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THE CHARACTERISATION OF OESTROGEN RECEPTORS BY GEL
FILTRATION IN HORMONE-SENSITIVE TISSUES: IMMATURE RAT
UTERUS, BRAIN AND THYMUS

A thesis submitted in partial satisfaction
of the requirement for the degree of
Doctor of Philosophy in the Faculty of Science

By

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Dedication

For my husband and family

ABSTRACT

The aims of this project were to investigate the binding characteristics of the cytosolic oestrogen receptor in the uterus, brain and thymus of immature Wistar rats. The specificities of the receptor in the uterus are well established. The specificities of the cytosolic receptor in the uterus and thymus of immature female Wistar rats were tested against a range of steroids and the values found for the thymus compared with those for the uterus. The concentrations and dissociation constant (K_d) of the cytosolic oestrogen receptor were determined in uterus, brain and thymus of male and female rats at 5, 18 and 30 days of age. Clomiphene citrate (CC), an oestrogen antagonist/partial agonist, oestradiol (E2), CC+E2 or 4-hydroxyandrostenedione (4-OHA), an aromatase inhibitor, were administered to animals at 15 days in order to study the effects of these compounds on receptor binding characteristics at 30 days. Significant differences in specificity were found between the thymus and uterus; the cytosolic oestrogen receptor in the thymus exhibited significantly higher affinity for corticosterone than it did in the uterus. Male animals were heavier than females at 30 days. Uterus and thymus weights increased exponentially between 5 and 30 days. The tissue-to-body weight ratio increased in uterus between 18 and 30 days and increased in the thymus in both sexes between 5 and 18 days. In males at 30 days, the tissue-to-body weight ratio of the thymus was significantly lower than in females of the same age. Cytosolic oestrogen receptor concentrations in the uterus, brain and thymus differed between some age and/or sex groups. Cytosolic oestrogen receptor concentrations increased exponentially in the uterus between the different age groups. Cytosolic oestrogen receptor concentrations in

both thymus and hypothalamus at 5 days were significantly higher in females than in males of the same age group. No differences in cytosolic oestrogen receptor concentrations were found between the sexes in the cortex at 5 and 18 days but at 30 days, receptors were not detectable in this brain area. The Kd for moxestrol, a synthetic oestrogen agonist that is not bound by alphafetoprotein present in the blood of immature rats, was similar in all tissues. E2 and CC+E2 treatments resulted in decreased body and thymus weight in both sexes, increased uterus weight and decreased thymus weight in both sexes but led to increased uterus weight. CC treatment decreased the concentration of the receptors in the female thymus only; E2 and CC+E2 treatments decreased the concentration of the receptor to levels that were undetectable in hypothalamus and thymus in both sexes; 4-OHA treatment increased thymus weight and cytosolic receptor concentrations in the hypothalamus and thymus of males only.

These results suggest that cytosolic oestrogen receptors in uterus, brain and thymus are similar and that sex differences in these tissues are mediated by differential exposure to oestradiol during the early postnatal period. The thymus is crucial to the development of the immune response. The finding that the cytosolic oestrogen receptor differed from the uterus receptor in its affinity for corticosterone and that sex differences in cytosolic oestrogen receptor concentrations were present in the thymus at 5 days could be relevant to the sex dimorphisms that exist in autoimmune disease manifestation.

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List of abbreviations

| | |
|----------------|---|
| 4-OHA | 4-hydroxyandrostenedione |
| B | Bound |
| BSA | Bovine serum albumin |
| CC | Clomiphene citrate |
| CD | Cluster of differentiation |
| DHEA | Dehydroepiandrosterone |
| E2, oestradiol | Oestradiol-17 β |
| ER α | Oestrogen receptor-alpha |
| ER β | Oestrogen receptor-beta |
| E2R | Oestrogen receptor |
| F | Free |
| GABA $_A$ | γ -aminobutyric acid receptor-alpha |
| hsp | Heat shock protein |
| Kd | Dissociation constant |
| LEW/N | Lewis rat |
| M | Molar |
| MHC | Major histocompatibility complex |
| NZB | New Zealand Black |
| NZW | New Zealand White |
| PVG/c | a strain derived from the hooded Lister rat |
| SLE | Systemic lupus erythymatosus |
| THDOC | Tetrahydrodeoxycorticosterone |
| THP | Tetrahydroprogesterone |

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Chapter one

Introduction

General introduction

1.1: Comparative studies of developmental effects of oestrogen

Oestrogen exerts a large number of subtle as well as dramatic effects in several tissues and organs in many species at different developmental stages. The brain and thymus of mammals as well as the uterus, the classical oestrogen target, are responsive to this hormone. Oestrogen alters brain development and behaviour in the adult animal (reviewed by Pilgrim and Hutchison, 1994) and causes changes in thymus structure and thymic cells (reviewed by Grossman *et al*, 1991). Moreover, the immune and neuroendocrine systems interact by the synthesis and release of thymic, hypothalamic, pituitary, adrenal and gonadal hormones. Stimulated T-cells and macrophages secrete lymphokines, which stimulate the hypothalamus to secrete corticotrophin-releasing hormone. The anterior pituitary is stimulated to release adrenocorticotrophic hormone which, at the adrenal level, stimulates the release of glucocorticoids. Glucocorticoids down-regulate the activity of T-cells and B-cells and also act on cells of the thymus epithelium to decrease the release of thymic hormones. The lower concentrations of thymic hormones, including thymosins, result in decreased activity of effector lymphocytes and at the level of the hypothalamus-pituitary may act to reduce the level of gonadotrophin-releasing hormone and luteinizing hormone (Rebar *et al*, 1981). Sex steroid levels are thereby reduced and the thymus responds by increased synthesis of thymic hormones. Homeostasis is thus maintained as thymic activity returns to its previous level (reviewed by Grossman, 1991).

There is much evidence that sex dimorphisms in brