserved among the steroid receptor superfamily. The DBD is globular in structure and divided into two distinct motifs, each contributing a `zinc-finger´- in which a zinc ion is co-ordinated to four cysteine residues with a tetrahedral geometry. The peptide loop thus formed is structurally similar to the *Xenopus* transcription factor, TFIIIA, where a zinc ion is co-ordinated by two cysteines and two histidines. Site directed mutagenesis revealed the absolute necessity of seven out of eight cysteines for proper receptor function (Schena *et al*, 1989). The first motif in the three dimensional DBD structure, called the P-box, contains the first Zn-finger; the peptide loop here starts with a short segment of antiparallel β-sheet and ends with an α-helical conformation. The constitution of the second Zn-finger, called the D-box is similar. The two α-helices lie perpendicular to one another and these along with the β-sheet helps to orient not

only the residues that contact the phosphate backbone but also the DBD to fit into the major groove of the double helical DNA (Wright *et al.*, 1993). The two motifs, though identical structurally, play distinct roles in the steroid action mechanism. To P-box is attributed the role of maintaining the fidelity of binding to cognate glucocorticoid response element (GRE) while the D-box has a role to play in the receptor dimerization process (Tsai and O'Malley, 1994).

Modulatory/immunologic domain

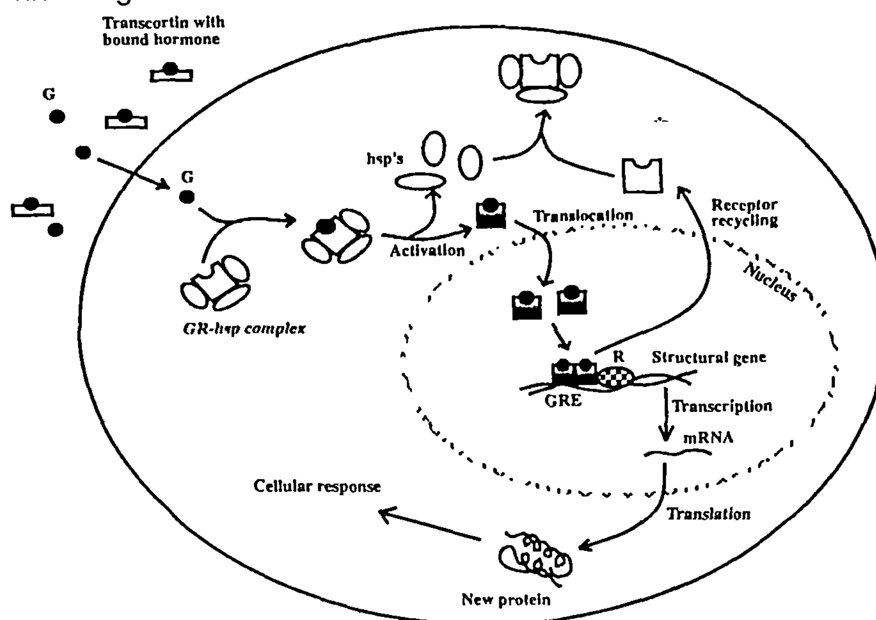
The other important portion of GR is the N-terminal region which is responsible for synergistic activation of transcription from adjacent binding sites. The tau1 and tau2 regions in hGR have been implicated in the transactivation process, the tau1 region contributing over 90% of the receptors' activity. Other functional constituents of this region are nuclear localization signals and the immunogenic epitopes. Most of the known antibodies generated against GR are from the antigenic epitopes localized in the N-terminal of the receptor protein (Dahlman-Wright, 1994; Cidlowski *et al.*, 1990)

Glucocorticoid action mechanism

The discovery of intracellular receptors for glucocorticoids in early 70s was followed by extensive work to decipher not only the role of the receptor in signal transduction process but also in understanding the exact mechanism of glucocorticoid action. Although a lot of questions remain unanswered, as yet, work over the years have contributed a lot and a number of schemes/models have been proposed for the glucocorticoid action mechanism.

Glucocorticoids synthesized and released from the adrenal cortex (zona fasciculata) is transported to the target organs by a protein- corticosteroid binding globulin (CBG) present in the blood. Most of the glucocorticoid released exists in a dynamic equilibrium between the bound and free form. Since glucocorticoid is lipophillic, the unbound hormone enters the target cell by a process of free diffusion and binds with high affinity and specificity to its cognate receptors (Brann *et al.*, 1995). The unliganded GR resides in the cytoplasm, where it exists as a large multiprotein complex consisting of a molecule of GR, two molecules of hsp90, a molecule each of hsp70 and hsp56 and other smaller proteins (Pratt, 1993; Czar *et al.*, 1994; Webster *et al.*, 1994). The association of other proteins especially the hsp90s is necessary to help the receptor attain high steroid binding affinity, prevent nuclear translocation by masking the nuclear localization signals and subsequent transactivation. Upon hormone binding, a conformational change occurs leading to the dissociation of all the receptor associated protein molecules. The hormone-bound receptor then translocates to the nucleus, where binding of the receptors directly to specific response element(s) or to other proteins, e.g., components of AP-1 signaling

pathway, lead to modulation of target gene activity. The nuclear bound receptors are subsequently degraded or cycled back to the cytoplasm (Litwack, 1988; Bamberger *et al.*, 1996). A schematic diagram representing the events in the glucocorticoid action mechanism is given below.



Glucocorticoid action mechanism

G, glucocorticoid; GR, glucocorticoid receptor; hsp, heat shock protein; GRE, glucocorticoid response element; R, RNA polymerase

Activation/Transformation

The process of activation (transformation) has been the subject of intense investigations over the years. However, the process remains ill defined till date although, many details of the events at molecular level have been elucidated in the recent past. As mentioned, the unliganded (unactivated) GR is present as a aporeceptor in association with a number of non-hormone binding proteins that enable the receptor to be in a conformational state capable of ligand binding. Activation is a response to an increase in the glucocorticoid concentration that drives ligand binding to the GR and consequently causes a conformational change in the HBD. This leads to the dissociation of all the protein components of the aporeceptor complex, a process termed activation or transformation (Truss and Beato, 1988; Hutchison *et al.*, 1993; Tsai and O'Malley, 1994). This process occurs *in vivo* under physiological conditions and is rate-limiting for nuclear binding, the entire process representing a normal step in signal transduction pathway (Munck and Foley, 1979;

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Markovic and Litwack, 1980; Htun *et al.*, 1996). While the hormone binding under cell free conditions occurs at 2-4 °C, activation is achieved at elevated temperatures of about 25-37 °C (Milogram *et al.*, 1979). Activation could also be achieved *in vitro*, by other means, e.g., dilution, acidic pH, high salt concentration, gel filtration, etc. (Goidl *et al.*, 1977; LeFevre *et al.*, 1979). Binding of the glucocorticoid hormone to its receptor is an absolute necessity to attain activation by any of the above mentioned factors and the process is time dependent (Denis *et al.*, 1988). A number of physicochemical changes occur upon GR activation in vitro-

- *increase in affinity for purified nuclei, chromatin or DNA.*
- *overall decrease in the charge content as evident from decreased binding affinity of activated receptors to anion-exchange resins.*
- *the sedimentation coefficient decreases from ~9S to ~4S.*
- *the molecular mass is reduced from ~300 kDa for the unactivated receptor to ~94 kDa upon activation. Consequently, the Stokes radius decrease from 60-70 Å to 20-26 Å.*
- *isoelectric point changes from pH 7.1 to 6.1.*

In order to gain a better understanding of the activation process, a number of approaches have been applied. These include interactions between unactivated/activated receptors with isolated nuclei, DNA, DNA-cellulose, etc. Consequently, several compounds have been identified that either enhance or inhibit the above mentioned interactions (Grody *et al.*, 1982; Moudgil *et al.*, 1984). Among these are transition metal ions, namely, molybdate and tungstate, thiol-modifying agents, e.g., N-ethylmaleimide. In addition, several endogenous heat-stable cytoplasmic and nuclear factors, phosphorylation/dephosphorylation processes have been identified that may have an important role in the glucocorticoid action mechanism.

Modulators of GR activation process

Initial studies on the use of molybdate reported it to be a stabilizer of glucocorticoid receptors (Nielsen *et al*, 1977), but was subsequently shown to block the *in vitro* activation process (Nishigori and Toft, 1980). However, no inhibitory effects are seen on the already activated hormone-receptor complexes. These effects were found to be reversible upon removal of molybdate from the system. The effects of tungstate and vanadate were also reported to be similar, albeit tungstate appeared to be a more effective agent for blocking receptor activation (Moudgil *et al.*, 1984). The ability to block the activation process allowed workers to purify the unactivated receptor complexes and study the structure and function in greater detail (Wrange *et al.*, 1986; Grandics *et al.*, 1984). Several workers have also reported the inhibitory effects of

thiol-modifying agents like N-ethyl maleimide, suggesting a possible role for thiol group(s) in the activation and/or DNA-binding processes of the glucocorticoid receptor (Simons and Pratt, 1995).

The possible role of pyridoxal-5′-phosphate (Vit B_6), as an endogenous modulator has also been investigated by many workers (DiSorbo *et al.*, 1980; Allgood *et al.*, 1990). Vit B_6. is an essential, water-soluble vitamin required for normal growth and development. Several biochemical properties if the GR is influenced by Vit B_6 including molecular conformation, polyanion binding, surface charge and susceptibility to exogenous proteolysis (Cidlowski, 1980; O′Brien and Cidlowski, 1981). In addition, subcellular localization and the DNA-binding capacity are also affected. Vit B_6 deficiency in experimental animals caused an increased translocation of GR from cytoplasm to nucleus, whereas the opposite was observed under conditions of elevated Vit B_6 concentration (Holley *et al.*, 1983; Bruce and Vessal, 1987). Vit B_6 also decreases the transcriptional efficiency of target genes by GR but the exact mechanism(s) of the process are not yet clear (Allgood *et al.*, 1993; Tully *et al.*, 1994).

Many other natural compounds, named as glucocorticoid action biomodulators, have been identified. These include compounds which are potent activators of Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C), e.g., 1,2-racemic dioctanoyl glycerol (1,2-DG), 12-O-tetradecanoyl-phorbol-13-acetate (TPA), guanisine 3′-diphosphate. epidermal growth factors (EGF) and interleukins. Based on these and other observations, a possible role for protein kinase C in glucocorticoid action mechanism has been proposed (Sharma, 1991).

Phosphorylation/dephosphorylation of GR

The role of phosphorylation/dephosphorylation in regulating the activities of many enzymatic and non-enzymatic proteins is well known, however, the persuasive importance of such processes on the functional ability of the GR is not very clear. Phosphorylation of the GR has been studied in a number of systems and it is seen that the basally phosphorylated GR becomes hyperphosphorylated, mostly on serine residues concomitantly with or shortly after dissociation from hsp complex, during the activation process (Hu *et al*, 1994). In most of these studies, phosphorylation has been shown to play a role in the binding of hormone to the receptor. Recently, Vivanco *et al.* (1995) proposed that changes in transcriptional activity of rat GR is due to alterations in receptor phosphorylation. The generation of `null receptor′ in ATP-depleted cells, which has low affinity for hsp and the hormone ligand, led researchers to propose that there exists both hormone-binding/non-binding GR forms depending on the phosphorylation status of receptor (Bodwell *et*

al, 1993). Moreover, the use of phosphatase inhibitors that stabilized the hormone-receptor complex and prevented its dissociation, pointed to the possibility that inactivation of receptors may result from dephosphorylation. The role of dephosphorylation in the activation process of glucocorticoid receptor was also studied. Alkaline phosphatase was reported to enhance the activation process whereas phosphatase inhibitors e.g., molybdate, tungstate and fluoride were seen to inhibit this process (Matic and Trajkovic, 1986; Schmidt and Litwack, 1982). The role of dephosphorylation has, however, been questioned and evidence presented to show that there is no dephosphorylation of GR during activation process (Mendel *et al.*, 1987, 1990; Tienrungroj *et al.*, 1987).

The role of protein kinase(s) and phosphatase(s) which mediate in the above processes has not yet been clearly defined. However, the role of protein kinase C in phosphorylation of GR seems interesting. Stimulation of cAMP-dependent protein kinase A (PKA) pathway has also been reported to augment GR activity in response to glucocorticoids. This change probably does not involve alterations in phosphorylation pattern of GR and is likely to be mediated by phosphorylation of factors interacting with the receptor (Rangarajan *et al.*, 1992; Moyer *et al.*, 1993; Reisfeld and Vardimon, 1994).

Thus, a consistent pattern of enhancement or inhibition of GR function via phosphorylation/dephosphorylation processes is lacking. It may be possible that the phosphorylation status of GR determines its subcellular localization rather than its overall activity (Orti *et al.*, 1993; Borror *et al.*, 1995).

Nuclear translocation of GR

Once the glucocorticoid receptors are transformed (activated) they translocate to the nucleus. The actual process of GR translocation has evaded numerous investigations and no conclusive evidence has been forwarded to explain the actual mechanism. It is known that the size of the GR is considerably larger than that of the nuclear pores to allow for passive diffusion (Lang *et al.*, 1986). A transport system, reported to be located in the nuclear membrane, called `transportosome´, is responsible for the facilitated transport of GR. In addition, Pratt (1993) has proposed a role for hsp90 along with the intracelluar microtuble system in the nuclear transport of receptor. This proposal is based on several observations that cited the association of hsp90 with GR and also of GR with tubulin (Wikstrom *et al.*, 1987; Sanchez *et al.*, 1988; Pratt, 1990). Moreover, observations suggest the co-translocation of GR and hsp90 to the nucleus under normal as well as stressed conditions. Howell *et al.* (1990) also reported the association of GR with nuclear envelopes, using [^{3}H] dexamethasone-mesylate affinity-labeling and immunological

techniques. They showed that this association of GR with nuclear envelopes is hormone responsive, suggesting that hormone binding causes the exposure of certain nuclear localization domains (signals) that helps in targeting of receptors to the nucleus. In support of the above observation, two nuclear localization signals (NLS), characterized by abundance of basic amino acids lysine and arginine, have been identified in the rat GR (Picard and Yamamoto, 1987). These signals probably permit interaction of activated GR with the nuclear transport machinery. The first nuclear localization signal (NL1) located at the C-terminal of GR, between amino acids 497-524 and has a short region of homology to the nuclear localization signal of SV40-T antigen. The NL2 lies within the HBD (Aa 525-795) and is probably masked by the bound hsps, since its function is revealed only upon hormone binding and subsequent dissociation of the hetero-oligomeric protein complex. Further support for the facilitated transport of GR into the nucleus came from an interesting observation by Htun *et al.* (1996), who used a chimeric protein to study the translocation process. A mutant form of the green fluorescent protein (GFP) was fused to GR, the unliganded chimera was then observed to reside in the cytoplasm that translocates to the nucleus only in response to glucocorticoid. The translocation process was ligand-, time- and dose-dependent and required energy, showing it to be a facilitated process.

Glucocorticoid response elements

Upon translocation to the nucleus, the hormone bound GR binds to specific DNA sequences, usually located 100-300 bp in the 5´-flanking regions of glucocorticoid regulated genes. These sequences, responsive to glucocorticoids, are termed glucocorticoid response elements (GREs) (Beato, 1989). Gene transfer techniques have helped in the identification of such GREs in many glucocorticoid inducible genes and any deletion or mutation of such sequences eliminated hormonal control (Yamamoto, 1985). It was also shown that this short oligonucleotide sequence is responsible for conferring glucocorticoid responsiveness, even to a heterologous promoter (Strahle *et al.*, 1987). The GRE has been identified as a 15-mer, consisting of two short inverted repeats separated by three nucleotides-5´GGTACAnnnTGTTCT3´. Such consensus DNA sequences have been observed for progesterone, mineralocorticoid, androgen, estrogen as well as for other members of steroid and non-steroid receptors. (Beato, 1989; Lucas and Granner, 1992). Recent work, also suggests that the mere availability of a GRE in isolation is not enough and the presence of multiple copies of GRE or its association with other cis-elements, constituting a complex unit- termed glucocorticoid response unit (GRU), is necessary for mediating the effect of glucocorticoids (Lucas and Granner, 1992). In

addition several other factors play a very significant role in the process of DNA-binding, a brief mention of a few will emphasize the complexity involved in regulating gene activity by glucocorticoids.

Receptor dimerization

Increasing evidence suggests that the glucocorticoid receptor binds to GRE as a homodimer (Kumar and Chambon, 1988; Hard *et al.*, 1990). This dimerization occurs prior to DNA-binding and the dimers interact with the GRE in a head-to-head configuration, each receptor contacting a single arm of the palindromic GRE. This observation is supported by studies on the interaction between DBD of GR with GRE using NMR, X-ray crystallography and other biochemical techniques (Hard *et al.*, 1990; Luisi *et al.*, 1991). As mentioned earlier, the dimerization motifs are located in the DBD but in absence of glucocorticoids are masked by the HBD. The role of hormone in DNA-binding is thus, seen as an agent that induces a conformational change in the receptor molecule leading to exposure of its dimerization function apart from NLS thereby, allowing high affinity binding to GRE and subsequent modulation of the cognate gene expression.

Interaction of GR with transcription machinery

The process of transcription is a complicated phenomena, involving a large number of factors which constitute the basal transcription machinery. Many trans-acting factors, including glucocorticoid receptor interact either directly or indirectly with the transcription machinery to modulate gene expression. The formation of transcription initiation complex is a sequential process and is a prerequisite for proper transcription of any gene. The activated GR is thought to either stimulate the formation and/or stabilization of this complex or help in the recruitment of preformed complexes at glucocorticoid responsive gene promoters. The role of tau1 and tau2 domains of GR in the above process(s) is currently under investigation (Truss and Beato, 1993; Onate *et al.*, 1995; Beato and Sanchez-Pacheco, 1996).

Synergism between different cis-acting elements

Many steroid response elements, including GREs are present in multiple copies or in conjugation with other cis-acting elements (Tsai and O'Malley, 1994). Mutation(s) in one of the GREs or in the adjacent cis-element produce a drastic decline in the promoter activity, suggesting the possibility of synergistic interaction between adjacent response elements in the transcription process. In the case of GR, it has been observed that binding of one receptor-dimer facilitates the binding of a second one thereby, allowing both the complexes to bind with higher affinity/specificity thus, promoting increased transcription (Schmid *et al.*, 1989). Such synergistic interactions probably through protein-protein interactions outside of DBD, between GR dimers

and other proteins like CCAAT- and CACCC-binding factors, NF1 or SP1 motifs have also been reported (Bamberger *et al.*, 1996; Ricousse *et al.*, 1996). The consequence of such synergistic interactions is not clear however, speculations about its role in nucleosome disruption that will provide access to other transcription factors is being researched upon.

Arrangement of cooperative binding sites

The magnitude of transcriptional regulation is also affected by the location of GREs relative to transcription initiation site. It has been reported that GREs located too far upstream of the TATA box have insignificant contribution on transcription as compared to those located just upstream of the initiation site (Bradshaw *et al.*, 1988; Schatt *et al.*, 1990). This distance-related synergism shows a cyclic pattern, with a period of about 10 bp which corresponds to one turn of the double helix, suggesting stereospecific requirement for protein-protein interactions. However, the relative arrangement of GREs and other cis-elements (either upstream or downstream of initiation site) showed no change in transcriptional ability of the respective genes (Schule *et al.*, 1988).

Alteration in chromatin structure

Genetic analysis using modern techniques has revealed a widespread involvement of the structural organization of DNA/chromatin in regulating gene expression (Weintraub, 1985). DNase I sensitivity of mouse mammary tumor virus (MMTV) promoter increases upon glucocorticoid administration, showing an alteration in the chromatin organization in the vicinity of glucocorticoid receptor binding sites (Zaret and Yamamoto, 1984). This change is believed to be due to either displacement or rearrangement of nucleosomes over GRE, causing a relaxation in the chromatin structure. This relaxation facilitates the binding of other transcriptional factors including nuclear factor, NF1 and the octamer transcription factor, OTF1 to MMTV promoter (Beato *et al.*, 1995). Furthermore, it has been shown that GR is able to bind naked DNA as well as reconstituted chromatin with equal affinity. In contrast, NF1 binds efficiently only with free DNA suggesting a requirement for structural relaxation in the chromatin structure to enable efficient binding of NF1. Also, secondary modifications of histones in the chromatin, e.g., acetylation, methylation in response to GR binding may be involved in such a displacement/rearrangement process (Bresnick *et al.*, 1990). It has also been reported that the specificity of DNA-binding by GR increases as the DNA obtains a higher ordered structure. This, probably augments the ability of GR to discriminate between specific- and random-binding sites in the genome (Perlman, 1992). Overall, the chromatin structure influences gene activity in three

ways- (i) by enhancing receptor-factor(s) interaction to promote transcription, (ii) by preventing the binding of trans-acting factors that can inhibit gene transcription and (iii) by masking the unwanted genes by packing them into a heterochromatin.

Role of other proteins in GR-DNA interaction

Recently, several proteins have been identified which possibly mediates in the GR-dependent transcription process. A set of proteins, termed GRIP (glucocorticoid receptor interacting protein) have been isolated, these do not influence basal promoter activity but does enhance GR induction of target genes (Eggart *et al.*, 1995). Similar receptor interacting proteins for estrogen, termed ERAP and T_3, termed TRAP have also been reported (Beato and Sanchez-Pacheco, 1996). Another protein called steroid receptor coactivator-1 (SRC), which stimulates trans-activation of all the steroid receptors have, recently been characterized (Onate *et al.*, 1995). Protein components of the SN1/SNF complex in yeast and its human homologs are reported to potentiate GR-mediated transactivation process (Bamberger et., 1996)

The above mentioned factors and their many possible interactions with GR, somehow stimulate the transcription machinery leading to induction of several glucocorticoid responsive genes, e.g., mouse mammary tumor virus, human metallothionein II_A, chicken lysozyme, growth hormone, maloney murine sarcoma virus, rat tyrosine aminotransferase, rat tryptophan oxygenase, phosphoenol pyruvate carboxykinase and α_1-acid glycoprotein. However, glucocorticoids are also involved in negative regulation of some physiologically relevant genes, e.g., α-subunit of glycoprotein hormone, rat prolactin, α-fetoprotein, urokinase, collagen, stromylesin and pro-opiomelanocortin (Beato *et al.*, 1989; Lucas and Granner, 1992). Two components, relevant to negative regulation have received much attention lately- one is the presence of negative GREs and the other is the presence of certain GR-binding proteins which interfere with the transcription process. Evidence for the presence of negative GRE came from the work of Sakai *et al.* (1988). Genetic analysis of the negative GREs did not yield a clear cut consensus, however a 15 nucleotide long sequence analogous to +ve GRE have been proposed. This sequence, which is an imperfect copy of the +ve GRE has the following arrangement, 5′-ATYACNNNTNTGATCN-3′. It allows high affinity binding of the activated GR, but appears to force the GR into a conformation that exposes domains which silence transcription. These sequences are orientation-independent and serve as a negative enhancer, the binding of GR may either block or in some way alter the action of other transcription factors that function through an overlapping site. The other mechanism mentioned above probably, involves the components of non-steroid hormone action pathways interacting with the steroid action component(s), the so called "cross-talk"

in signal transduction. Of particular interest is the role of AP-1 and NF-κB in interfering with the transcriptional activity of genes regulated by GR. Interaction of GR with the components of AP-1 (Fos/Jun) leads to the formation of a complex unable to bind DNA, consequently down regulating target genes (Tsai and O'Malley, 1994). This interaction takes place even in the absence of the DBD of GR, suggesting a role for other domains in this process. Moreover, the induction of either of the AP-1 components, responsive to phorbolesters leads to repression of GR expression. Similarly, the induction of GR represses the expression of AP-1 components. This mutual repression requires the N-terminal sequences of GR and the basic leucine zipper region of Jun suggesting conformational change as the basis of altered activity. Similar interaction between p65, one of the transcriptionally active subunits of NF-κB (an important element of the inflammatory response) and GR have also been reported (Beato *et al.*, 1995).

The above observations clearly emphasize the complexity involved in the transcriptional regulation of genes by glucocorticoid receptors and also the persuasive role steroid hormones play in a vast number of physiologic and pathologic processes.

<u>GR cycling</u>

An interesting feature of the glucocorticoid signal transduction pathway is the nucleo-cytoplasmic cycling of GR. It was observed that the DNA bound hormone-receptor complexes get dislodged following gene modulation, the free receptors then migrate back to the cytoplasm where they are mostly recycled or sometimes degraded (Munck and Holbrook, 1988). Receptor recycling can also be gauged from the observation of the so-called 'null' receptors, which are either newly translated ones or most likely, are those recycled from the nucleus. These null receptors need to be associated with the components of the protein folding system to attain a conformation suitable for steroid binding. This cycle is ATP-dependent and the involvement of phosphorylation/dephosphorylation steps cannot be ruled out (Mendel *et al.*, 1990; Bohen, 1995). Observations have suggested the presence of export signals similar to nuclear localization signals, that help in receptor recycling.

Regulation of glucocorticoid responsiveness

Tissue responsiveness to glucocorticoids is dependent upon a number of parameters: (i) the concentration of free hormone available to a cell, (ii) the intracellular receptor concentration and (iii) the concentration and availability various factors integral to the transduction of the hormonal signal. The regulation of plasma and tissue glucocorticoid concentration is achieved mainly by the interplay between hypothalamo-hypophyseal system and has been relatively well studied and

understood. This concentration is also influenced by the plasma and tissue levels of corticosteroid-binding globulin (CBG), which are themselves under complex regulatory control (Orth *et al.*, 1992; Dhabhar *et al.*, 1993). An interesting mechanism by which kidney cells in the distal tubules are rendered unresponsive to physiological concentrations of glucocorticoids, is achieved by the presence of high levels of 11β-hydroxysteroid dehydrogenase (11β-HSD). In these cells, the incoming glucocorticoids are rapidly metabolized by this enzyme, thus rendering the cells primarily mineralocorticoid responsive. The expression level and/or activity of this enzyme in different tissues may therefore, modulate glucocorticoid responsiveness (Funder *et al.*, 1988; Brown *et al.*, 1996). A transporter protein, recently reported by Kralli *et al.* (1995) that actively and specifically exports glucocorticoids can regulate intracellular levels of this steroid.

There is a tissue- and age-specific variation in the GR expression level, thymus being the tissue showing the highest number of receptors per cell (Miller *et al.*, 1990). A number of factors have been reported to cause such a change-glucocorticoids themselves appear to be the most potent regulator and has been shown to cause down-regulation of its receptor in many cell lines and tissues from animals and humans. This regulation is probably at the transcriptional level through the inhibition of GR mRNA generation, by interfering with the mediators of transcriptional activation of the GR gene, AP-1 and/or AP-2 (Burnstein and Cidlowski, 1992; Barrett *et al.*, 1996). Inhibition can also occur via binding of activated GR to sites within the coding DNA and/or mRNA, thus reducing mRNA stability and translatibility. Moreover, in the presence of glucocorticoids, the half-life of the GR protein has been reported to be reduced (Burnstein *et al.*, 1990; McIntyre and Samuels, 1985). The effects of estrogens, neurotransmitters and of heterozygous microdeletions in an exon-intron splice site in one GR allele, on GR expression add to the complexity of GR transcription and expression (Bamberger *et al.*, 1996).

The structural integrity of the HBD, and hence, the hormone-binding affinity also determines the potency of GR as a transcriptional regulator. The role of hsp90 in assembly, folding and maintenance of the GR HBD in a ligand-friendly, high-affinity conformation is vital. The intracellular levels of hsp90 and its structural integrity can influence GR function. In yeast low levels of hsp90 leads to impaired signal transduction and in the thymus, which is highly sensitive to glucocorticoids, hsp90 levels are also high (Hu *et al.*, 1994; Bohen, 1995).

In addition, numerous other endogenous factors have been identified that affect the various stages of this signal transduction process and any intra-/inter-individual variations in one or more of these factors can, therefore, have profound influence on

the GR activity. Many of these factors have already been discussed before and a few need a brief mention here to stress their possible importance in glucocorticoid responsiveness. A heat-stable stimulator protein and a inhibitory modulator, a 1500 Da phosphoglyceride that binds both the GR and hsp and stabilize the complex has been reported (Schmidt *et al.*, 1985; Bodine and Litwack, 1988). Also, variations in a tissue- and age-specific manner of the phosphorylation/dephosphorylation, nuclear translocation, DNA/GRE binding and many other factors associated with these processes can play an important role in regulating GR activity (Orti *et al.*, 1993; Hsu and DeFranco, 1995; Beato *et al.*, 1995). An important, physiologically relevant observation made recently shows the presence of two isoforms of the GR- GRα and GRβ that are generated by alternate splicing of the primary transcript. These two isoform proteins have the first 727 amino acids in common but differ only in the C-terminus with replacement of the last 50 amino acids by a unique 15 amino acid in GRβ. This difference renders the GRβ unable to bind hormone and is thus, transcriptionally inactive. The GRβ is located primarily in the nucleus and its over expression led to an antagonizing effect on the GRα activity, causing upto 90% reduction in reporter gene activity. The mechanism of this inhibitory effect is proposed to occur via occupation of GRE target sites by GRβ/GRβ homodimers or GRα/GRβ heterodimers. These block the binding of GRα/GRα homodimers, necessary for GR-mediated transcription (Hollenberg *et al.*, 1985; Encio and Detera-Wadleigh, 1991; Oakley *et al.*, 1996)

The thorough understanding of the mechanisms regulating glucocorticoid sensitivity in target tissues, necessitates the determination of relative amounts of all these factors and the regulatory processes, if any, which control their expression/production levels. Many pathophysiological states are potentially associated with tissue-specific and/or acquired glucocorticoid resistance or hypersensitivity (Bamberger *et al.*, 1996). Therefore, such studies will help in our understanding the actual mechanisms involved in glucocorticoid sensitivity of target tissues.

All the above observations aptly accentuate the importance of glucocorticoid receptor-mediated signal transduction process in the growth, development and in the maintenance of the overall homeostatic balance in higher organisms. Keeping in view the data generated over the years on GR structure and action mechanism, we decided to direct our attention to the study of physicochemical changes in the receptor during the postnatal development of male mice.

The work was divided into the following phases:

i) *Determination of age- and tissue-specific concentration of the GR at various postnatal ages (10- to 60-day), to see changes, if any, in the level of GR. Such changes could also be due to alterations in the receptor's affinity for the ligand. Therefore, scatchard analysis of the binding data was performed to see if there is any change in the dissociation constant, which might correlate to changes in the receptor level.*

ii) *The activation process of the receptor, by temperature and salt, at two ages (10- and 60-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. Furthermore, nuclear-exchange assays were performed to determine the role of nucleus in GR binding. The effects of various inhibitors (molybdate, tungstate and N-ethylmaleimide) of the activation process were studied to observe the tissue- and age-specific sensitivity of GR to these inhibitors. Salt extraction of the nuclear bound hormone-receptor complexes was performed to see the strength of receptor-chromatin interaction at two ages. Moreover, DNase I digestion of the nuclear chromatin was done to ascertain the change in chromatin organization, which might have a significant role to play in tissue responsiveness to glucocorticoids.*

iii) *Finally, other properties of the unactivated and activated glucocorticoid receptor, e.g., molecular weight, stoke's radii and charge content were determined to see if there is any age-related change in these parameters. Also, the antigenic property of the unactivated receptor was assessed by immunoadsorption technique.*

Experimental Procedures

Materials

Animals

Swiss albino mice (balb/ c strain) were used for all the experiments. The animals were housed in polycarbonate cages with light husk bedding, under normal colony conditions at 24±2°C and a photoperiod of 12h light/ dark cycle. Pelleted mice feed (Amrut laboratories, Pune, India) and tap water were made available *ad libitum*. Male mice of different postnatal ages (10-, 15-, 30-, 45-, and 60-day old) were used for the experiments.

Chemicals

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

Amersham, England: [1,2,4,6,7-^{3}H]dexamethasone (specific activity- 87, 89 and 90 Ci/ mmol).

Sigma Chemical Co., USA: [^{1}H]dexamethasone, Tris, sodium molybdate, sodium tungstate, EDTA, dithiothretriol, β-mercaptoethanol, Triton X-100, DNase I, diphenylamine, dextran T-70, DNA-cellulose, deoxyribonucleic acid, bovine serum albumin, Molecular weight markers, N-ethylmaleimide, Sephadex G-100, G-200, protein A-Sepharose.

Qualigens, India: Sucrose, glycerol, sodium chloride, potassium chloride, sodium hydroxide, hydrochloric acid, acetic acid, orthophosphoric acid.

Merck, India: Calcium chloride, magnesium chloride, activated charcoal.

HiMedia, India: Potassium di-hydrogen phosphate and di-potassium hydrogen phosphate.

Bengal chemicals and Pharmaceuticals, India: Ethanol

Whatman, England: Filter papers and anion exchanger DE-52.

Sisco Research Laboratories, India. Scintillation cocktail-W (1% PPO, 0.025% POPOP, 10% Naphthalene in 1,4-dioxan).

Instrumentation

All the centrifugation work were carried out in a Hitachi (Himac CR20B2 model) high speed refrigerated centrifuge at 2°C. Absorbance measurements were done on a Hitachi U-2000 double beam spectrophotometer, using glass and quartz cuvettes of 1 cm path length. The radioactivity measurements were done in a Beckman LS1801 scintillation counter with 65% efficiency for tritium.

Buffers

The buffers used in the experimentation were prepared in glass double-distilled water, the pH adjusted at room temperature and stored at 4°C until use.

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 DTT/ 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_2PO_4, pH 7.5/ 1 mM EDTA/ 1 mM β-mercapto ethanol/ 20 mM Na_2MO_4

(ii) 100 mM KH_2PO_4, pH 7.5/ 1 mM EDTA/ 1 mM β-mercapto ethanol/ 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.

E Immunological studies:

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 DTT/ 10 mM NaCl

Methods

[^{3}H]dexamethasone binding studies

Tissue preparation

Male mice of different postnatal ages, as mentioned above were killed by cervical dislocation at a fixed time of the day (11:00 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, twice in ice-cold saline (0.9% NaCl in distilled water). Tissues (Liver, Kidney, Brain, Heart and Skeletal muscles) were then blotted dry and stored separately at -70°C until use. No significant change in the receptor level/ activation was observed upon such storage, for a period of upto two weeks. All the experimental procedures were carried out at 2-4°C, unless otherwise mentioned.

Preparation of cytosol

The tissues were properly thawed and minced, these were then used for preparation and assay of the glucocorticoid receptors. A 20% (w/ v) homogenate of the tissues were prepared in buffer A, using a motor driven teflon-coated pestle and a glass homogenizer. Homogenization was carried out in the cold for ~1 min at a moderate speed (1000 rpm). 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 27,500*g* for 60 min. The fatty layer on the surface were aspirated using pasteur pipette and the clear cytosols thus obtained were used for the assay of glucocorticoid receptors.

Concentration of saturable [^{3}H]dexamethasone required

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 2.5-120 nM [^{3}H]dexamethasone (final concentration) only. 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 binding values (NS). All the tubes were incubated for 6 hr in crushed 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. 100 μl of the supernatant, devoid of any charcoal particle was carefully pipetted out into scintillation tubes and to it 5 ml of scintillation cocktail added. The contents were thoroughly mixed and the radioactivity measured in a Beckman LS1801 counter with an efficiency of 65 % 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.

Incubation time required

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 40 nM. The hormone-binding was allowed to occur out for different periods of time- 0.5 hr to 10 hr. Specific binding, at each time interval was determined

after the 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 used to determine the level of the glucocorticoid receptor in different tissues and at different postnatal ages of mice.

Determination of the glucocorticoid receptor level

Glucocorticoid receptors levels in various tissues of mice and at different postnatal ages were obtained from 5-6 separate mice of each age group. The assays were performed under identical conditions to minimize the effects of any external variation. Cytosols from different tissues of mice were obtained as above, and used for determination of specific saturable binding of [^{3}H]dexamethasone at various postnatal ages. The assays were performed according to the method of Kalimi et al (1983), in a total volume of 1.5 ml and consisted of the following:

For total binding	*For non-specific binding*
100 μl cytosol	100 μl cytosol
15 μl [^{3}H]dexamethasone	15 μl [^{3}H]dexamethasone
(to give a final concentration of 40 nM)	
15 μl buffer	15 μl dexamethasone (final concentration- 500-fold excess to that of [^{3}H]dexamethasone)

The contents were mixed by gentle vortexing and incubated for 4 hr at 2°C, with vortexing at regular intervals to ensure proper binding. At the end of the incubation period, 50 μl of ice-cold DCC was added to each tube, the charcoal particles kept in suspension by gentle vortexing and allowed to stand for 10 min. The charcoal was then pelleted by centrifugation at 2000*g* for 10 min. 100 μl of the clear cytosol was carefully pipetted, avoiding any charcoal particle into scintillation vials and 5 ml of scintillation cocktail-W added. The contents were thoroughly mixed and the bound radioactivity (CPM) measured. The protein content in the final reaction mixture (after DCC treatment) from each set of assay was determined according to Bradford's dye-binding method (Bradford, 1976) (Appendix I). Specific binding was calculated, as described above and expressed as fmol/ mg protein (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 3-4 mice of the same age group and the cytosol obtained as described above. The experimental setup was similar to that described above except the assays were performed with increasing concentration of $[^3H]$dexamethasone (from 2.5-120 nM). Parallel tubes contained 500-fold molar excess of cold dexamethasone to that of the respective $[^3H]$dexamethasone, at each concentration point. Specific binding, $[S]_{bound}$ was determined for each concentration of $[^3H]$dexamethasone used. The total hormone concentration ($[S]_{total}$) added to each tube was also determined. The value of free hormone concentration ($[S]_{free}$) was obtained by subtracting $[S]_{bound}$ from $[S]_{total}$. The ratio of $[S]_{bound}/[S]_{free}$ values was plotted against the specific bound ($[S]_{bound}$) values and a linear regressed curve plot drawn. The slope of the plot provided the dissociation constant (K_d) and the intercept on the x-axis provided the maximum specific binding values.

Activation studies

Activation studies were performed in two tissues, liver and kidney and at two postnatal ages, 10-day and 60-day old male mice. The magnitude of glucocorticoid receptor activation was judged using DNA-cellulose as well as purified nuclei binding assays.

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

Mice were killed by cervical dislocation 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 3-4 mice of each age group were 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 27,500*g* for 60 min and to the clear, fat free cytosols was added $[^3H]$dexamethasone to a final concentration of 40 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 $[^3H]$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 20 mM $CaCl_2$ at 0°C

Aliquots of the cytosol were also kept at 0°C for 45 min to provide the unactivated receptor complexes.

DNA-cellulose binding assay

DNA-cellulose binding assays were performed according to the method of Kalimi *et al.* (1975). Commercially available DNA-cellulose (4.2 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 a homogenous slurry, from which aliquots (50 μl) containing 25 μg DNA were transferred to 1.5 ml eppendorf tubes and a further 1 ml ice-cold buffer added to each tube. The cellulose was pelleted by centrifugation at 2000*g* for 10 min and the supernatant discarded. Aliquots of the activated hormone-receptor complexes, from the two ages, containing approximately 30,000 CPM were added, in duplicate to the cellulose pellets and the contents mixed by gentle vortexing. The reaction was allowed to occur for 1 hr at 2°C with regular vortexing of the tubes to keep the cellulose in suspension, thus ensuring proper binding of the hormone-receptor complexes to DNA-cellulose. The reaction was stopped by addition of 1 ml chilled buffer followed by centrifugation at 2000*g* for 10 min. The cellulose pellets thus obtained were washed twice more in the same buffer. The supernatants in each stage were carefully discarded avoiding any loss of cellulose particles. The final pellets obtained were suspended in 1 ml of cocktail-W and transferred to scintillation vials to which a further 3 ml cocktail was added. The radioactivity bound in the pellets were counted and results expressed as CPM/ 100 μg DNA in DNA-cellulose.

Nuclear binding assay

The nuclear pellets obtained as mentioned above, containing cellular debris and cytoplasmic contaminations were further processed according to the procedure of (Eberhardt *et al.*, 1978). To the pellets was added 1 ml of chilled buffer B (ii) and the contents gently homogenized and then centrifuged at 2000*g* for 10 min. The supernatants were discarded and the pellets washed thrice in buffer B (i) followed by centrifugation. The final pellets were suspended in the same buffer to give a homogenous slurry, aliquots of which containing 200-250 μg DNA were pipetted to eppendorf tubes and 1 ml of buffer B (i) added to each tube. The tubes were centrifuged at 2000*g* for 10 min to give the purified nuclear pellets, the supernatants being discarded. For **nuclear binding assay**, aliquots of the activated hormone-receptor complexes containing approximately 30,000 CPM were added, in duplicate and the nuclear pellets suspended by gentle vortexing. **Nuclear exchange assays**

were also performed, wherein the thermally activated (25°C for 45 min) hormone-receptor complexes from 60-day were allowed to interact with the nuclei of 10-day and *vice versa*. The nuclear binding was allowed to occur for 1 hr at 2°C, regular mixing of the contents being done to ensure proper interaction. The pellets were subsequently processed and the bound radioactivity counted as described for DNA-cellulose assay. The amount of nuclear DNA was determined according to the diphenyl method of Burton (1956, 68) (Appendix III). The data obtained were statistically analysed and the results expressed as CPM/ 100 μg nuclear DNA.

DNase I digestion studies

DNase I digestion studies were performed on the purified nuclei obtained from liver and kidney of mice at two postnatal ages (10- and 60-day), according to a modified procedure of Chaturvedi and Kanungo (1983). Temperature activated [^{3}H]dexamethasone-receptor complexes, from the two age groups, were allowed to interact with their respective purified nuclei, as described above. After washing off the unbound complexes, the final 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. The nuclear pellets were properly suspended by gentle vortexing at regular intervals and the reaction stopped by addition of 1 ml of buffer B (i) followed by centrifugation at 2000*g* for 10 min. 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.

Salt extraction of the bound hormone-receptor complexes

Extraction of the hepatic nuclear bound, thermally activated hormone-receptor complexes were also done using different concentrations of sodium chloride. The extracting solution (100 μl) contained 0-0.5 M NaCl, added from a 1 M stock solution prepared in buffer B (i). The extraction was allowed to occur for 45 min at 2°C, with vortexing at regular intervals of time. The reaction was stopped by addition of 1 ml buffer B (i) and the extracted receptor complexes were removed by washing the pellets, thrice with the same buffer. The radioactivity bound in the pellets was then determined and the results expressed as %[^{3}H]dexamethasone-receptor complexes bound to nuclei.

Inhibition of activation studies

Inhibition of the activation process was studied in the liver and kidney of mice at two different ages, 10- and 60-day. The magnitude of inhibition was judged using DNA-cellulose and purified nuclei, the inhibitors used were sodium molybdate, sodium tungstate and N-ethylmaleimide. These were prepared as 1 M stock in buffer B (i)

and added to aliquots of the [^{3}H]dexamethasone-receptor complexes to give a final concentration of 20 mM. These complexes were then subjected to the activation process by heat at 25°C for 45 min. For the low-temperature salt mediated activation process, only molybdate and tungstate were used as the inhibitors. The binding assays, subsequent processing of the DNA-cellulose/ nuclear pellets and the determination of bound radioactivity were done as described above. The results obtained were expressed as %[^{3}H]dexamethasone-receptor complexes bound to DNA-cellulose/ nuclei, 100% binding being attributed to hormone-receptor complexes activated in absence of inhibitors.

Physical characterization of GR

Physical properties of glucocorticoid receptors were studied in the liver of mice at two postnatal ages (10- and 60-day) to see if there is any age-related differences in various physical parameters of the receptor. To determine the molecular weight and stokes radii of the unactivated as well as thermally 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. Immunological properties of the unactivated receptors from two age groups were studied using immunoadsorption technique. All the gel filtration and ion-exchange 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 supernatant. A thick gel slurry was prepared and degassed for 1 hr, this was then poured into a glass column (1.8 x 45 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/ hr and equilibration was continued till the pH of the eluant 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/ hr. [^{3}H]dexamethasone-receptor complexes from the liver tissues of 10- and 60-day old mice were prepared in buffer A, as mentioned and the unbound hormone removed using DCC. 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/ hr flow rate. 2 ml

fractions 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 the stokes radii of unactivated receptors, from both the ages were calculated according to the theoretical treatments of Andrews (1970), Porath (1963) and Laurent and Killander (1964) from the following plots:

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. R_S

Gel filtration analyses of the activated receptor

The physical properties of thermally activated glucocorticoid receptors were analysed on a sephadex G-100 column (1.8 x 42 cm). Gel was prepared and the column set up and equilibrated with buffer C(ii) at a flow rate of 20 ml/ hr. 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/ hr. The [^{3}H]dexamethasone-receptor complexes from the liver of 10- and 60-day animals were prepared in buffer B (i) and activated at 25°C for 45 min. Aliquots (2 ml), containing approximately same radioactivity were loaded and eluted with buffer C(ii). 2 ml fractions were collected and radioactivity of 100 μl, from each fraction counted. The apparent molecular weights and the stokes radii were determined as that for the unactivated receptors.

ion-exchange analyses of unactivated and activated receptors

To determine the difference in the net charge content of unactivated and thermally-activated glucocorticoid receptors, anion exchange chromatography on DEAE-cellulose (DE-52) were done according to the procedure of Grandics *et al.* (1984). The ion-exchange resin was washed thoroughly with 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 (≈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 height of 3 cm. The resin was then washed extensively with buffer D, in the cold at a flow rate of 25 ml/ hr till the pH of the eluant equals that of the buffer. Unactivated [^{3}H]dexamethasone-receptor complexes, from the liver were prepared in buffer B(i) and 2 ml of it loaded onto the column. Upon 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/ hr flow rate. 1 ml fractions 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 experiments provided the data for the thermally activated receptors.

immunological studies on the inactivated receptors

Immunological studies were performed with the unactivated glucocorticoid receptors from the liver of 10- and 60-day old mice. The polyclonal antibody was raised against a specific amino acid sequence in the N-terminal region of the rat glucocorticoid receptor (Sharma *et al.*, 1991).The unactivated receptors were prepared in buffer A, as mentioned above and 200 μl aliquots of cytosol, containing the [^{3}H]dexamethasone-receptor complexes were incubated with 5 μl of either the pre-immunized serum or immunized serum. The incubation was carried out for 18 hr at 2°C. At the end of this period, 50 μl of protein A-sepharose (25% slurry) was added to each tube and the contents mixed thoroughly by gentle vortexing. After an incubation period of 1 hr at 2°C, the sepharose was pelleted by centrifugation at 2000*g* for 10 min. The sepharose pellets were further washed, thrice with ice-cold buffer and the final pellets obtained were suspended in cocktail-W and transferred to scintillation vials. 3 ml more of the cocktail was added and the bound radioactivity measured.

Results

The results obtained were plotted as line and bar diagrams, each point representing the mean value ± standard deviation. The results are also presented in tabular form, wherever necessary to summarize the data obtained from a series of experiments. All the data generated were statistically analysed and the level of significance (p-value) between two sets of data was calculated according to paired student's t-test, taking the value of $p<0.05$ as significant.

Glucocorticoid receptor binding studies

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

Fig. 1. depicts the kinetics 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 40 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 ~40 nM of [^{3}H]dexamethasone.

The time course for [^{3}H]dexamethasone binding is shown in Fig. 2. As can be seen, the maximum saturable binding, using 40 nM [^{3}H]dexamethasone was attained by about 4 hr of reaction time. Each data point in the figures represent the mean value obtained from three 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 (heart), skeletal muscle and the cerebral hemispheres at various postnatal ages (10-day to 60-day) of male mice. In the **liver**, the receptor level is low at early postnatal ages (10- and 15-day) and reaches a peak value by day 30 of postnatal age, which is significantly higher (about 2-fold) than the values observed for earlier ages. The receptor concentration value then shows a sharp decline by day 45 and 60, where the values are similar to those at day 10 and 15 (Fig. 3A). In the **kidney** however, the receptor level shows a peak value at day 45, which is significantly higher (~1.5 fold) than the receptor concentration at day 10, 15 and 30. By day 60 the receptor level declines to a value similar to those observed for early postnatal ages (Fig. 3B). The glucocorticoid receptor concentration in the **cardiac muscle** (heart) shows a trend similar to that observed in the liver, with a peak value at day 30 (Fig. 3C). However,

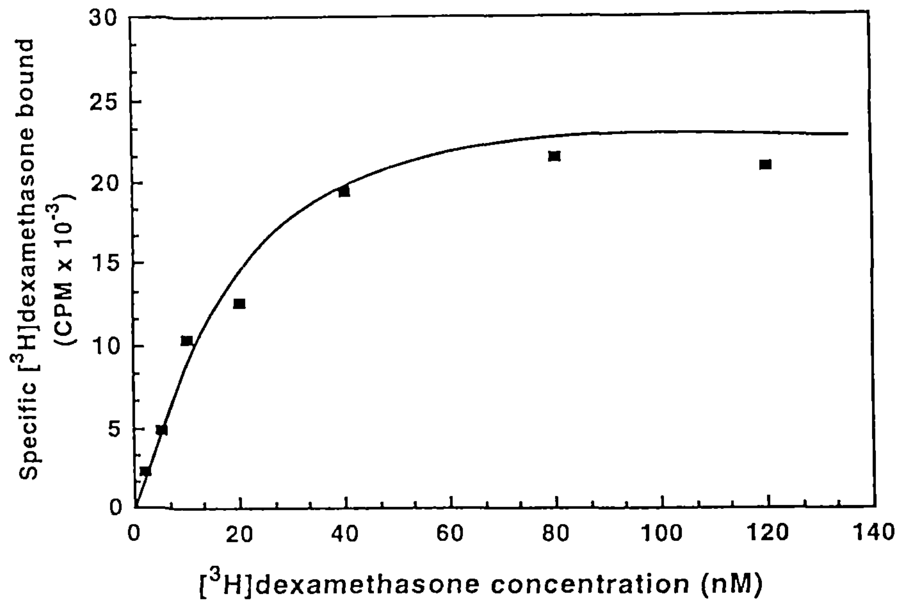


Fig. 1. Concentration dependence of [^{3}H]dexamethasone binding to glucocorticoid receptors in the liver of mice. Aliquots of cytosol (100 µl) were incubated with increasing concentrations of [^{3}H]dexamethasone ± 500-fold excess non-radioactive dexamethasone for 6 hr at 2°C. Unbound hormones were removed by dextran coated charcoal (DCC) treatment as described in the Materials and Methods section. Specific binding was calculated as the differnce 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.

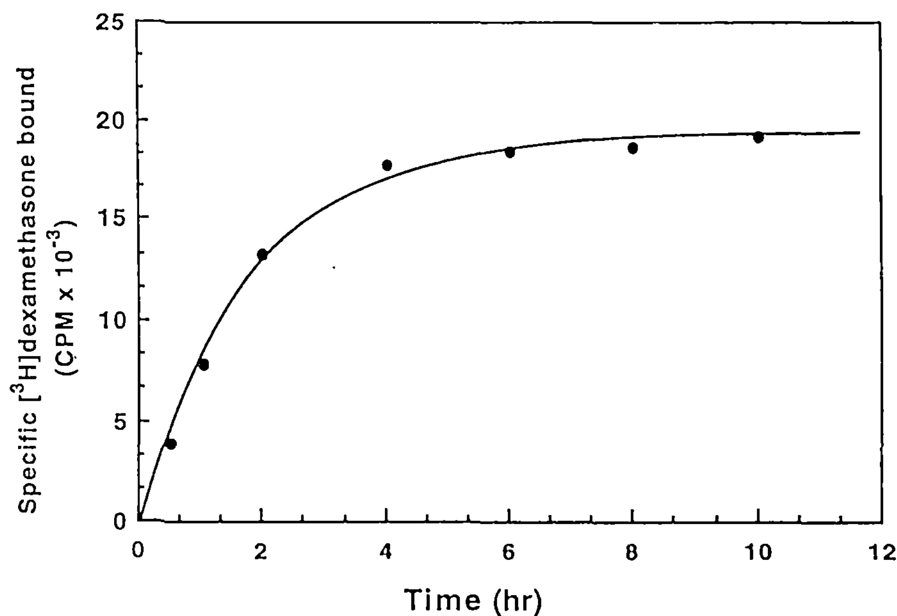
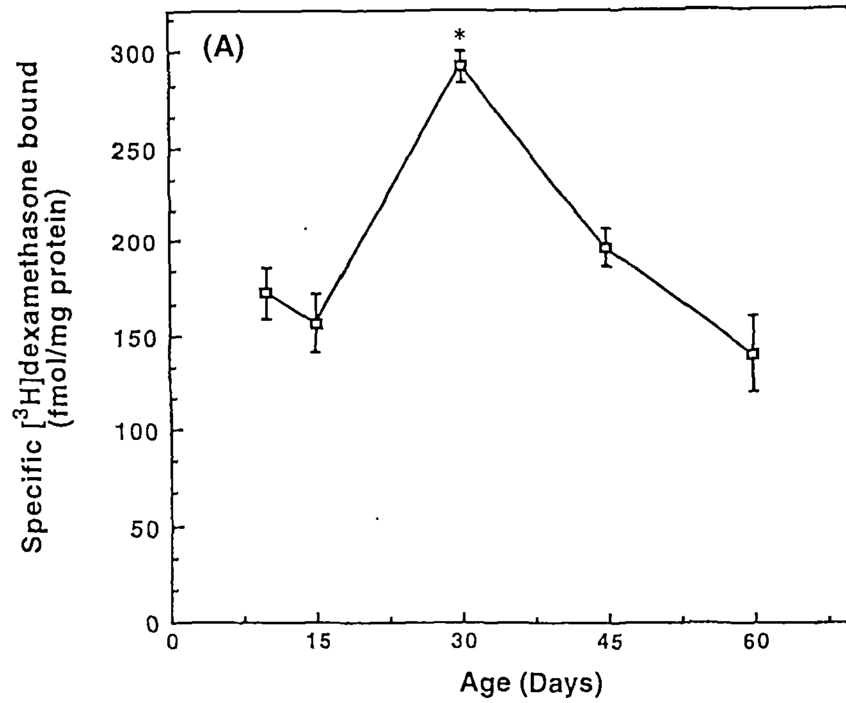


Fig. 2. Time course of [^{3}H]dexamethasone binding to hepatic glucocorticoid receptor. Cytosols were incubated with 40 nM [^{3}H]dexamethasone ± 500-fold excess cold dexamethasone at 2°C. At the indicated intervals of time, specific binding was determined as mentioned in the Materials and Methods section. Each data point is the mean of 4 separate sets of experiments.



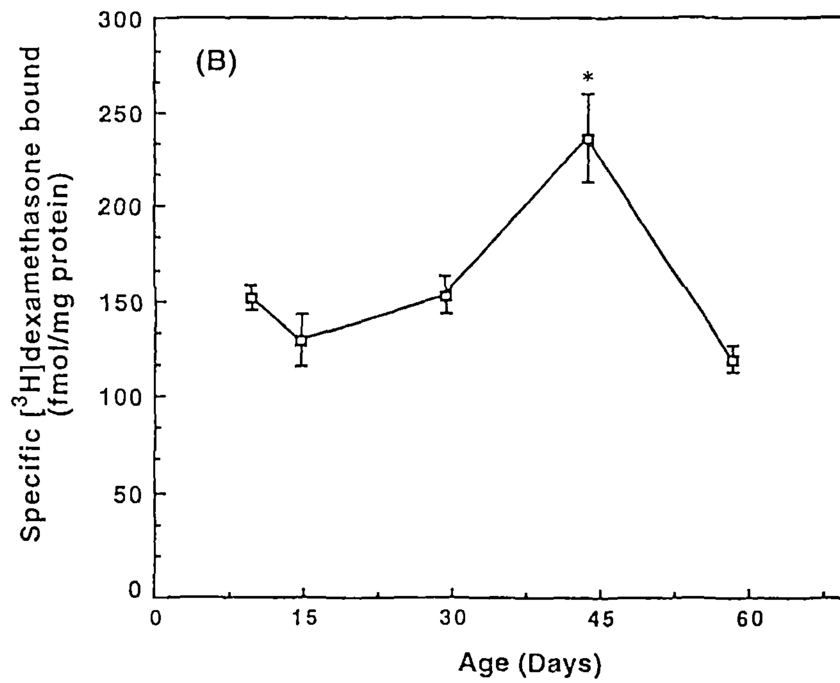
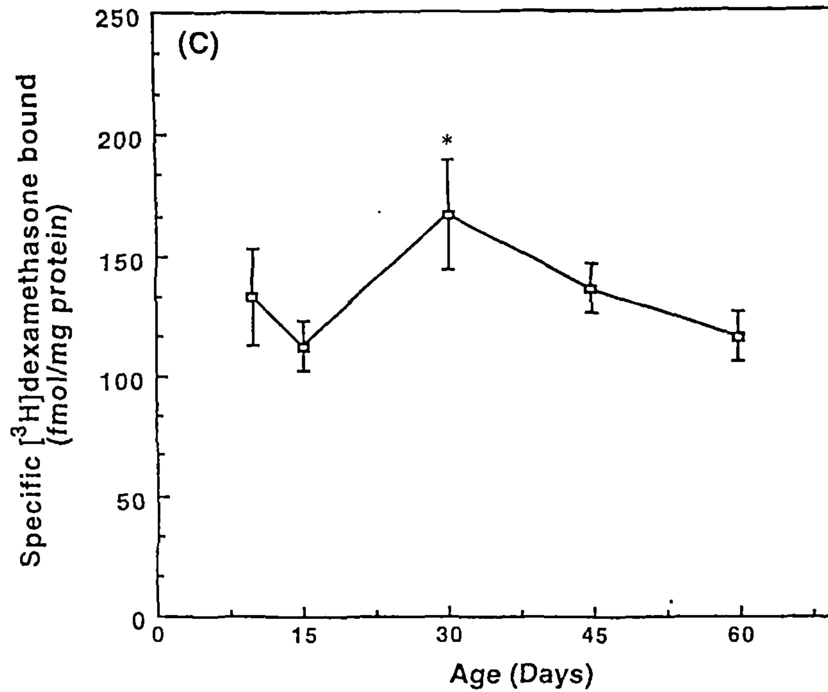


Fig. 3. Specific saturable binding of [^{3}H]dexamethasone in various tissues of mice at different postnatal ages. Tissue fractionation and receptor assay conditions are as described in the Materials and Methods section. Values are mean ± standard deviation from 4-5 mice of each age group. Liver (A) and Kidney (B).

* statistically significant as compared to other age groups



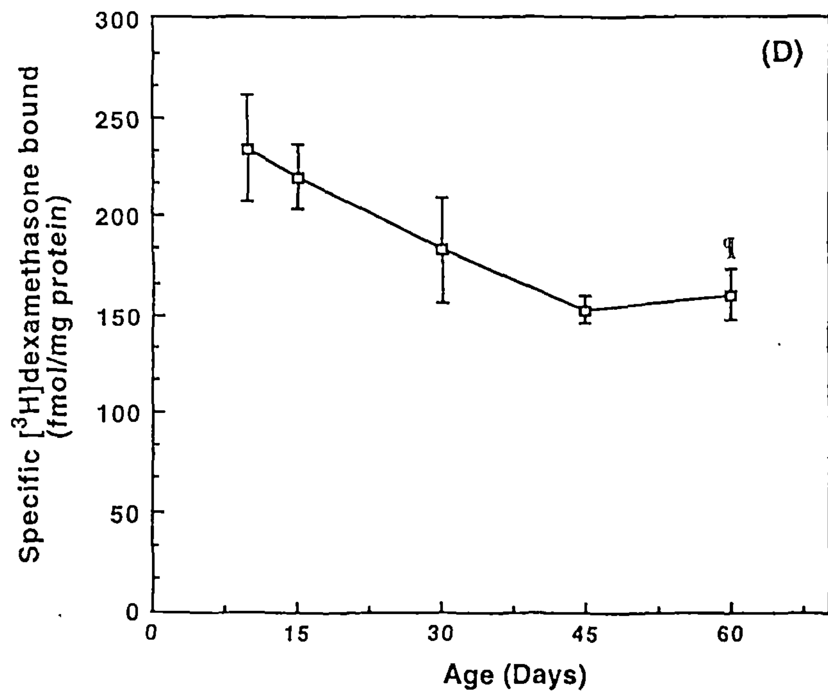


Fig. 3. Specific saturable binding of [^{3}H]dexamethasone in various tissues of mice at different postnatal ages. Tissue fractionation and receptor assay conditions are as described in the Materials and Methods section. Values are mean ± standard deviation from 4-5 mice of each age group. Cardiac muscle (C) and Skeletal muscle (D).

* *statistically significant ($p<0.05$) as compared to15- and 60-day age groups*

¶ statistically significant ($p<0.05$) as compared to 10-day only

the change in receptor concentration at various ages seen here are not as significant as in the case of liver. In contrast to the above observations, the **skeletal muscle** glucocorticoid receptor concentration is high in the early postnatal age (10-day). Thereafter, the receptor concentration shows a gradual decline upto day 45 and remains unchanged at day 60, where the value is significantly lower than that of day 10 (Fig. 3D). A similar trend in the receptor concentration values is observed in the **cerebral hemispheres** of mice, except that the receptor concentration is quite low at all the ages studied (Fig. 3E). Moreover, the decrease is noticeable upto day 30 and remains unchanged thereafter till day 60 of the postnatal age. The glucocorticoid receptor concentration in different tissues at various postnatal ages are also summarized in Table I.

Scatchard analysis

Scatchard analysis of the binding data was performed in the liver and kidney of mice at two age groups, where the change in receptor concentration was maximum. Scatchard plot of the binding data in **liver** reveals no difference in the slopes of the linear-regressed plots obtained from 15- and 30-day old mice (Fig. 4A). This shows that the dissociation constant (K_d), the value of which is ~2.5 nM remains unchanged at these two ages. However, the intercept of the plots on the x-axis showed that the value of specific binding sites in 30-day old mice (310 fmol/ mg protein) is significantly higher than that at day 15 (180 fmol/ mg protein). In the **kidney**, scatchard data from 15- and 45-day old mice, revealed no age-associated change in the dissociation constant value, which is ~2.9 nM for both the age groups studied (Fig. 4B). However, as seen in the liver, here too the specific binding sites at day 45 (236 fmol/ mg protein) is significantly higher than that at day 15 (180 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 pre-weaned (10-day) and post-weaned (60-day), to see changes if any, that occur in the activation properties during the developmental period of mice. 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

In the **liver**, the magnitude of activation of [^{3}H]dexamethasone-receptor complexes by heat (25°C for 45 min) are significantly higher (~3-fold) as compared to the unactivated receptor complexes, incubated at 0°C for 45 min. However, the degree of

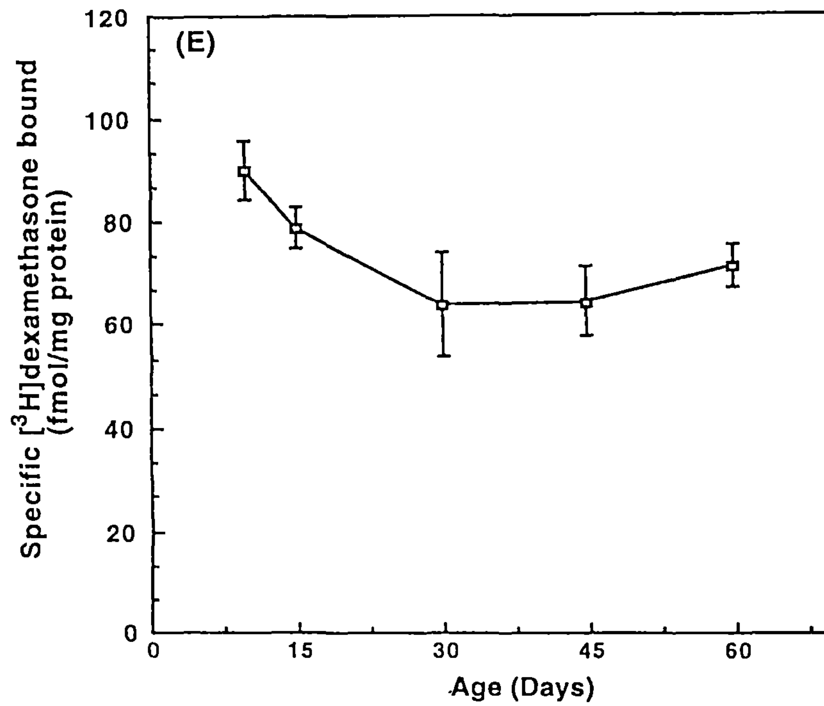


Fig. 3. Specific saturable binding of [^{3}H]dexamethasone in cerebral hemispheres of mice at different postnatal ages. Tissue fractionation and receptor assay conditions are as described in the Materials and Methods section. Values are mean ± standard deviation from 4-5 mice of each age group.

* statistically significant ($p<0.05$) as compared 10-day value

Table I

Level of Glucocorticoid Receptor at various postnatal ages of mice

Tissue	*Age (Days)* 10	15	30	45	60
Liver	174±13	157±15	293±10 *	197±10	140±20
Kidney	152±7	130±13	153±10	237±23 *	120±7
Cardiac muscle	132±20	112±11	166±23 ¶	135±11	115±10
Skeletal muscle	232±27	218±17	182±26	152±7	159±13 †
Cerebral hemispheres	90±6	79±4	64±10	64±7	72±4 †

Glucocorticoid 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 15- and 60-day only

† statistically significant ($p<0.05$) as compared to 10-day only

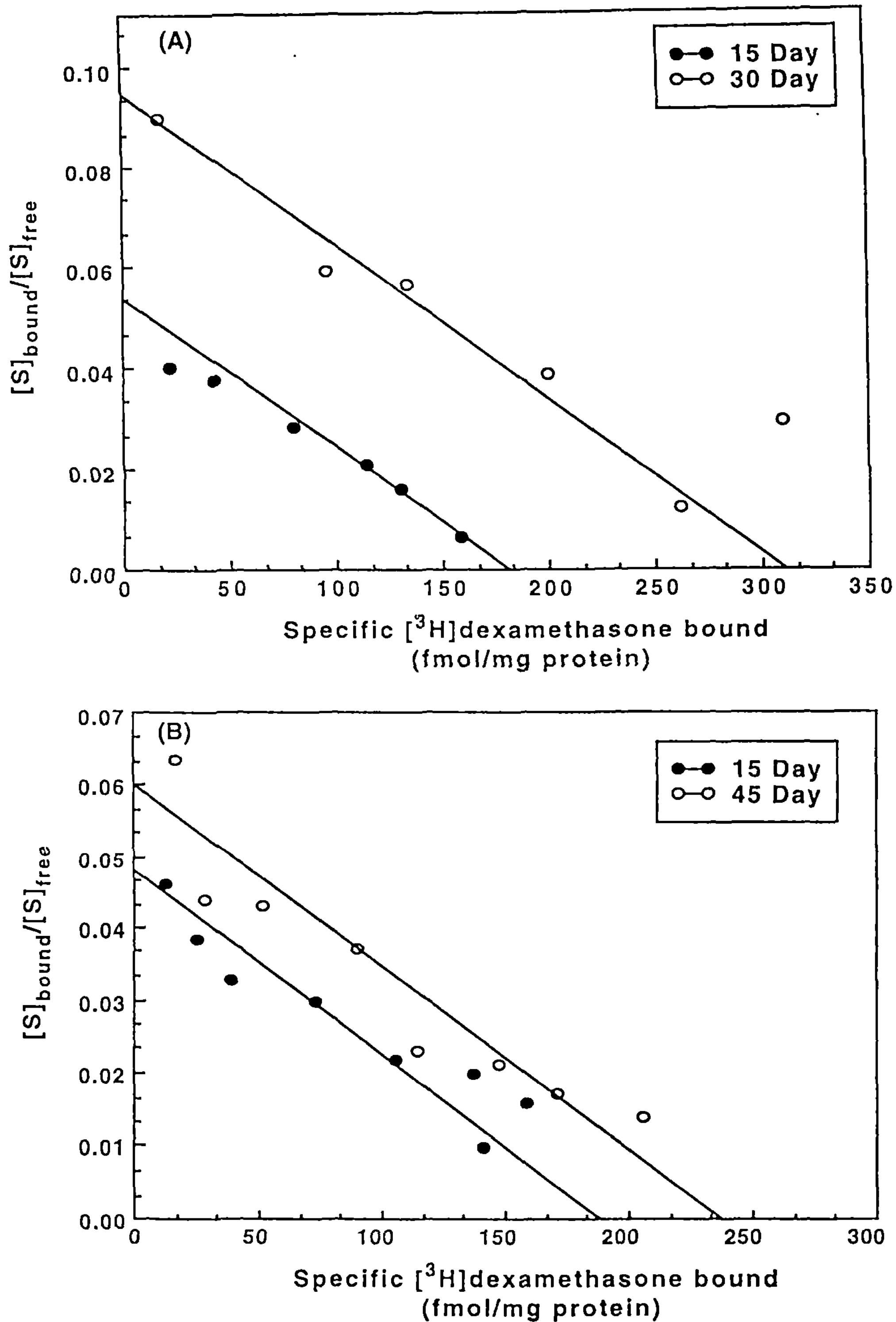


Fig. 4. Scatchard plot of the [^{3}H]dexamethasone binding data. Cytosols from liver (A) and kidney (B) were incubated with 2.5-120 nM [^{3}H]dexamethasone ± 500-fold excess cold dexamethasone for 4 hr at 2°C. Specific binding at each concentration point was calculated as the difference between total 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 value (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 3-4 mice of each age group. $[S]_{bound}$, the amount of specifically bound [^{3}H]dexamethasone; $[S]_{free}$, the amount of free [^{3}H]dexamethasone in the assay medium.

Table II

Concentration and affinity of [^{3}H]Dexamethasone-receptors in the liver and kidney of mice

Parameters	Liver		Kidney	
	15-day	30-day	15-day	45-day
Number of specific binding sites (fmol/mg protein)	180±6.3	310±9.5*	180±5.4	236±8.2*
Dissociation constant, Kd (nM)	2.5±0.3	2.3±0.3	2.9±0.2	2.9±0.4

Data were collected from three sets of experiments with pooled tissues from 4-5 mice of each age group and analysed using scatchard plot as given in the Materials and Methods section. Values are mean±standard deviation.

* statistically significant ($p<0.01$) as compared to the other age group

thermal activation is similar for hepatic glucocorticoid receptors obtained from 10- and 60-day old mice. In the case of low temperature, Ca^{2+}-mediated activation too, the magnitude of activation is similar for both the age groups studied (Fig. 5A).

In the **kidney**, the degree of thermal- as well as Ca^{2+}-mediated activation processes were significantly higher (~2.7-fold) as compared to the unactivated hormone-receptor complexes. But, no differences were observed in the degree of activation between the age groups studied (Fig. 5B).

Nuclear binding assay

As observed in the case of DNA-cellulose binding studies in the **liver**, the degree of thermal activation (25°C for 45 min) of the [^{3}H]dexamethasone-receptor complexes is ~2.5-fold higher than the unactivated receptor complexes (0°C for 45 min) in the 10-day old mice. But, in contrast to DNA-cellulose binding assay in the 60-day mice, the magnitude of thermal activation is low (~1.3-fold) as compared to unactivated receptors from the same age group. However, the low temperature, Ca^{2+}-mediated activation of 10- and 60-day liver glucocorticoid receptors is similar (Fig. 6A). Nuclear exchange assay, where the thermally activated receptors from 60-day were allowed to interact with the nuclei of 10-day and *vice versa*, showed a significantly higher binding by the hepatic nuclei of 10-day as compared to those from 60-day mice (Fig. 6A).

In **kidney**, the pattern of nuclear binding by thermally activated receptor complexes is similar to that observed in liver. The binding of heat activated [^{3}H]dexamethasone-receptor complexes is approximately 3-fold higher for 10-day and 1.7-fold higher for 60-day as compared to the binding by their respective unactivated receptor complexes. In contrast to the finding in liver, the Ca^{2+}-mediated activation too, is higher in the kidney of 10-day as compared to 60-day mice. Also the nuclear exchange assay showed a pattern similar to that observed in the case of liver (Fig. 6B).

DNase I digestion assay

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 10-day as compared to 60 day nuclei. These findings indicated a change in nuclear property(s) that contributed to the higher binding of hormone-receptor complexes in immature mice. 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 extracted from the nuclei. This will reflect the change in chromatin organization that might be responsible for the observed changes in the nuclear binding at the two ages. DNase I digestion of

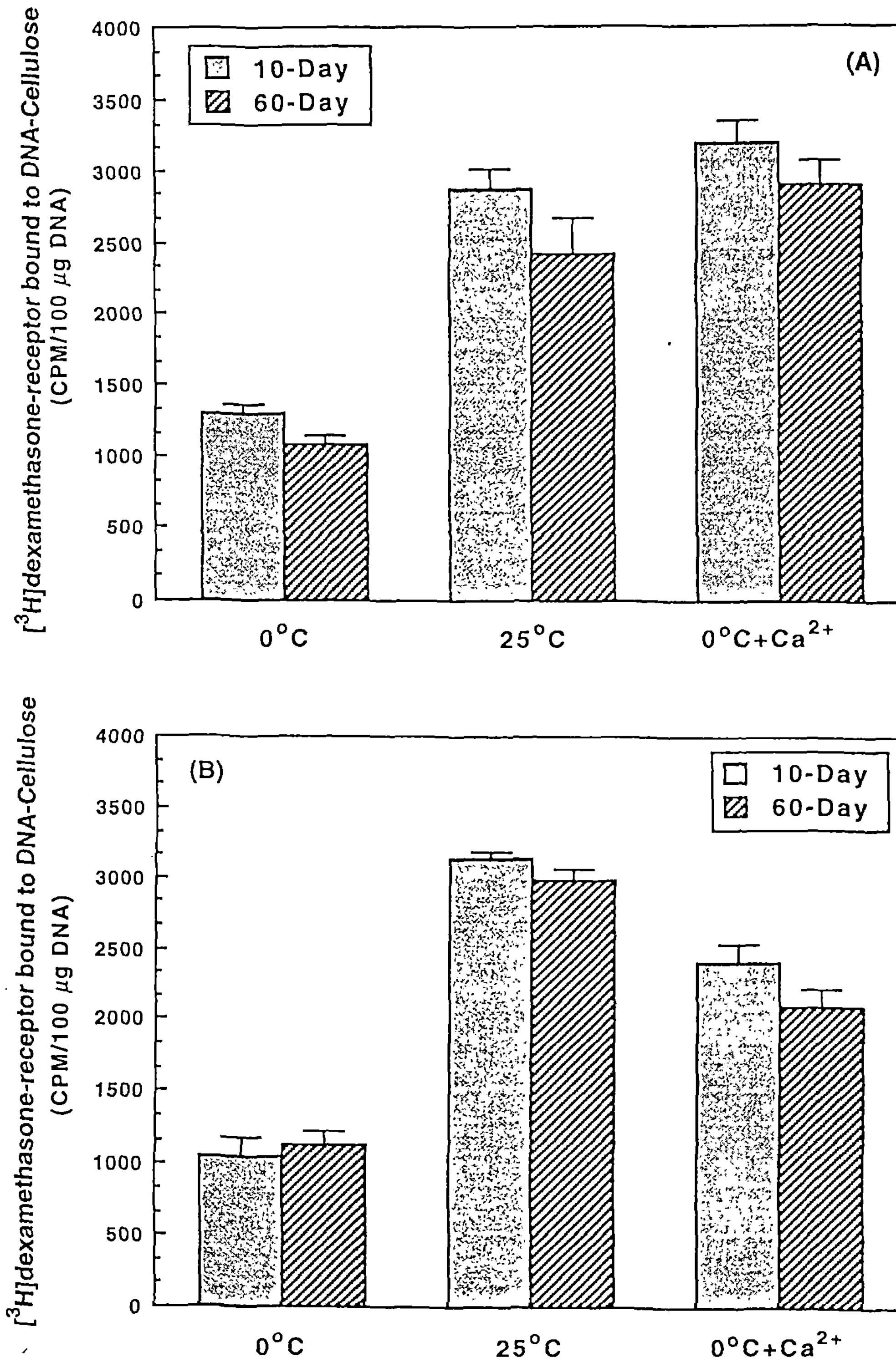


Fig. 5. **Binding of [^{3}H]dexamethasone-receptor complexes to DNA-cellulose.** Cytosols from liver (A) and kidney (B) were prepared in a buffer without molybdate and the hormone-receptor complexes obtained by incubating with 40 nM [^{3}H]dexamethasone for 4 hr at 2°C. Activation, binding to DNA-cellulose and furthur processing of the pellets were done as described in the text. The results are mean ± standard deviation of 3 experiments, each set done in triplicate with pooled tissues from 3-4 mice of same age-group.

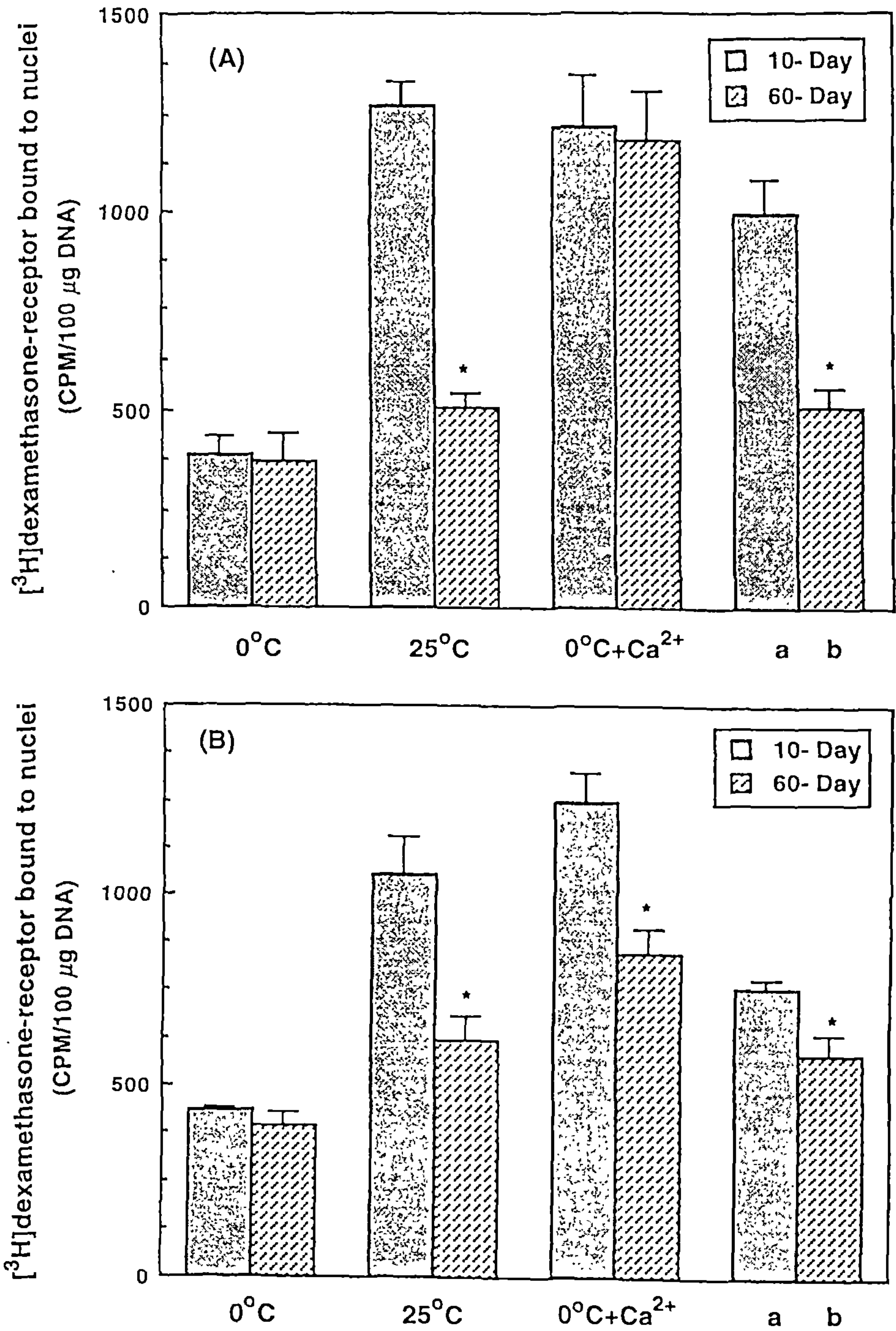


Fig. 6. Binding of [^{3}H]dexamethasone-receptors to purified nuclei. The hormone-receptor complexes were prepared and nuclear binding of the receptors activated by different means was performed in the liver (A) and kidney (B). Details of processing and determination of the bound complexes are as described in the Materials and Methods section. The **a** and **b** barograms in the above figures, represent nuclear-exchange assay in which 25°C activated receptor complexes from 60-day mice were incubated with 10-day old mice nuclei *(a)* and vice versa *(b)*. The values are mean ± standard deviation for 3 sets of experiment, performed each time with tissues from 3-4 mice of the same age-group.

* statistically significant ($p<0.05$) with respect to the other age-group.

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 10-day with respect to 60-day old mice. The degree of extraction of the nuclear bound receptors in the **liver** is 70% (10-day) and 46% (60-day) as compared to their respective controls taken as 100% (Fig. 7A). In the **kidney** too, the percentage of extraction was observed to be 63% for 10-day and 43% for 60-day mice (Fig. 7B).

Salt extraction of the bound hormone-receptor complexes

Salt-dependent release of thermally activated, nuclear bound [^{3}H]dexamethasone-receptor complexes from hepatic nuclei showed no age-associated variations as depicted in Fig. 8. The percent extraction of the complexes in both the ages were similar at the various concentrations of sodium chloride used. As compared to the control, 50% extraction occurred at ~0.11 M NaCl. Moreover, almost 65% extraction of the bound complexes, from both the ages occurred at 0.2 M NaCl and thereafter the degree of extraction was lowered so much so that ~70-80% extraction was possible at a very high salt concentration of 0.5 M NaCl.

Inhibition of activation studies

The *in vitro* activation process could be inhibited by the use of known inhibitors of this process, molybdate, tungstate and N-ethylmaleimide. These substances lowered the thermal activation (25°C for 45 min) significantly in both liver and kidney, as judged by DNA-cellulose and nuclear binding assays. The percentage of inhibition, with respect to the activated (non-inhibited receptor complexes taken as 100 %) hormone-receptor complexes, ranged from 40-60% and showed no differences in the two ages of mice, 10- and 60-day (Fig. 9 & 11). In the case of salt-mediated (Ca^{2+}, 0°C for 45 min) activation process, where molybdate and tungstate only were used as inhibitors, data obtained did not reveal any tissue- and age-specific difference in the magnitude of inhibition. Moreover, the percentage of inhibition, compared to the uninhibited control, revealed a pattern similar to that observed in the liver (Fig. 10 & 12).

Studies on the physical parameters of glucocorticoid receptor

To study some of the physical characteristics of the unactivated as well as activated glucocorticoid receptor from immature (10-day) and mature (60-day) mice, gel filtration analyses, anion-exchange chromatography and immunological studies were performed only on the receptors obtained from the liver of mice.

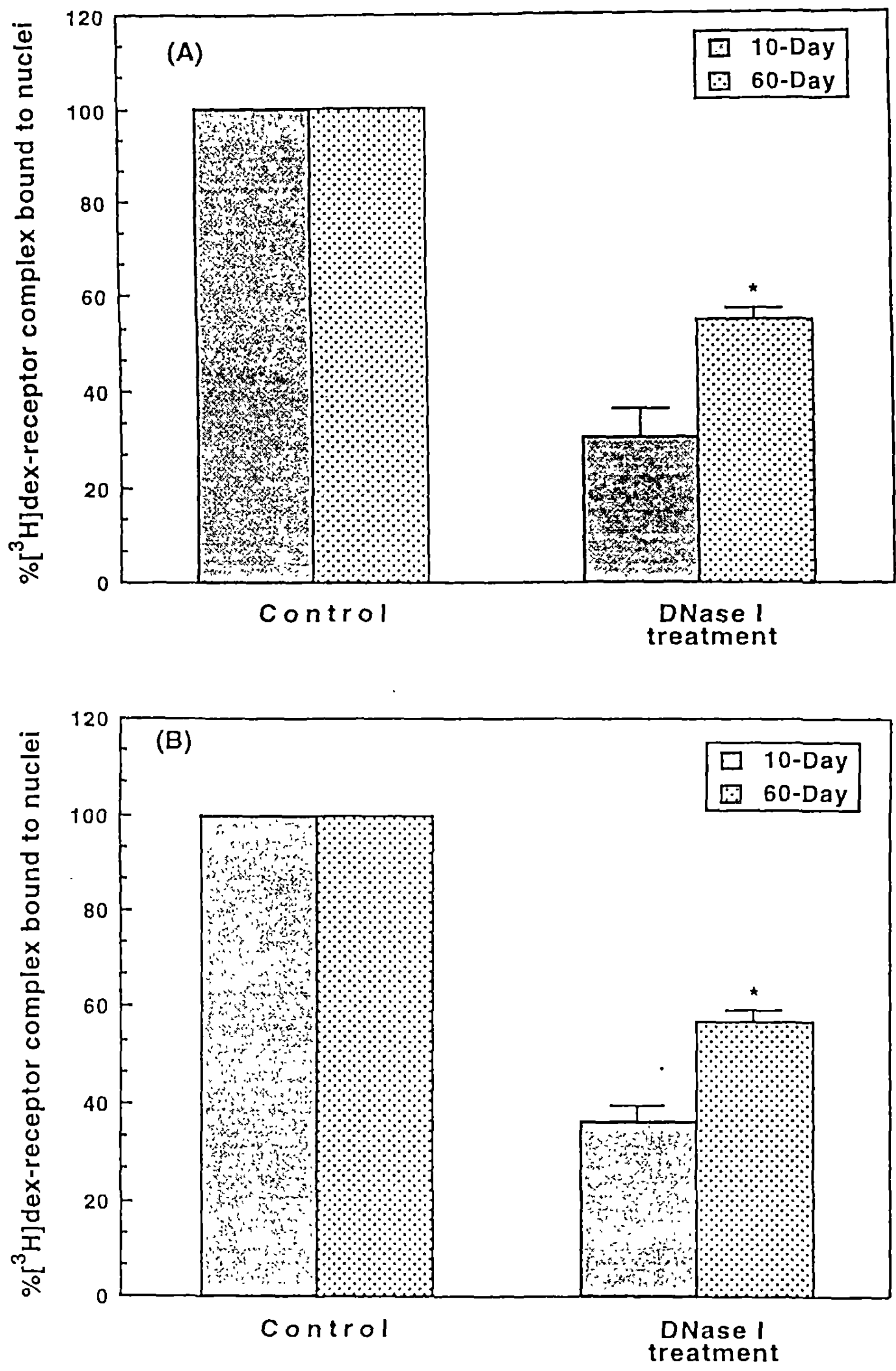


Fig. 7. Extraction of the nuclear bound [^{3}H]dexamethasone-receptor complexes by DNase I. Thermally activated, nuclear bound hormone-receptor complexes were extracted using DNase I (150 μg/100 μg DNA) in the liver (A) and kidney (B), for 45 min at 2°C. Details of the procedure and determination of the bound radioactivity are given in the text. The values are mean ± standard deviation of 3 experiments. * statistically significant with respect to the other age-group.

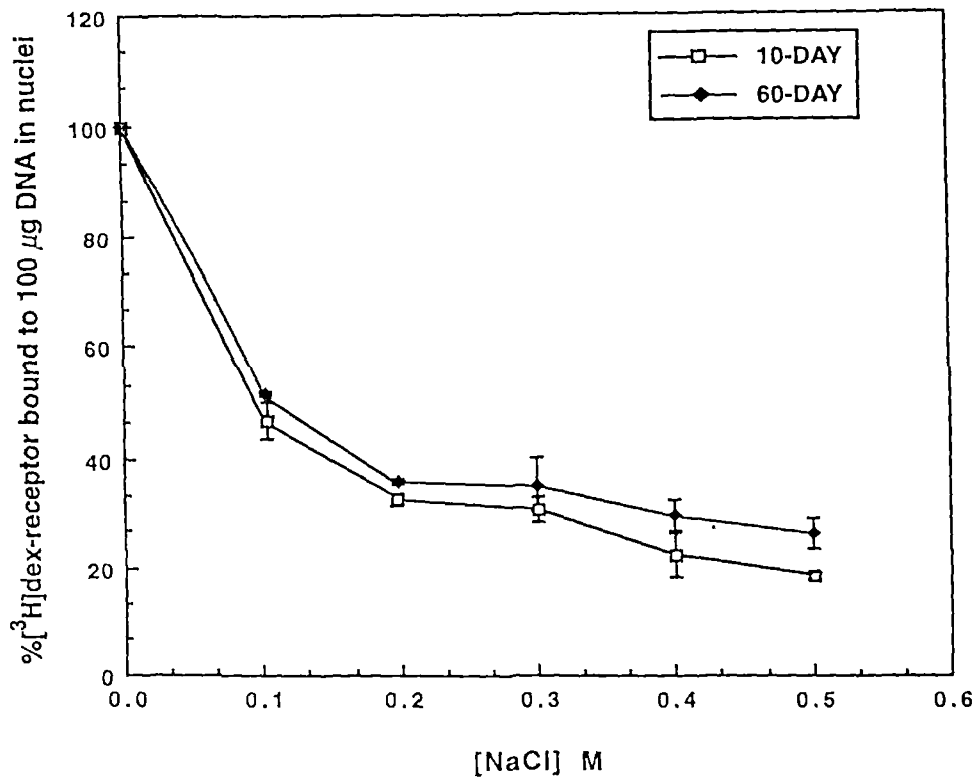


Fig. 8. Extraction of the nuclear bound [^{3}H]dexamethasone-receptor complexes by salt. Thermally activated, hepatic nuclear bound receptor complexes were extracted using increasing concentrations of NaCl, for 45 min at 2°C. Details of the proedure are as mentioned in the Materials and Methods section. The values are mean ± standard deviation of three experiments.

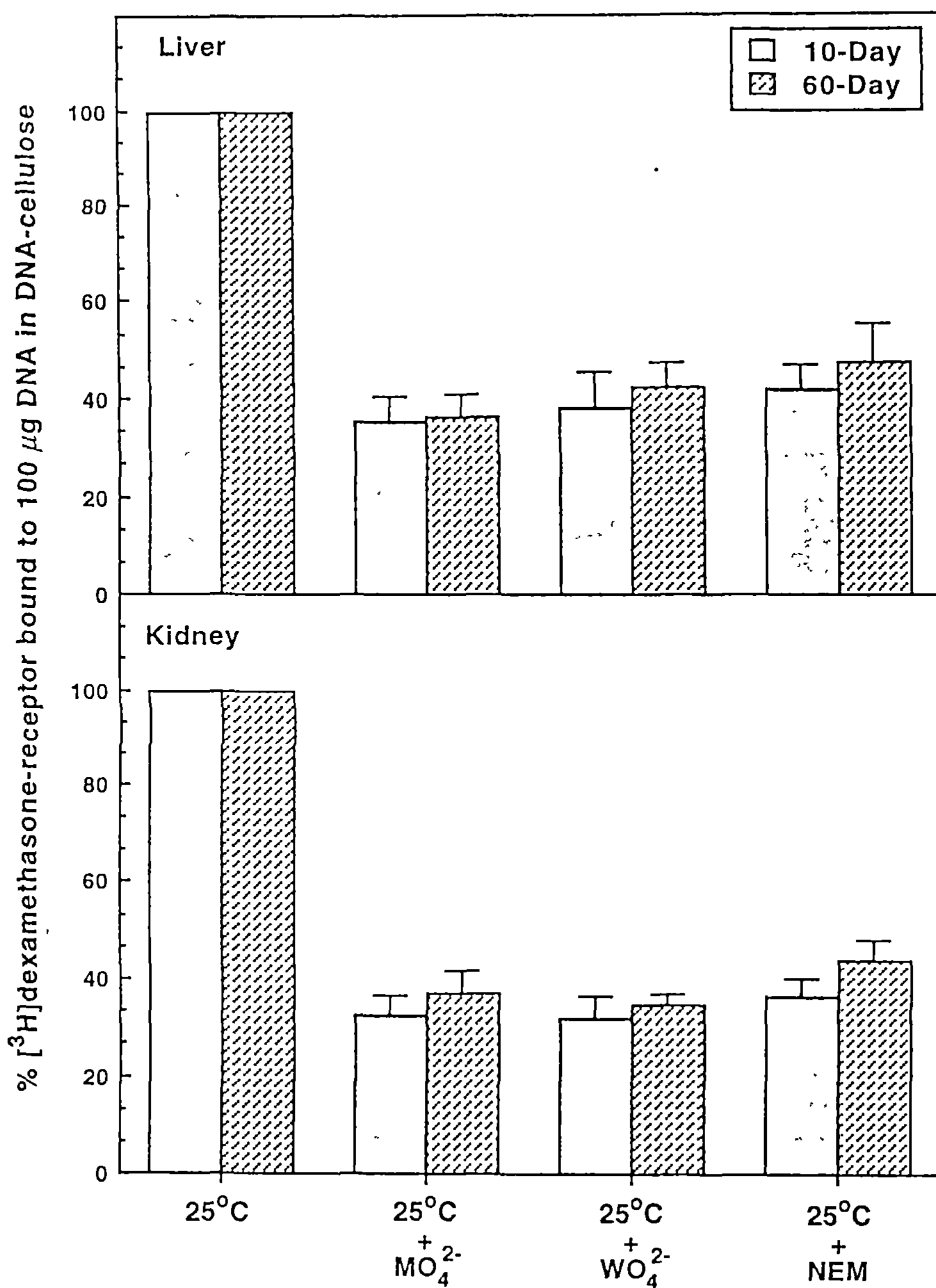


Fig. 9. Effect of various inhibitors on the thermal activation of glucocorticoid receptors. Freshly prepared cytosols from liver and kidney were complexed with 40 nM [^{3}H]dexamethasone for 4 hr at 2°C. After DCC treatment, the cytosols were divided into aliquots and activated, either in absence or in presence of 20 mM of inhibitors, for 45 min at 25°C. The extent of activation was then determined using DNA-cellulose, taking the binding of receptors activated in absence of inhibitors as 100%. Details of the assay procedure are as mentioned in the Materials and Methods section. The inhibitors used were sodium molybdate (MO_4^{2-}), sodium tungstate (WO_4^{2-}) and N-ethylmaleimide (NEM). The results are mean ± standard deviation of three experiments.

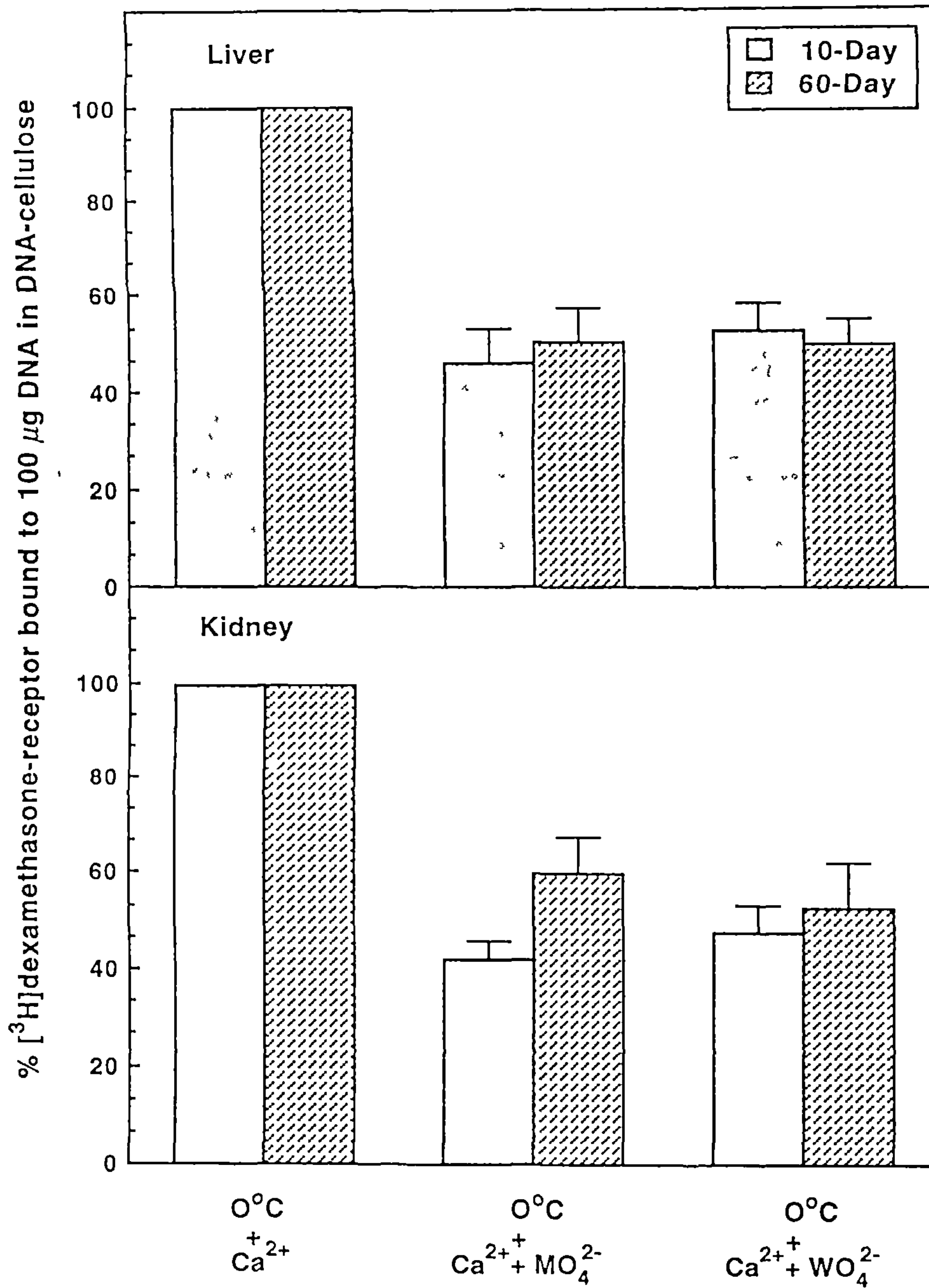


Fig. 10. Effect of inhibitors on the salt activation of glucocorticoid receptors Liver and kidney cytosols were incubated with 40 nM [^{3}H]dexamethasone for 4 hr at 2°C and then treated with DCC Aliquots of the cytosol were activated with 20 mM Ca^{2+} for 45 min at 0°C either in absence or in presence of 20 mM of the inhibitors, sodium molybdate (MO_4^{2}) and sodium tungstate (WO_4^{2}) The percentage of activation was determined by binding to DNA-cellulose, taking as 100% the binding by receptors activated in absence of inhibitors Details of the assay procedure are given in the text The values are mean ± standard deviation for three experiments

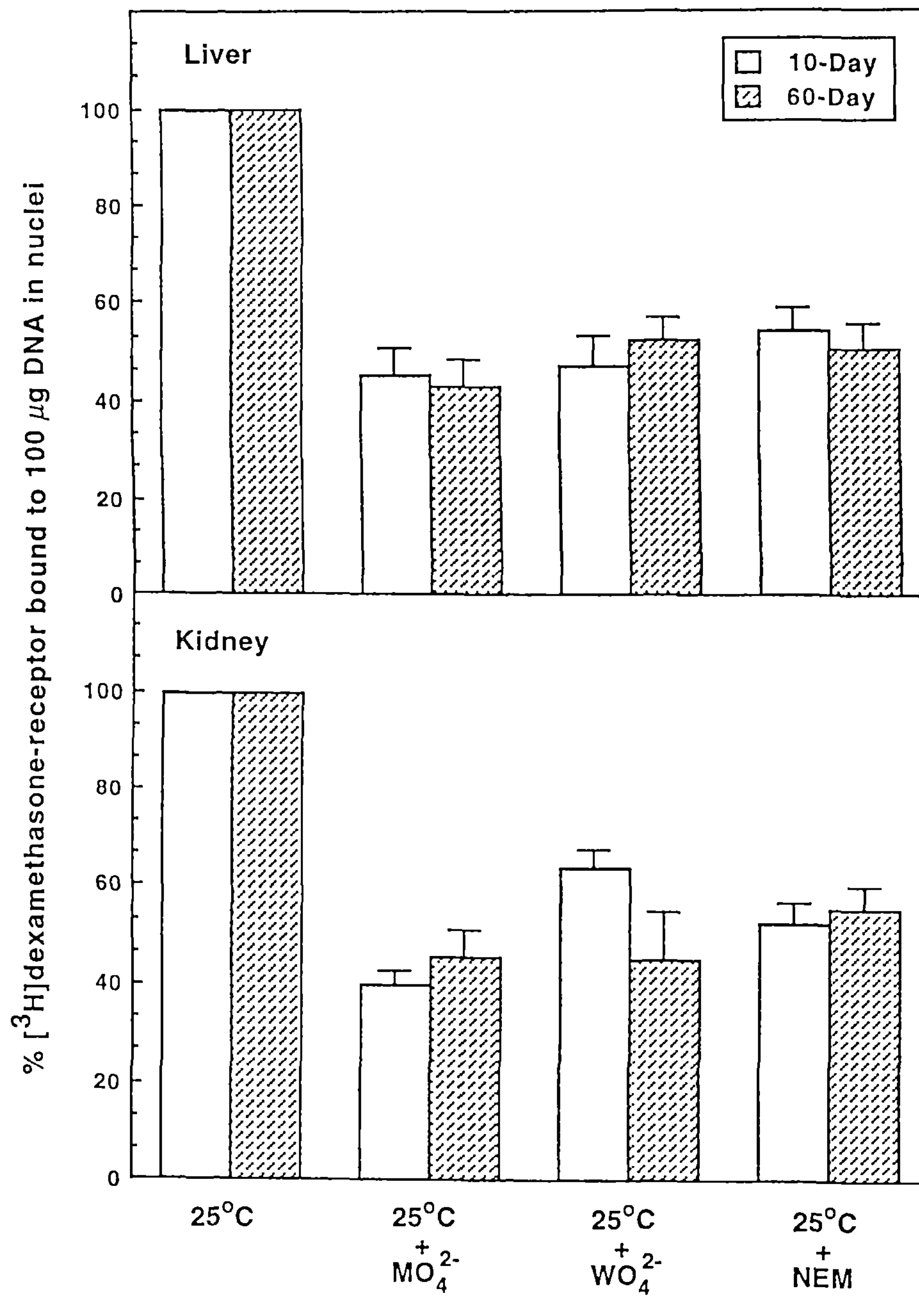


Fig. 11. Effect of inhibitors on the thermal activation of glucocorticoid receptors. Glucocorticoid receptor complexes from liver and kidney were generated by incubating cytosols with 40 nM [^{3}H]dexamethasone for 4 hr at 2°C. Activation of the hormone-receptor complexes were performed as given in Fig. 9. The magnitude of activation in absence and in presence of the inhibitors was determined using nuclear binding assay as described in the Materials and Methods section. The values depicted represent the mean ± standard deviation of 3 separate experiments.

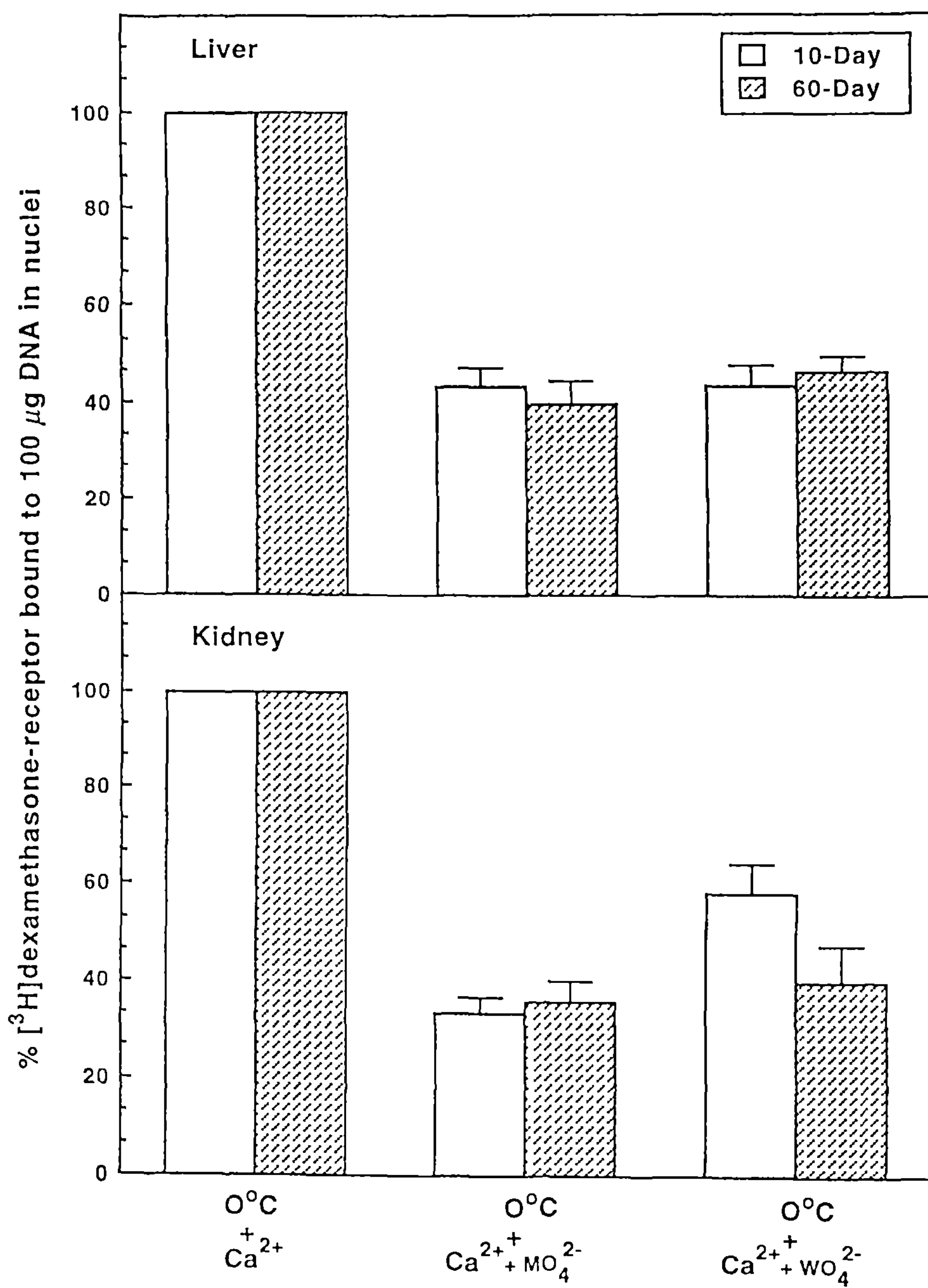


Fig. 12. Effect of inhibitors on the salt activation of glucocorticoid receptors [^{3}H]dexamethasone recptor complexes from the liver (A) and kidney (B) were generated and activated using Ca^{2+}, in absence or in presence of inhibitors as mentioned in the legend to Fig 10 Nuclear binding assay was used to determine the percentage of activation, details of the procedure are described in the text Results are mean ± standard deviation of 3 experiments

Gel filtration analyses

Fig. 13. depicts the elution profile of the **unactivated** [^{3}H]dexamethasone-receptor complexes analysed on a sephadex G-200 column. The receptors from both the ages (10- and 60-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 the molecular weight and the stokes radii (R_S) were calculated. The plot of log M vs. V_e/ V_o gave a molecular weight of 285 kDa and 283 kDa for 10- and 60-day receptor, respectively (Fig. 14). The Porath plot of $M^{1/3}$ vs. $K_d^{1/3}$ gave the values 306 kDa and 304 kDa for the receptors of the two ages (Fig. 15). The stokes radii were calculated from a plot of -(log K_{av})$^{1/3}$ vs. R_S and calculated to be 5.81 and 5.76 nm for the receptors of 10- and 60-day, respectively (Fig. 16). From the plot of $K_d^{1/3}$ vs. R_S, the values were calculated to be 5.86 and 5.93 nm for the two ages (Fig. 17).

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. 18). 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 weights were calculated to be 92 kDa (10-day) and 88 kDa (60-day) (Fig. 19), while the plot of $M^{1/3}$ vs. $K_d^{1/3}$ gave the values as 89 kDa (10-day) and 87 kDa (60-day) (Fig. 20). The stokes radii, calculated from the plot of -(log K_{av})$^{1/3}$ vs. R_S gave the values as 3.55 nm (10-day) and 3.52 nm (60-day) (Fig. 21). The values of stokes radii, 3.65 and 3.67 nm, for 10- and 60-day liver receptor, respectively were obtained from the plot of $K_d^{1/3}$ vs. Rs (Fig. 22).

Ion-exchange analyses

To study the charge content of the unactivated as well as activated [^{3}H]dexamethasone-receptor complexes from liver of 10- and 60-day mice, ion-exchange analyses were performed. The elution profile of the unactivated receptors, shown in Fig. 23A. does not reveal any age-related differences 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 ~240 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 ~240 mM of KCl (Fig. 23B). 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. However, data did not reveal any differences in these parameters in the two ages of mice.

The data obtained from the above studies are also presented in a tabular form in Table III.

Immunological studies

Studies were performed to see differences if any, in the immunological epitopes of the glucocorticoid receptor with development. Our data showed that the antibody against rat glucocorticoid receptor was able to recognize the mice glucocorticoid receptor. The amount of unactivated [^{3}H]dexamethasone-receptor complexes immunoadsorbed by protein A-sepharose remained almost similar in the liver of 10- and 60-day old mice (Fig. 24). Data revealed no differences in these properties at the two ages studied.

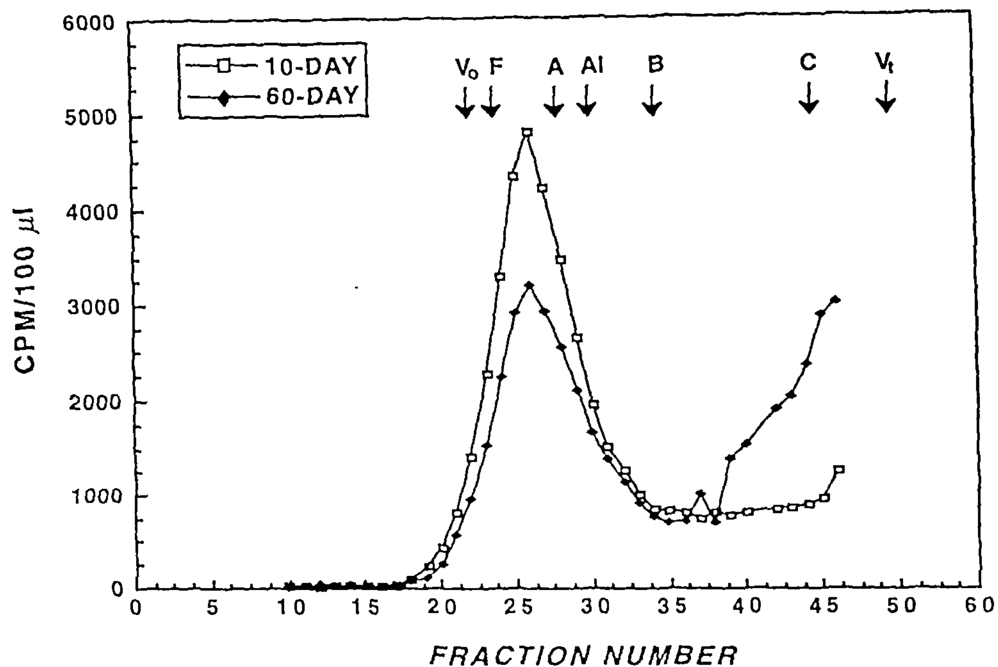


Fig. 13. Gel permeation column chromatography of hepatic unactivated glucocorticoid receptors. A column (1.8 x 45 cm) of sephadex G-200, equlibrated with buffer (100 mM potassium phosphate, pH 7.5/ 0.25 M sucrose/ 1 mM EDTA/ 1 mM mercaptoethanol/ 20 mM sodium molybdate) at 2-4°C was used. Cytosol prepared in buffer containing molybdate was made 40 nM [^{3}H]dexamethasone and incubated for 4 hr at 2°C. After DCC treatment, 2 ml of the cytosol was loaded onto the column and eluted with the above buffer. 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; A, β-amylase; Al, aldolase; B, bovine serum albumin and C, cytochromé C. V_0 and V_t represent the elution volume of blue dextran and [^{3}H]dexamethasone, respectively. Each point in the elution profile represent the mean value of three experiments.

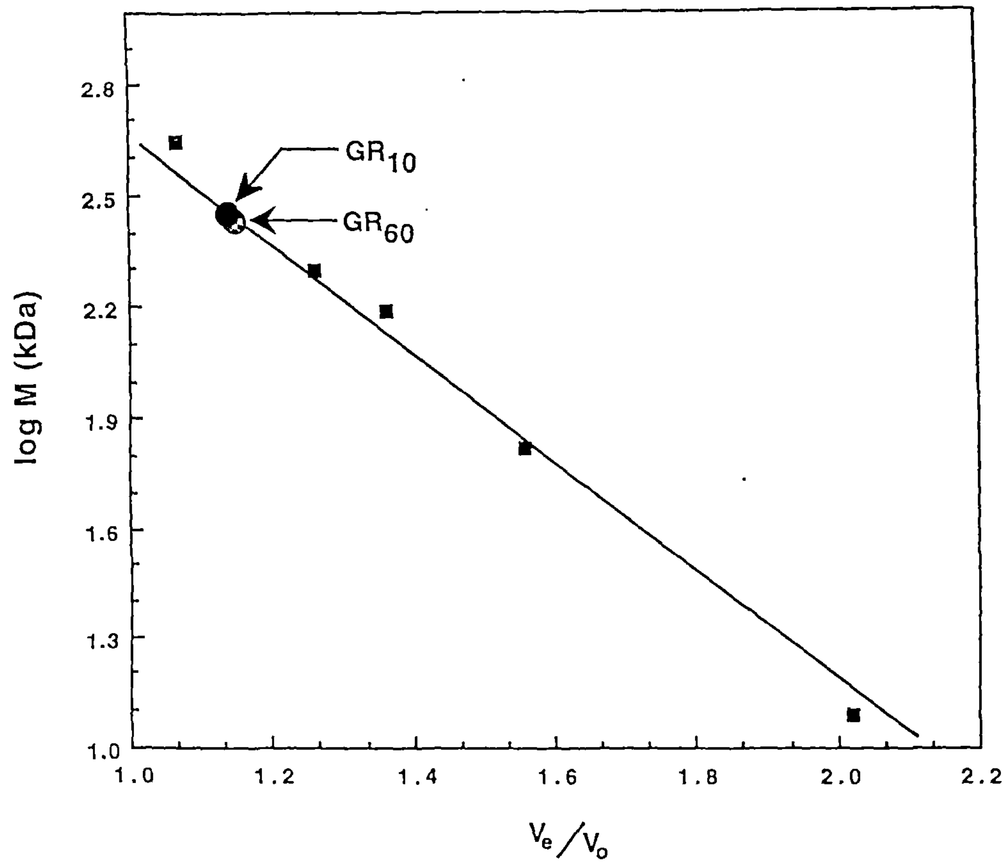


Fig. 14. Plot of log M vs. V_e/V_o for the determination of molecular weight of unactivated receptors. The data from sephadex G-200 gel chromatography were plotted to obtain a linear-regressed curve. The standard protein markers used were ferritin (443 kDa), β-amylase (200 kDa), aldolase (156 kDa), bovine serum albumin (67 kDa) and cytochrome C (12.5 kDa). GR_{10} and GR_{60} represent the positions of unactivated hepatic glucocorticoid receptors from 10- and 60-day old mice, respectively. The data presented is the mean of three independent experiments.

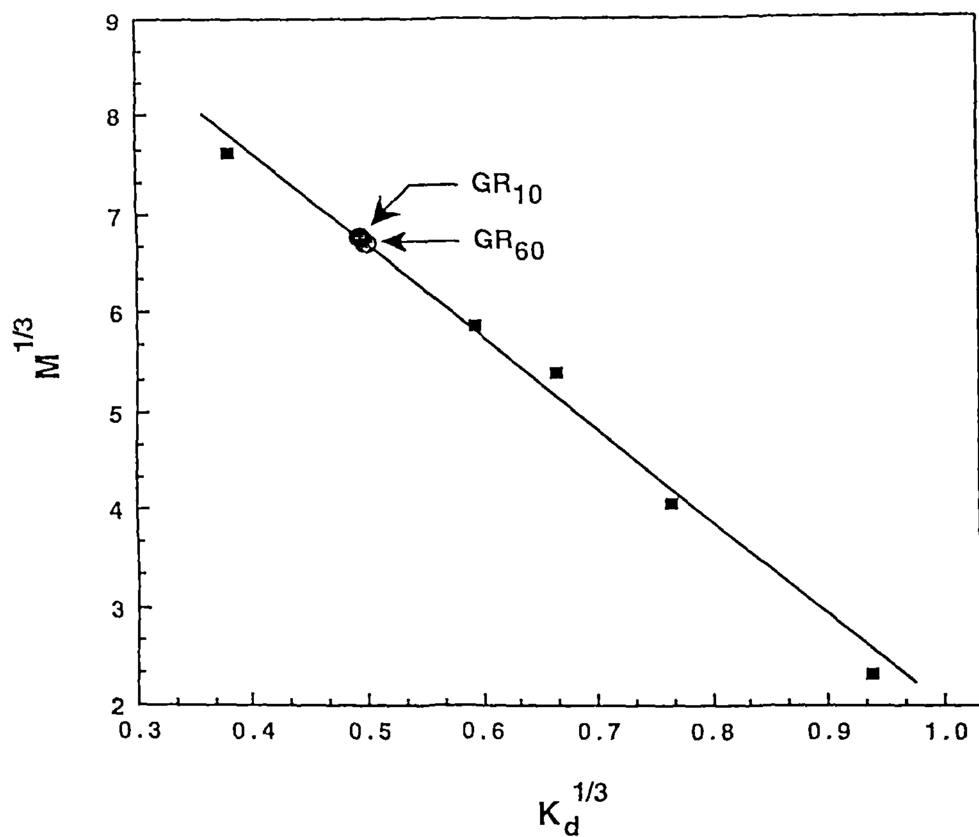


Fig. 15. **Porath plot of $M^{1/3}$ vs. $K_d^{1/3}$ for the determination of molecular weight of unactivated receptors.** Data from sephadex G-200 chromatography were used to generate the linear-regressed plot. Standard marker proteins were the same as mentioned in the legend to Fig. 14. The position of the receptors from the liver of 10- and 60-day old mice are indicated as GR_{10} and GR_{60}, respectively. The values are mean of 3 experiments.

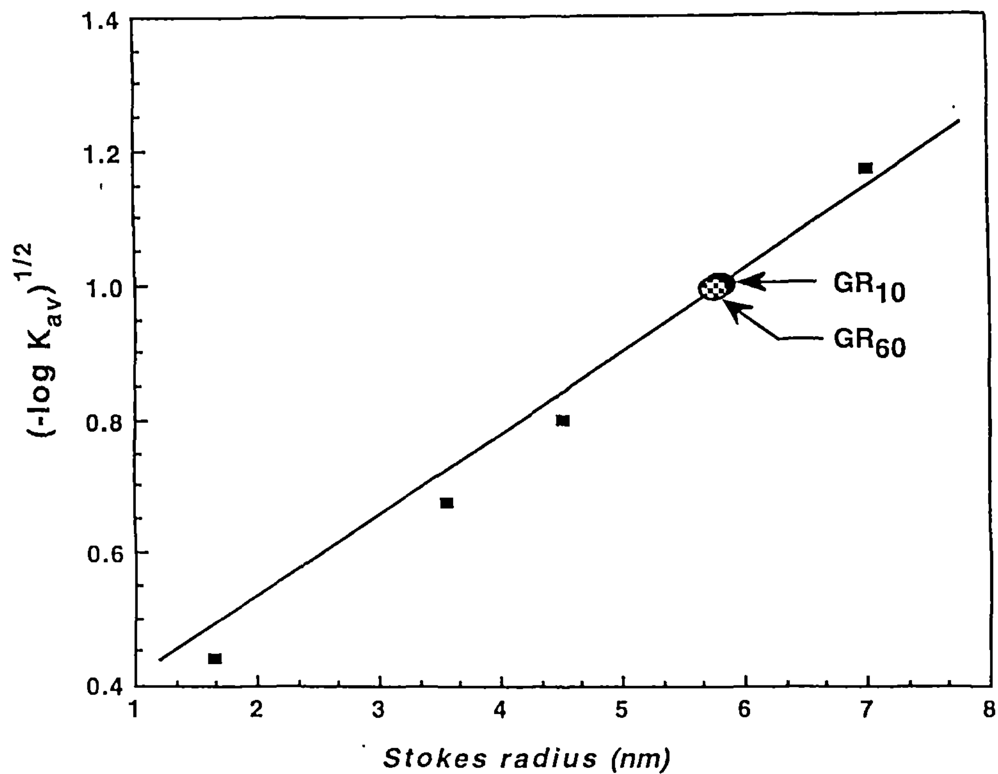


Fig. 16. Plot of $(-\log K_{av})^{1/3}$ vs. R_s for the determination of stokes radii of unactivated receptors. The plot was generated using data from G-200 gel chromatography. The molecular markers used were ferritin (7.0 nm), aldolase (4.5 nm), bovine serum albumin (3.55 nm) and cytochrome C (1.64 nm). The positions of the receptor from the two ages are indicated as GR_{10} and GR_{60}, respectively. The values are mean of 3 experiments.

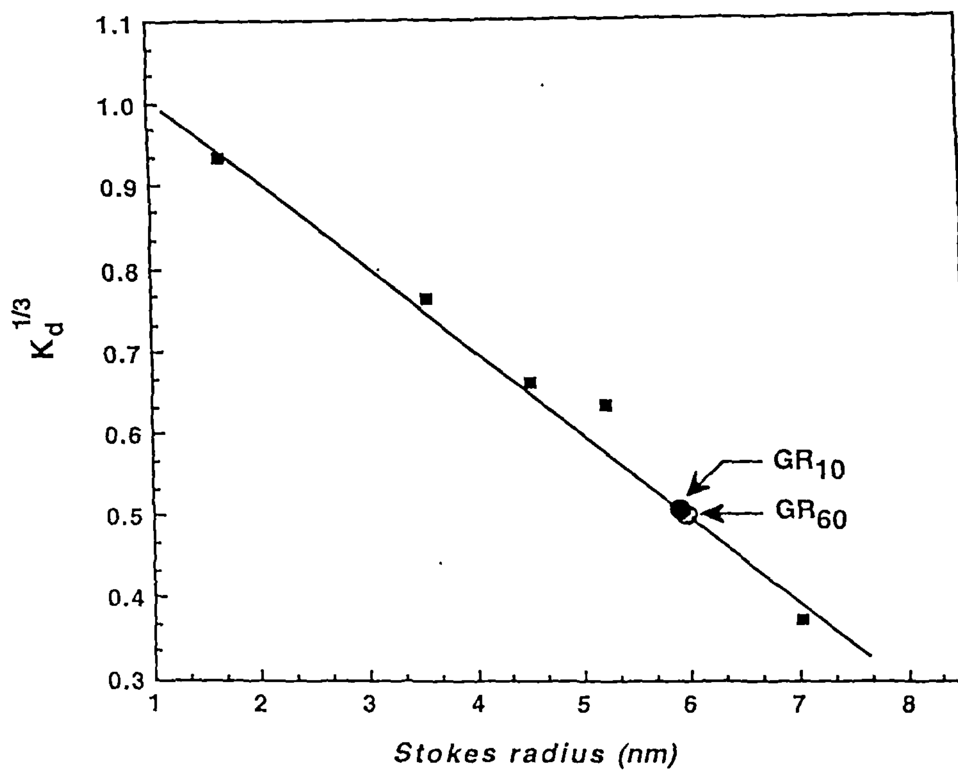


Fig. 17. Plot of $K_d^{1/3}$ vs. R_s for the determination of stokes radii of unactivated receptors. Sephadex G-200 gel chromatography data were used to obtain the plot. Standard markers and other details are the same as given in the legend to Fig. 16.

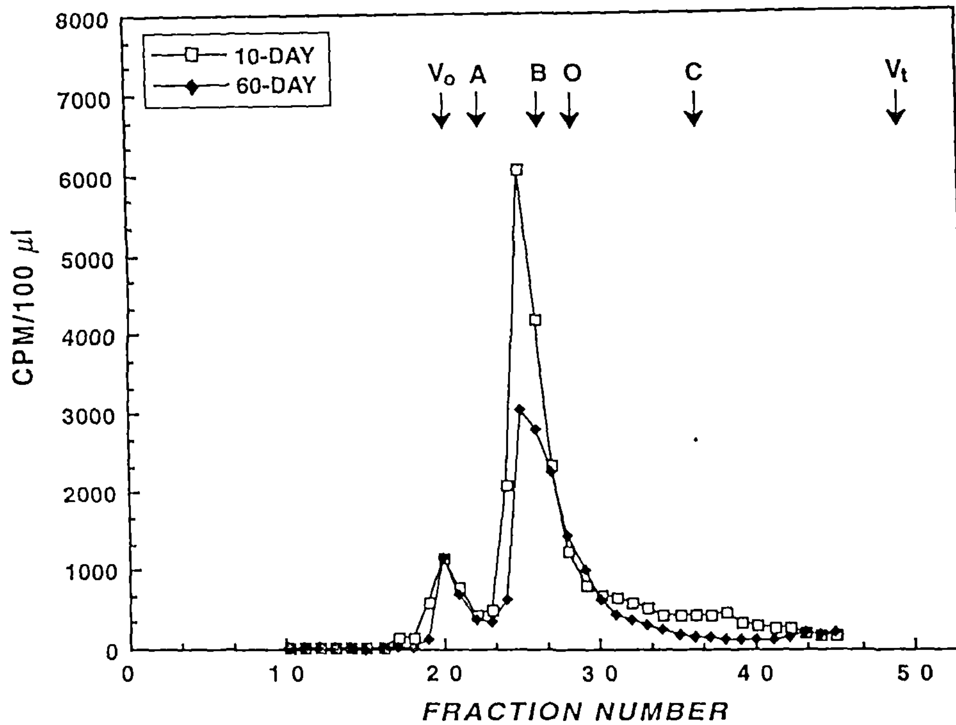


Fig. 18. Gel filtration column chromatography of activated glucocorticoid receptors from the liver of mice. A column (1.8 x 42 cm) of sephadex G-100, equlibrated with the buffer containing 300 mM KCl, at 2-4°C was used. After DCC treatment, the hormone-receptor complexes were activated at 25°C for 45 and 2 ml of it loaded onto the column and eluted with the above buffer. Fractions of 2 ml each were collected and 100 μl from each counted for bound radioactivity. Standard protein markers used were alcohol dehydrogenase (156 kDA); bovine serum albumin (67 kDa); ovalbumin (45 kDa) and cytochrome C (12.5 kDa). V_0 and V_t represent the elution volume of blue dextran and [^{3}H]dexamethasone, respectively. Each point in the elution profile represent the mean value of three experiments.

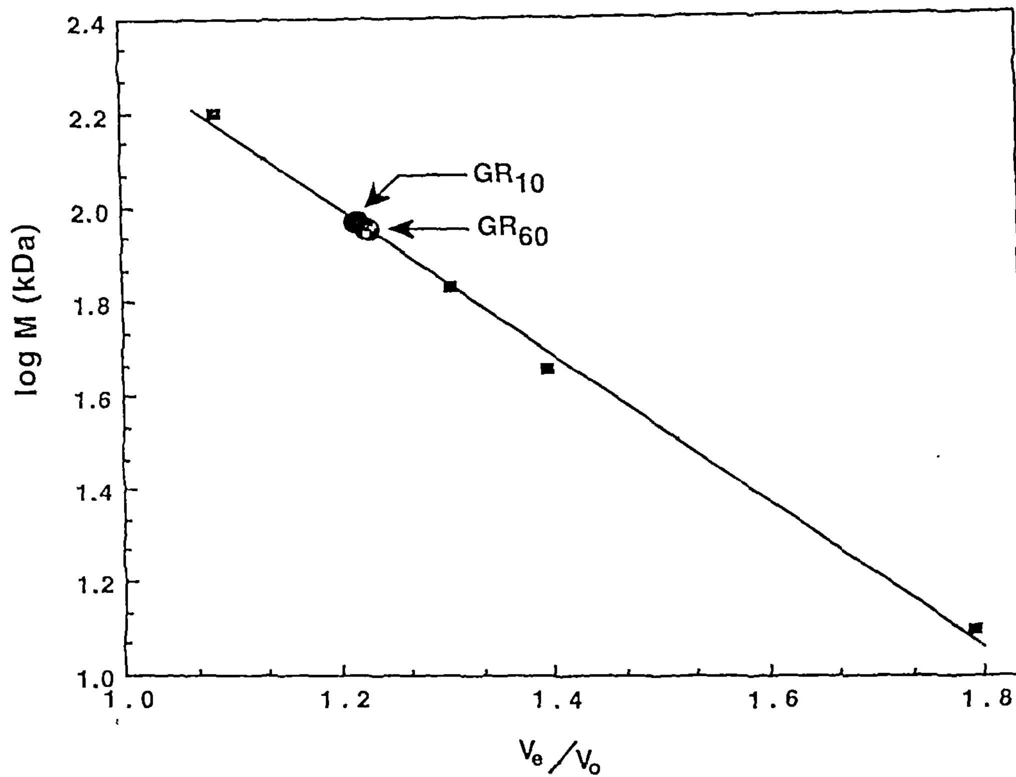


Fig. 19. Plot of log M vs. V_e/V_o for the determination of molecular weight of activated receptors. Sephadex G-100 gel chromatography data were plotted to obtain a linear-regressed curve. The standard protein markers used were alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (46 kDa) and cytochrome C (12.5 kDa). GR_{10} and GR_{60} represent the positions of activated hepatic glucocorticoid receptors from 10- and 60-day old mice, respectively. The data presented is the mean of three independent experiments.

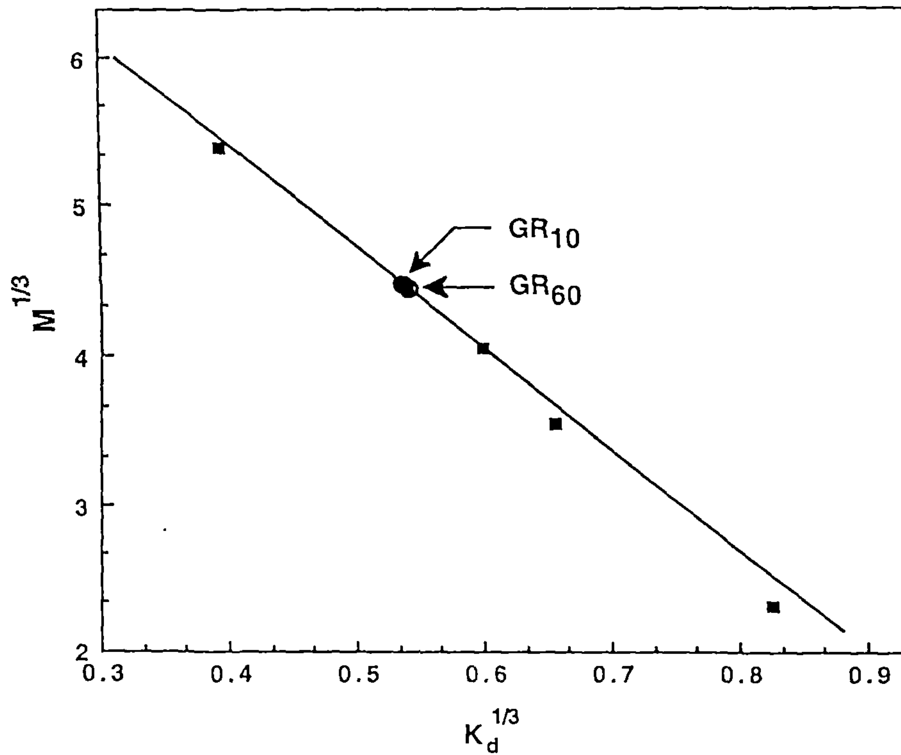


Fig. 20. **Porath plot of $M^{1/3}$ vs. $K_d^{1/3}$ for the determination of molecular weight of activated receptors.** Data from sephadex G-100 gel chromatography were used to generate the linear-regressed plot. Standard marker proteins were the same as mentioned in the legend to Fig. 18. The position of the receptors from the liver of 10- and 60-day old mice are indicated as GR_{10} and GR_{60}, respectively. The values are mean of 3 experiments.

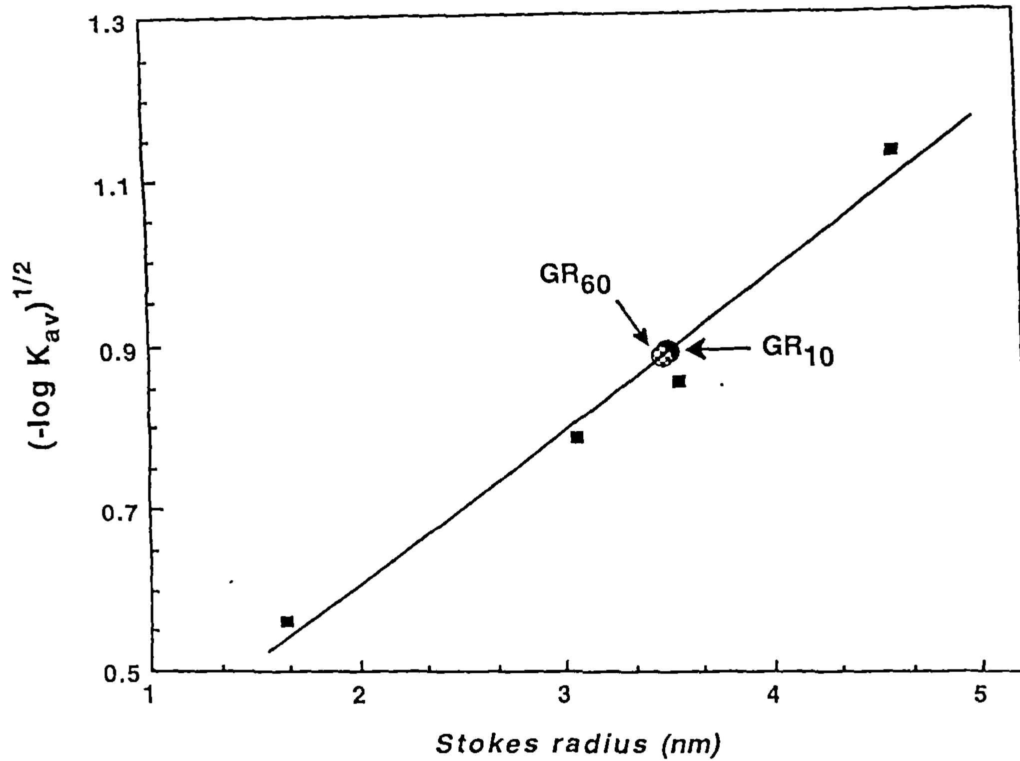


Fig. 21. Plot of $(-\log K_{av})^{1/3}$ vs. R_S for the determination of stokes radii of activated receptors. The plot was generated using data from G-100 gel chromatography. The molecular markers used were alcohol dehydrogenase (4.6 nm); bovine serum albumin (3.55 nm); ovalbumin (3.05 nm) and cytochrome C (1.64 nm). The positions of the receptor from the two ages is indicated by GR_{10} and GR_{60}, respectively. The values are mean of 3 experiments.

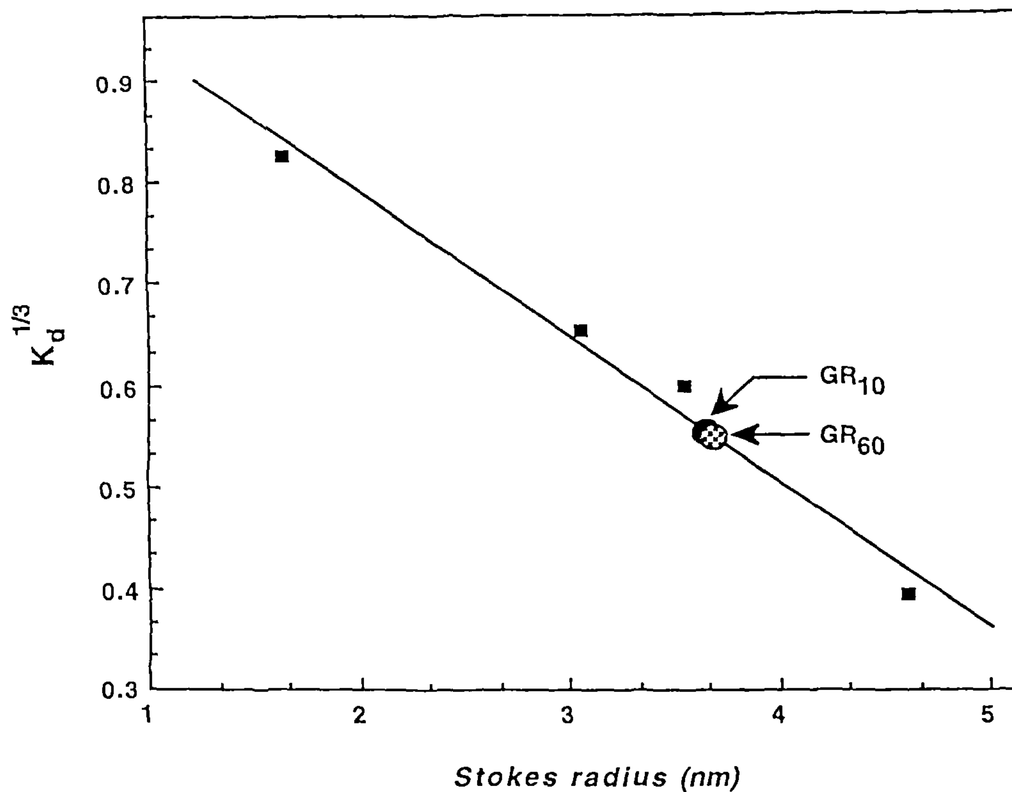


Fig. 22. Plot of $K_d^{1/3}$ vs. R_s for the determination of stokes radii of activated receptors. Sephadex G-100 gel chromatography data were used to obtain the plot. Standard markers and other details are the same as for the legend to Fig. 21.

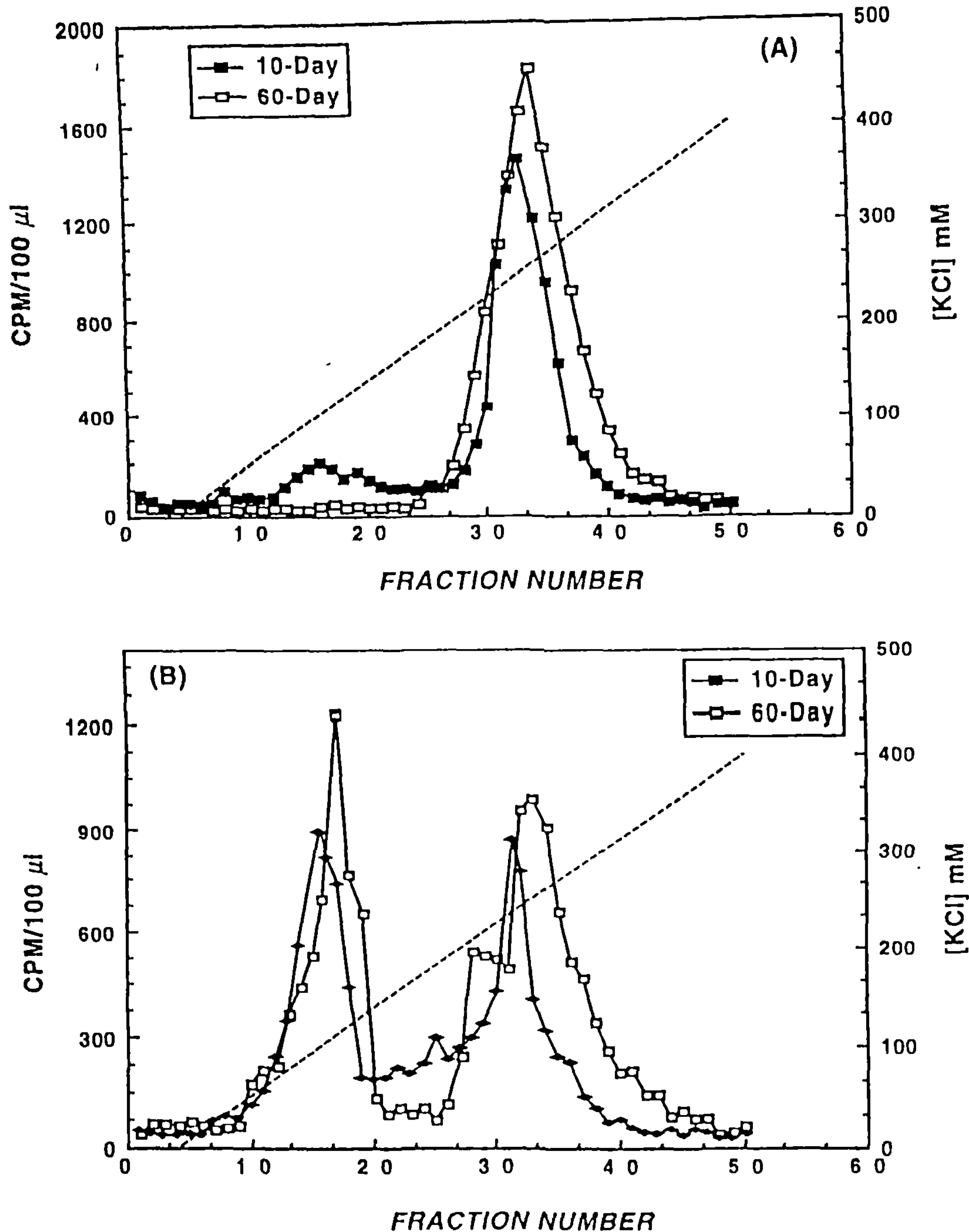


Fig 23. Elution profile of the glucocorticoid receptors from anion-exchanger DE-52. A 5 ml glass syringe containing 3 cm high gel-bed layered over a 2 mm thick DCC layer was used. The gel was equilibrated at 2-4°C with buffer (10 mM potassium phosphate, pH 7.5/ 1 mM β-mercaptoethanol/ 5 mM sodium molybdate). Unactivated and thermally activated [^{3}H]dexamethasone receptor complexes from liver were prepared as given in the Materials and Methods section and 500 μl loaded onto the column. After washing off the unbound proteins, the bound proteins were eluted with a salt gradient (0-400 mM KCl in the above buffer). 1 ml fractions were collected and 100 μl from each fraction counted for radioactivity. (A) elution profile of the unactivated receptors and (B) that of the activated receptors. Each point in the two profiles represent the mean of three experiments each.

Table III

Physicochemical properties of hepatic glucocorticoid receptor from immature (10-day) and mature (60-day) mice

Parameters	10-Day	60-Day
Unactivated receptor		
Molecular weight (kDa)	293±17	286±20
Stokes radius (nm)	5.85±0.04	5.84±0.09
Elution from DE-52 by KCl (mM)	245±5	252±4
Activated receptor		
Molecular weight (kDa)	90±2	88±1
Stokes radius (nm)	3.61±0.04	3.58±0.1
Elution from DE-52 by KCl (mM)	100±6	112±7

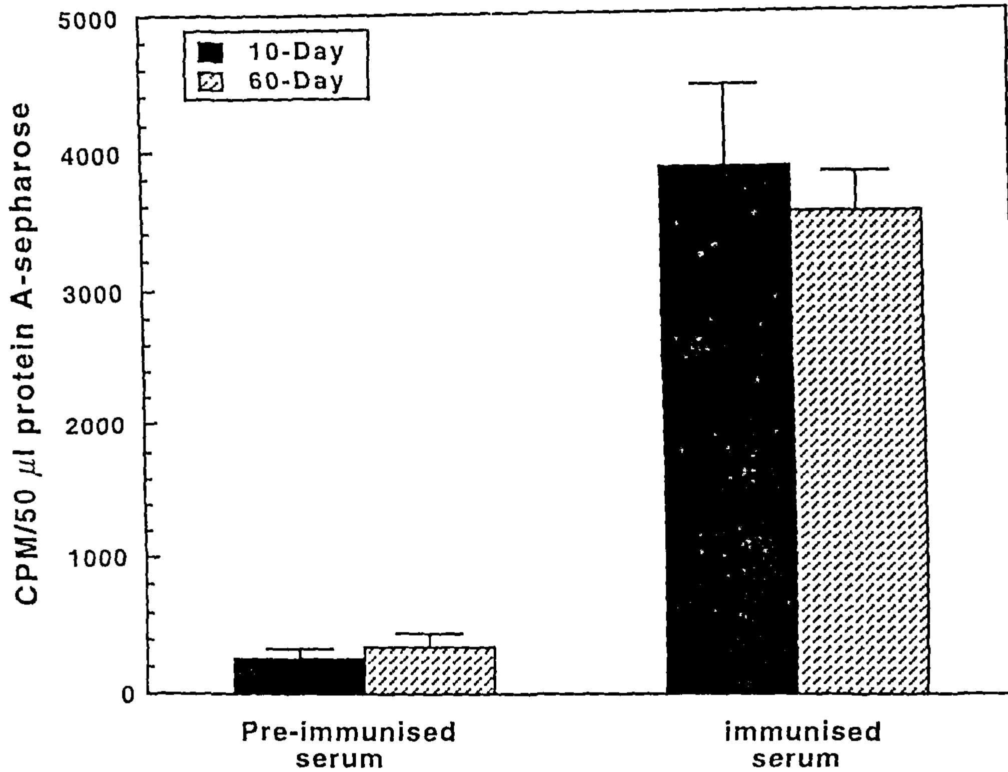


Fig. 24. Immunoadsorption of glucocorticoid receptors by protein A-sepharose. 200 μl of unactivated [^{3}H]dexamethasone receptor complexes from the liver of mice were incubated with 5 μl of the preimmunised or immunised serum for 18 hr at 2°C. To it 50 μl of protein A-sepharose was added and theincubation was allowed to occur for 1 hr at 2°C. At the end of this period sepharose was pelleted and washed thrice with buffer, the final pellets obtained were suspended in cocktail and bound radioactivity measured. The results are mean ± standard deviation of three separate experiments.

DISCUSSION

Development as a discrete period of time, is an intrinsic part of the lifespan in higher multicellular organisms. It is characterised by distinct set of events leading to the evolution of specialized functions that enable the organism to lead an independent existence and help in propagation of the species (Kanungo, 1994). A definite boundary to distinguish the various phases of lifespan is hard to define. However, it is safe to assume the developmental period as one which encompasses the total prenatal phase and extends to a considerable period of the postnatal life too, until the attainment of reproductive maturity in an organism. Most of the major morphological, physiological, biochemical and psychological changes take place during the developmental period and it is important to decipher these changes as they might have a profound role in the advent of senescence and aging in an organism. Senescence and aging are characteristic of all multicellular organisms, wherein the functional abilities of most bodily systems decline concurrent with a decrease in adaptability to internal and external stresses. Overall, the ability of an organism to maintain optimum homeostatic conditions declines gradually leading to an increased susceptibility to diseases and ultimately death (Timiras, 1988).

The path to proper development rests primarily on the messages encoded in the genetic material of an organism. During this period of lifespan, an intricate display of genes are seen, as some are switched on and others, switched off. The rate and duration of each of these processes dictate the production of a whole array of biomolecules necessary to support growth and development (Kanungo, 1994). There are a number of well-documented processes by which control of gene expression is achieved. One of the important means by which such a control is brought about is through chemical signals acting via cell-to-cell interactions. This involves the transmission of messages by molecules, known as hormones, acting through their cognate receptors in target cells (Zubay *et al.*, 1995). Information can thus, be transferred to the ultimate cellular control centres allowing a proper response to be generated. Moreover, during this phase of lifespan the organism is greatly influenced by stresses- ranging from changing environment to diet, and hormones provide a mechanism by which adaptation to these stresses is achieved. In addition, hormones play an important role in maintaining the overall homeostatic balance necessary for optimal bodily functions of the organism (Timiras, 1994).

Hormones in general influence the overall metabolic status in an animal. Since metabolic events that occur during development have been shown to influence the latter stages of lifespan, a detailed study of the hormones/ hormone receptors is necessary to better understand their role in development and subsequent aging process. Among the numerous hormones identified and studied so far, glucocorticoids appear important as its presence is required for proper fetal cellular and tissue differentiation. Also, glucocorticoids are responsible for the overall development as they regulate intermediate metabolism of glucose, the principal energy providing metabolite in higher organisms. In addition, glucocorticoids influence a number of physiologically important processes namely, suppression of immunologic and inflammatory response, general protein and amino acid metabolism and most importantly it acts as a `stress-hormone´, in response to stressful conditions (Norman and Litwack, 1987).

Glucocorticoids, secreted by the adrenocortical cells under the regulatory influence of ACTH, act on the target cells via the presence of specific, high-affinity intracellular proteins, called glucocorticoid receptors (GR). These receptors are located in the cytoplasm in a non-covalent association with a number of non-hormone binding proteins, forming a large multiprotein complex having a molecular mass of ~300 kDa and sedimentation coefficient of 9-10S. This complex consists of a molecule of the receptor, two molecules of hsp90, and a molecule each of hsp70, hsp56 and a 23 kDa acidic protein. The association of these molecules, primarily that of hsp90's, keeps the GR in a ligand-friendly conformation and also helps to repress the transactivation and nuclear localization signals present in the receptor molecule. Upon hormone binding, the protein complex dissociates due to a conformational change in the GR, a process termed transformation/ activation. The activated receptor has a molecular weight of ~94 kDa and sedimentation coefficient of 4-5S. Activation allows the hormone bound receptor to translocate to the nucleus where they bind directly to specific DNA sequences called GREs or interact indirectly with other nuclear proteins, both the processes leading to expression modulation of the cognate genes (Pratt, 1993; Tsai and O'Malley, 1994; Beato *et al.*, 1995).

Over the past three decades, since the discovery of receptor for glucocorticoids, numerous strides have been made in unraveling the entire process of glucocorticoid action mechanism in greater detail. Moreover, the use of powerful molecular genetic techniques in the recent past have enabled workers to get details at the molecular level of the GR structure, translocation process and its interaction with numerous protein factors and chromatin. These findings have not only provided a clearer picture but have also added much complexity to the process of glucocorticoid

action. Glucocorticoid receptors belong to a highly conserved family of nuclear hormone receptors that includes receptors for other steroid hormones, thyroid hormone, retinoic acid, oncogene products and the recently discovered orphan receptors, whose ligand requirement is not yet known. The members of this superfamily constitute the largest known group of eukaryotic transcription factors, of which glucocorticoid receptor is the one to be studied in maximum detail (Mangelsdorf *et al.*, 1995).

Among the voluminous data on glucocorticoid receptors are those directed towards understanding the alterations in the glucocorticoid receptor-mediated action during development and aging. Changes in the adaptive response to hormonal stimuli are characteristic of developing animals (Roth, 1989). These show a marked decline *and is characterised by a decreased ability of organism to respond to stress. Since* response to stress is mediated to a great extent by the glucocorticoids, knowledge about changes in the physicochemical characteristics of the glucocorticoid receptor, during development is of considerable importance. There are many reports on the age-associated alterations in the glucocorticoid action mechanism that is reflected, among others, by a decreased ability to induce many hepatic enzymes (Sharma, 1988). This decreased responsiveness may, either be due to a decline in the receptor concentration and/ or changes in the molecular events subsequent to hormone binding by the receptor (post-receptor events). Moreover, the internal milieu of a cell is likely to undergo changes, with age, that may affect the physical and functional attributes of the glucocorticoid receptor-mediated signal transduction processes. The potential association of many pathophysiological states with tissue-/ age-specific and/ or acquired glucocorticoid resistance or hypersensitivity, necessitates studies that will help in understanding the mechanism involved in altered glucocorticoid responsiveness (Bamberger *et al.*, 1996). The work embodied in this thesis was planned to study some of the physicochemical properties of glucocorticoid receptors during development of mice. In order to explain our data, the findings will be discussed under the following headings:

i) studies on the binding of [^{3}H]dexamethasone to glucocorticoid receptor

ii) studies on the activation of [^{3}H]dexamethasone-receptor complexes

iii) studies on the physical and chemical properties of glucocorticoid receptors

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

It has been reported that the ability of cells to respond to glucocorticoids is tissue-specific and altered during development. This altered responsiveness may, in part,

be explained by quantitative changes in the receptor molecule (Roth and Hess, 1982; Kalimi, 1984; Gehring *et al.*, 1984; Roth *et al.*, 1987)). Our findings, using [^{3}H]dexamethasone binding studies, demonstrate changes in the glucocorticoid receptor concentration in a tissue- and age-specific manner during the developmental period of mice. In the liver, the receptor level increases significantly at day 30 and by day 60 the level returns to values observed for the early postnatal ages (10- and 15-day) (Borbhuiya and Sharma, 1995a). This pattern of GR expression is similar to that reported by Kalinyak and Perlman (1987), who have shown that the rat liver GR concentration reached a peak value by the 3rd week of postnatal age. Our findings are also consistent with earlier reports of higher GR concentration in the hepatic cytosol of weaning rats as compared to mature (Parchman *et al.*, 1978; Kitkari *et al.*, 1984; Sharma and Timiras, 1987).

In the kidney too, the concentration of receptor is minimal at early postnatal ages. However, in contrast to liver, in this tissue the receptor level shows a peak value at day 45 of postnatal age. This observation is supportive of Kalinyak *et al.* (1989), who observed an initial increase in GR mRNA level which then decreased to adult levels by 2 months of postnatal age. A similar decrease in GR levels is observed in our study (Borbhuiya and Sharma, 1995b), which is also consistent with the published studies of Aperia *et al.* (1985) and Sharma *et al.* (1988).

The observed increase in receptor concentration, both in the liver and kidney could be due to an increase in the receptors affinity for the hormone. This in addition to an increase in the receptor number *per se*, could account for our findings of higher receptor number at day 30 and 45 in the liver and kidney, respectively. Scatchard analysis of the binding data were, therefore performed to see which of the two possibilities contributed to our observations. The data obtained confirmed our findings of elevated GR concentration in the liver and kidney. 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. Our results are in agreement with earlier reports that there is no apparent change in the receptor dissociation constant during development (Kalimi, 1984; Roth, 1988

In the cardiac muscle, the receptor concentration is low as compared to liver and kidney. The pattern of GR expression level is similar to that seen in liver, albeit the increase at 30-day is not significant as compared to receptor levels in other postnatal ages studied.

In the case of skeletal muscle and cerebral hemispheres, the GR expression level shows a pattern quite different to that observed in the other tissues, mentioned above. In the skeletal muscle, the level of GR is high in the early postnatal period (10-day), which declines gradually upto day 60, where the concentration of GR is significantly lower than that of day 10. This finding of ours in the skeletal muscle supports the observation of Roth (1974), Mayer *et al.* (1981) and Sharma and Timiras (1987), while it contradicts the report by Kalimi (1983), who observed no such age-related differences in the receptor concentration. The Data on cerebral hemispheres contradicts the findings of Clayton *et al.* (1977), Olpe *et al.* (1976) and also that of Kalinyak *et al.* (1989).

The ontogenic regulation of glucocorticoid receptor level in a tissue- and age-specific manner needs to be looked at in the context of- (a) a change in circulatory glucocorticoid levels and (b) the tissue effects of glucocorticoids. Such a regulation may be physiologically significant in providing adaptive competence to developing animals. The basal circulating levels of glucocorticoid is low in early postnatal ages, upto about 2 weeks. Thereafter, there is an increase in circulating levels of glucocorticoids, the adult levels being achieved by 4-5 weeks of postnatal age (Henning, 1978). Glucocorticoids have a multitude of effects on the various tissues and organ systems which together produce a pattern of metabolic changes. In liver and kidney the actions of glucocorticoid are mostly anabolic whereas in the muscle, skin and in lymphoid, connective and adipose tissues, its action is mostly catabolic (Baxter and Forsham, 1972). Therefore, our observation of increasing receptor concentration in the liver and kidney may be necessary to increase tissue responsiveness to increasing glucocorticoid levels. This will promote anabolic actions of glucocorticoids necessary for proper growth and development during this period of lifespan. Moreover, the gradual increase in receptor concentration in the kidney, where it peaks at day 45 in contrast to liver, may be due to tissue-specific ontogenic regulation of GR. It may also be due to the fact that kidney is primarily mineralocorticoid responsive that influence glomerular filtration rate, ion-transport and other metabolic functions much more than glucocorticoids (Fanestil and Park, 1981; Funder *et al.*, 1988). Therefore, the need to express mineralocorticoid receptors probably takes precedence over glucocorticoid receptors in the early ages of lifespan.

In the heart, glucocorticoids act in a permissive fashion to potentiate the circulatory and myocardial effects of cathecholamines such as tropic effect on heart muscle and increase in arterial pressure (Baxter and Forsham, 1972; Boer and Oddos, 1979). Since these actions are critical for the development and survival of animal, they need to be carefully balanced. Our findings of the glucocorticoid receptor

concentration pattern, which does not alter significantly during development argues well with the permissive nature of glucocorticoid action in this tissue. However, a slight increase of the receptor concentration at day 30, probably allows the tissue responsiveness to adjust to the overall increase in metabolic demands distinctive of the early developmental period.

In the skeletal muscle, the catabolic actions of glucocorticoids result in decreased synthesis and increased degradation of proteins and RNA, resulting in muscle wasting and myopathy (Baxter, 1976; Dahlberg *et al.*, 1981). Therefore, concurrent with increase in plasma glucocorticoid level a decrease in the receptor concentration would render the tissue less responsive to the hormone, thus reducing catabolic effects. Moreover, during this period of lifespan the metabolic status of the organism is primarily anabolic that encourages the growth of most organs and tissues, including skeletal muscles. Our findings of a gradual decline in receptor number with age thus, supports the above argument.

In the brain, glucocorticoids acting via their cognate receptors appear to be an essential link in the brain-pituitary-adrenal regulatory loop. Glucocorticoids also play a permissive role in the maintenance of normal taste, and olfactory and auditory thresholds. Moreover, a number of brain enzymes are reported to under the control of glucocorticoids (Baxter, 1976; McEwen, 1982; Sapolsky *et al.*, 1986). Reports also suggest that sustained exposure to glucocorticoids can reduce neural number and increase the vulnerability of neurons to a variety of metabolic insults (Sapolsky *et al.*, 1985; Kerr *et al.*, 1989). The gradual decline in glucocorticoid receptor number during development, as observed by us, may help the cells of the cerebral hemispheres avoid the deleterious effects of glucocorticoids.

The above observations demonstrate the age- and tissue-specific regulation of glucocorticoid receptor number during development of mice. This may provide a means of adapting to stress due to changing dietary and metabolic status of the animals during this period of lifespan.

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

Activation studies were performed in the liver and kidney of mice at two age-groups (10- and 60-day) only, to determine the physicochemical changes in the receptor molecules at the activation and nuclear binding stages. The age-groups were so chosen on the rationale that- (i) they delineate the two extreme ends of the developmental period in our study and (ii) the glucocorticoid receptor concentration is similar, so that 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 (25°C for 45 min) and salt (Ca^{2+}, 0°C for 45 min) were judged using DNA-cellulose and purified nuclei. 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.

The magnitude of heat- and salt-mediated activation, as compared to the unactivated receptors (0°C for 45 min) was similar in the liver and kidney and revealed no age-associated differences, as judged by DNA-cellulose binding assay. Earlier reports suggested a difference in thermal activation but not in salt-dependent activation in the liver and *vice versa* in the kidney of immature and mature male rats (Sharma and Timiras, 1987, 88).

Binding of the activated receptor complexes to purified nuclei exposed differences in the degree of nuclear binding in the age-groups studied. The magnitude of thermal activation is significantly higher, as compared to the unactivated receptors, in the liver and kidney of 10-day old mice as compared to those of 60-day. These findings are in contradiction to earlier reports of Kalimi and Gupta(1982) and of Kalimi *et al.* (1983), of unaltered nuclear binding of thermally activated receptors in the liver and kidney of immature and mature Sprague-Dawley rats. However, Sharma and Timiras (1987) reported an increased binding of thermally activated receptors in the liver of mature Long-Evans rats. Interestingly, no such differences in thermal activation were reported in the kidney (Sharma and Timiras, 1988) and skeletal muscle (Sharma and Timiras, 1987) of rats. This suggests the possibility of tissue- and species-specific regulation of the activation processes. Our findings are however, similar to reports of decreased efficiency of nuclei with age, to bind estrogen and progesterone receptors (Chuknyiska *et al.*, 1985; Konoplya *et al.*, 1986; Belisle *et al.*, 1986).

In contrast, low temperature Ca^{2+}-mediated activation does not show any age-related alterations in the liver, but in the kidney the activation is slightly higher in immature mice (10-day). These findings are similar to the observations of Sharma and Timiras (1987, 88). The exact mechanism(s) of this salt-mediated activation process are not well understood. The enhanced nuclear and DNA-cellulose binding may be due to direct interaction of the Ca^{2+} with the receptor molecule and/ or receptor transforming factors, leading to a conformational change capable of exposing DNA-binding domains in the receptor molecule (Grody *et al.*, 1982; Kalimi *et al.*, 1983).

The decreased nuclear binding of the heat activated [^{3}H]dexamethasone-receptor complexes in mature mice tissues could be, either due to a decline in the affinity of the receptor for nuclear components (receptor property) or due to changes in the DNA/ chromatin (nuclear property), that allowed lower binding of the activated

receptors in developed mice. To determine which of these two properties contributed to the observed differences in nuclear binding, nuclear exchange assays were performed, wherein thermally activated receptors from mature (60-day) mice were allowed to interact with the nuclei of immature (10-day) mice and *vice versa*. Some of the earlier reports cited no apparent variation in either of the two parameters involved in nuclear binding (Kalimi *et al.*, 1983; Sharma and Timiras, 1987, 88). Others have reported a defect in the cytosolic fraction as being largely responsible for these age-related differences (Kalimi, 1984). In contrast to the above observations, 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 candidate for the decreased nuclear binding in mature mice (Borbhuiya and Sharma, 1995a, b).

An important modulatory role in gene expression *in vivo*, is played by chromatin structure and organization and any alteration in these could affect the interaction of transcription factors with cis-acting elements (Tsai and O'Malley, 1994; Beato and Sanchez-Pacheco, 1996; Bamberger *et al.*, 1996). Reports exist of changes, primarily in the conformational status of the chromatin as reflected by a decline in the degree of post-translational covalent modifications of histones like acetylation, methylation and phosphorylation (Kanungo, 1994). Change in the nuclear chromatin organization, with age seems to be the most probable reason for the observed decrease in nuclear binding of activated receptors in mature mice. Using DNase I, an endonuclease that digests DNA at inter- as well as intra-nucleosomal regions at intervals of 10.4 bp (Prunell *et al.*, 1979), we found a decreased extractability of the nuclear bound, thermally activated receptors in the liver and kidney of 60-day mice as compared to 10-day (Borbhuiya and Sharma, 1995a, b). Our findings are consistent with that of Chaturvedi and Kanungo (1983), who reported a decreased digestibility of nuclear chromatin with age, by DNase I in the brain of rats. These findings reflect a change in the chromatin organization to a higher ordered structure as development proceeds, thereby limiting the access to DNase I and probably also to trans-acting factors like glucocorticoid receptors. Moreover, alterations in the binding affinity of receptors to chromatin, primarily via non-covalent interactions could not be ruled out. Salt extraction of the bound receptor complexes from the hepatic nuclei, however did not reveal any age-related differences in the percentage of extraction at various concentrations of salt used. Also, about 70-80% extraction of the nuclear bound receptors could be achieved even at a high salt concentration of 0.5 M NaCl in both the ages studied. This suggests that a small percentage of the activated receptors are involved in high affinity (specific) binding whereas, a major fraction of it are involved in low affinity (non-specific) binding with

the nuclear chromatin. Non-specific DNA-binding facilitates the binding of trans-acting factors to specific sequences and this probably acts as a buffer to prevent saturation of a small number of high-affinity GREs, thereby preventing full induction or repression of cognate genes, over a narrow concentration of steroid (Winter and von Hipple, 1981; Cavanaugh and Simons, 1990). Data, however revealed no differences in these parameters during development of mice.

To study the tissue-specific sensitivity of the glucocorticoid receptors and also additional changes in the receptor activation process with development, the actions of certain chemicals on the activation process were studied. Among the numerous modifying agents reported for glucocorticoid receptor are chemicals which inhibit the *in vitro* activation process These include metal ions like molybdate and tungstate and sulfhydryl modifying reagents like N-ethylmaleimide (NEM) (Moudgil *et al.*, 1984; Simons and Pratt, 1995). Our findings are consistent with the earlier reports on inhibition of the activation process by these chemicals (Kalimi *et al.*, 1983; Zakula and Moudgil, 1991). Both the thermal as well as Ca^{2+}-mediated activation processes were inhibited to a similar magnitude in the liver and kidney of mice. 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 chemicals. This agrees well with the observations 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 supports the opinion that alterations in the receptors vis-a-vis their chemical constitution does not occur with age.

Studies on the physical parameters of glucocorticoid receptors

Studies to determine the physical nature of the glucocorticoid receptors did not reveal any differences in the liver receptor from 10- and 60-day old mice. Gel permeation analyses indicated the molecular weight and stokes radii values of the unactivated as well as thermally activated glucocorticoid receptors that are consistent to the reported values of crude as well as purified receptors from different tissues. (Kalimi *et al.*, 1983; Grandics *et al.*, 1984).

It is also evident from our observations that the elution pattern from DE-52 did not reveal any charge differences in the glucocorticoid receptors in the two ages. Moreover, the concentration of salt required to elute both the unactivated as well as activated receptors are similar to that reported by other workers (Schmidt *et al.*, 1985; Bodine and Litwack, 1988).

The immunological studies too, did not expose any variation in the immunological epitopes of the receptor during development. However, the high

affinity of anti-rat GR antibody to the mice GR, reflects a high degree of structural homology as far as the immunologic domain is concerned. Taken together, these findings did not convey any fundamental differences in the physical and chemical properties of the glucocorticoid receptor during development of mice.

In conclusion, we would like to emphasize that the glucocorticoid receptor number and not the hormone binding affinity alters during development. In addition, a decreased nuclear binding of the thermally activated receptors in developed animals is seen. This is probably due to a shift in the chromatin organization to a higher ordered, more compact conformation thereby limiting the availability of nuclear binding sites in mature animals. Other physicochemical properties studied remain unaltered during this period of lifespan. These changes are possibly responsible for the changes in the tissue responsiveness to glucocorticoids, characteristic of developing animals. The altered tissue responsiveness may provide a better adaptational capacity to the changing demand made upon growing animals during development.

Summary

An inherent component of lifespan in higher multicellular organisms is the developmental period. It is characterised by major morphological, physiological, biochemical and psychological changes that empower the organism to lead a self-reliant existence and help in propagation of the species. Moreover, the developing organism is greatly influenced by factors, internal as well as external that can influence the ability of an organism to maintain optimum homeostatic conditions. In this respect, hormones play a vital role in providing adaptational power and in the *maintenance of homeostatic equilibrium in developing animals.*

Amid the many hormones identified and studied so far, glucocorticoids appear important as its presence is required for the overall development and maintenance of homeostatic balance. Glucocorticoids regulate the intermediary metabolism of glucose and influence a wide variety of physiological processes namely, suppression of immunologic and inflammatory response, general protein and amino acid metabolism and most importantly it acts as a `stress-hormone´, in protecting against stressful conditions.

Glucocorticoids act on the target cells through the presence of specific, high-affinity intracellular proteins, called glucocorticoid receptors (GR). These receptors are located in the cytoplasm in association with a number of non-hormone binding proteins. Upon hormone binding, the protein complex dissociates due to a conformational change in the GR, a process termed activation/transformation. Activation allows the hormone bound receptors to translocate to the nucleus where they bind directly to specific DNA sequences called GREs or interact indirectly with other nuclear proteins, both these processes leading to modulation of cognate gene expression.

Among the voluminous data on glucocorticoid receptors are those directed towards understanding the alterations in the glucocorticoid receptor-mediated action during development. Since response to stress is mediated largely by the glucocorticoids, knowledge about changes in properties of the glucocorticoid receptor during development is of considerable importance. This will provide a better insight into the mechanisms involved in altered glucocorticoid responsiveness. The endeavor of our work was to study changes in some of the physicochemical properties of glucocorticoid receptors during development of mice. These will be summarized under the following headings:

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 developmental period of mice. In the liver, the receptor level increases significantly at day 30 and by day 60 the level returns to values observed for the early postnatal ages (10- and 15-day). In the kidney too, the concentration of receptor is minimal at early postnatal ages. However, in contrast to liver, in this tissue the receptor level shows a peak value at day 45 of postnatal age. The observed increase in receptor concentration, both in the liver and kidney could be due to an increase in the receptors' affinity for the hormone. This besides an increase in the receptor number *per se*, could account for our findings of elevated receptor number at day 30 and 45 in the liver and kidney, respectively. Scatchard analysis of the binding data confirmed our findings of elevated GR concentration in the liver and kidney. However, there is no age-associated alteration in the dissociation constant (K_d) values in both the tissues. In the cardiac muscle, the receptor concentration is low throughout, but the pattern of GR concentration is similar to that seen in liver, albeit the increase at 30-day is not significant as compared to receptor levels in other postnatal ages studied. In the case of skeletal muscle and cerebral hemispheres, the GR level shows a pattern quite different to that observed in the other tissues. In these tissues, the level of GR is high in the early postnatal period (10-day), which declines gradually upto day 60, where the concentration of GR is significantly lower than that of day 10.

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

The magnitude of heat- (25°C for 45 min) and salt- (Ca^{2+}, 0°C for 45 min) mediated activation, as compared to the unactivated receptors (0°C for 45 min) was similar in the liver and kidney and revealed no age-associated differences, as judged by DNA-cellulose binding assay. However, the magnitude of thermal activation is significantly higher than the unactivated receptors in both the tissues of 10-day old mice as compared to those of 60-day. In contrast, low temperature Ca^{2+}-mediated activation does not show any age-related alterations in the liver, but in the kidney the activation is slightly higher in immature mice (10-day).

Nuclear exchange assays showed an identical pattern of binding by the mixed as well as the non-mixed groups that indicated a change in the nuclear property as a probable candidate for the decreased nuclear binding in mature mice. Using DNase I digestion studies, we found a decreased extractability of the nuclear bound, thermally activated receptors in the liver and kidney of 60-day as compared to 10-day old mice. These findings reflect a change in the chromatin organization to a

higher ordered structure as development proceeds, thereby limiting the access to DNase I and also possibly, to trans-acting factors like glucocorticoid receptors. Salt extraction of the bound receptor complexes from the hepatic nuclei however, did not reveal any age-related differences in the percentage of extraction at various concentrations of salt used. Also, about 70-80% extraction of the nuclear bound receptors could be achieved even at a high salt concentration of 0.5 M NaCl in both the ages studied. This suggests that a small percentage of the activated receptors are involved in high affinity (specific) binding whereas, a major fraction of it are involved in low affinity (non-specific) binding with the nuclear chromatin. Data revealed no differences in these parameters during development of mice.

Both the thermal as well as Ca^{2+}-mediated activation processes were inhibited by molybdate, tungstate and N-ethylmaleimide to a similar magnitude in the liver and kidney of mice. 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 modulators. The above observations support the opinion that alterations in the receptors vis-a-vis their chemical constitution do not occur with age.

Studies on the physical parameters of glucocorticoid receptors

Studies to determine the physical nature of the glucocorticoid receptors did not reveal any difference in the liver receptor from 10- and 60-day old mice. Gel permeation analyses indicated the molecular weight and stokes radii values of ~290 kDa and ~5.8 nm, respectively for the unactivated receptors. For the thermally activated receptors the values were ~90 kDa and ~3.6 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. The unactivated receptors from both the age-groups eluted at ~250 mM KCl, whereas the activated receptors eluted at a salt strength of ~100 mM KCl. The immunological studies too, did not expose any variation in the immunological epitopes of the receptor during development. However, the high affinity of anti-rat GR antibody to the mice GR, reflects a high degree of structural homology as far as the immunologic domain is concerned. Taken together, these findings entail no differences in the physical and chemical properties of the glucocorticoid receptor during development of mice.

From the overall data, we conclude that the glucocorticoid receptor number and not the hormone binding affinity alters during development. In addition, an increased nuclear binding of the thermally activated receptors, probably due to relaxed and unordered chromatin organization, is seen in immature animals. Whereas, nuclear binding of activated receptors is decreased in mature mice due to a more ordered

organization of nuclear chromatin in these animals. Other physicochemical properties studied remain unaltered during this period of lifespan. These changes in receptor concentration as well as nuclear chromatin organization may be responsible for the changes in the tissue responsiveness to glucocorticoids, characteristic of developing animals. The altered tissue responsiveness might provide a better adaptation to the changing demands made upon animals during development.

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Appendix I

Determination of protein concentration by the dye-binding method of Bradford

The protein concentrations of the samples were determined according to the dye-binding method of Bradford (1976), using bovine serum albumin as the standard.

Preparation of the color reagent

100 mg of Coomassie Brilliant Blue G-250 was dissolved thoroughly in 50 ml of 95% ethanol and to this solution was added 100 ml of orthophosphoric acid (85% w/v). The contents were properly mixed and stored in a brown bottle, this stock solution was kept refrigerated.

At the time of use, 15 ml of the stock solution was made upto 100 ml with distilled water and filtered through Whatman (No.1) filter paper. This working solution has a bench life of approximately two weeks at room temperature.

Standard protein solution

Bovine serum albumin was dissolved in distilled water to give a final concentration of 1 $\mu g/\mu l$. From this, aliquots containing 10-100 μg of the protein were pipetted out and made upto 100 μl with distilled water. To this 5 ml of the Bradford working solution was added and mixed thoroughly. Color was allowed to develop for 10 min at room temperature and the intensity of the color was determined at 595 nm against a reagent blank. A linear regressed curve was plotted from the above data.

10-50 μl of the cytosol, in which the protein needed to be estimated was pipetted out and made upto 100 μl with water. 5 ml of the working solution was added to each tube and the absorbance determined as above. From the standard curve the concentration of protein in the samples were determined.

Appendix II

Determination of the factor for converting CPM into concentration (mmol)

5 μl of [^{3}H]dexamethasone obtained from Amersham (Specific Activity, 87-89 Ci/mmol) was diluted to 50 μl with the homogenization buffer. From this, 5 μl in triplicate was pipetted into scintillation vials and 4 ml of scintillation cocktail-W added to each vial. The radioactivity (CPM) in each vial was counted in a Beckman LS1801 counter with 65% efficiency for tritium. The average CPM was calculated and the value obtained (X) was converted to concentration unit (mmol), using the following formula:

$$\frac{\text{Avg. CPM (X)}}{\text{Volume of [}^3\text{H]dex. solution takrn (ml)}} \times \frac{1}{\text{Radioactive concentration (Ci/ml)}} \times \text{Specific Activity of [}^3\text{H]dex. (Ci/mmol)} \times \text{dilution factor}$$

After putting the values in the above equation, a value of X CPM/mmol was obtained. It was inverted to give $^1/_X$ mmol/CPM and 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

Determination of DNA concentration by diphenylamine method

The reaction between deoxyribose and diphenylamine is the most frequently used color reaction for the determination of DNA concentration in animal tissues. The methodology described below (Burton, 1968) is a slightly modified version of the original procedure described by Burton (1956)

Diphenylamine reagent solution

l.5 g of analytical grade diphenylamine was thoroughly dissolved in 100 ml of glacial acetic acid. To this solution was added 1.5 ml of concentrated H_2SO_4 and the reagent stored in a brown bottle. Just before use, 0.5 ml of an aqueous solution of acetaldehyde, from a 1.6% stock was added.

Standard DNA solution

To 2 mg of DNA (sodium salt, type I from calf thymus) was added 5 ml of 5 mM NaOH and this was kept overnight at room temperature to allow the DNA to dissolve completely. To this was added 5 ml of 1 N $HClO_4$ and the solution incubated at 70°C, in a water bath for 15 min. The solution was cooled and made exactly 10 ml by adding 1 N $HClO_4$. This gave a standard DNA solution having concentration of 200 μg/ml.

A standard curve (linear regressed) was prepared by pipetting 0.1 to 1 ml (20-200 μg DNA) in clean test tubes and the volume made to 1 ml by adding 0.5 N $HClO_4$. To each tube was added 2 ml of the diphenylamine reagent and the solution mixed thoroughly by vortexing. The tubes were incubated at 30°C for 17 hr in a water bath, after which the solutions were brought to room temperature and the absorbance read at 600 nm against a reagent blank.

Estimation of DNA in nuclei

From each set of experiments, 50 μl of the purified nuclear preparation was pipetted out and made upto 1 ml with 0.5 N $HClO_4$ and the solution incubated for 15 min at 70°C. The solution was allowed to cool and 2 ml of diphenylamine reagent was added to each tube, followed by incubation at 30°C for 17 hr in a water bath. The absorbance was read at 600 nm and the concentration of DNA in the samples calculated from the standard curve.

List of Abbreviations

Å	: Angstrom unit
Aa	: Amino acid
ADH	: Antidiuretic hormone
ADP	: Adenosine 5′-diphosphate
ATP	: Adenosine 5′-triphosphate
BSA	: Bovine serum albumin
$CaCl_2$	: Calcium chloride
cAMP	: cyclic 3′,5′-adenosine monophosphate
Ci	: Curie
CPM	: Counts per minute
DBD	: DNA binding domain
DHEA	: Dehydroepiandrosterone
DNA	: Deoxyribonucleic acid
DNA-cellulose	: Deoxyribonucleic acid Cellulose
DNase I	: Deoxyribonuclease I
DTT	: DL-Dithiothreitol
EDTA	: Ethylenediaminetetraacetic acid
FSH	: Follicle-stimulating hormone
g	: Gravitational force
GnRH	: Gonadotropin-releasing hormone
GR	: Glucocorticoid receptor
GRE	: Glucocorticoid response element
GRU	: Glucocorticoid response unit
HBD	: Hormone binding domain
HCl	: Hydrochloric acid
hsp	: Heat shock protein
KCl	: Potassium chloride
kDa	: Kilo Dalton
LH	: Luteinizing hormone
$MgCl_2$	: Magnesium chloride
MNase	: Micrococcal nuclease
Na_2MO_4	: Sodium molybdate
Na_2WO_4	: Sodium tungstate
NaCl	: Sodium chloride
NaOH	: Sodium hydroxide
NEM	: N-ethylmaleimide
POPOP	: 1,4-bis(5-phenyl-2-oxazolyl)benzene;2,2′-p-phenylene-bis(5-phenyloxazole)
PPO	: 2,5-diphenyl oxazole
R_S	: Stokes radius
S	: Svedberg unit
Thr	: Threonine
Tris	: Tris(hydroxymethyl)-amino methane
v/v	: Volume/volume
w/v	: Weight/volume

MD. MONSUR AHMED BORBHUIYA

Mailing address	**Home address**
Department of Biochemistry North Eastern Hill University Umshing, Shillong-793 022 Meghalaya. Tel: (0364) 250107 Fax: (0364) 250076	Jackson Trace Road Lower Lumparing P.O. Laban Shillong-793 004 Meghalaya.

Bio Data

Date of birth — 6th. December 1970

Education

B.Sc. (Honours)	Chemistry, North Eastern Hill University, 1991. 68.3%
M.Sc.	Biochemistry, North Eastern Hill University, 1993. 65.8%
Specialization in M.Sc.	Protein Chemistry
M.Phil (course work)	Life Sciences, North Eastern Hill University, 1994. Grade A

Field of Research — Cell Biology; Development and Aging; Steroid Hormone Signal Transduction Mechanism.

Research Experience — Three and half years.

Specific Research Project carried out — Worked in a DST sponsored project titled "Biochemical changes in glucocorticoid receptor during development and ageing of mice," 1994-'95.

Experience

Biochemical — Radio-receptor assay, modern techniques of biochemical work using centrifugation, spectrophotometry, protein fractionation and purification using gel filtration, ion-exchange chromatography, polyacrylamide gel electrophoresis, western blotting. Working knowledge of HPLC, GC, Spectroflurometer, PCR.

Computational

- MS Word, Aldus PageMaker, Claris Works Aldus Free Hand, 3.1 (Macintosh Ver.)
- Spreadsheet- Lotus, Excel, Cricket Graph (IBM& Mac Ver.)
- Windows 3.1, Multimedia.

Research Publications

1. Monsur A. Borbhuiya and R. Sharma (1995) **Postnatal changes in Kidney glucocorticoid receptor of mice.** *Indian Journal of Biochemistry and Biophysics. 32, 125-129.*
2. Monsur A. Borbhuiya and R. Sharma (1995) **Regulation of Hepatic glucocorticoid receptor during development of mice.** *Biochemistry and Molecular Biology International. 37, 645-652.*

Conferences/Symposiums attended

1. Monsur A. Borbhuiya and R. Sharma (1994) **Hepatic glucocorticoid receptor during development of mice: its level and binding to DNA-Cellulose and Nuclei.** *International Conference on Molecular Biology of Development and Ageing*, A Satellite conference of 16th ICBMB, Bhubaneshwar, Sept. 24-26.
2. Attended the *65th. Annual meeting of SBC (I) held at IISc., Bangalore* from the 20-23rd. November, 1996 and presented a poster titled **Physicochemical characterization of the glucocorticoid receptor from liver of pre- and post-weaned mice.**

Additional qualification

GATE-1994. Percentile score-89.72

Awards/Scholarships

- Prof. S. N. Paul award, St. Anthony's College, Shillong, 1991.
- Meghalaya State Junior Merit Scholarship, Govt. of Meghalaya, 1986-1988.
- NEHU Merit Scholarship, 1991-1993.
- DST Junior Research Fellowship, April 1994-July 1995.
- NEHU-UGC Research Fellowship, August 1995 to present.

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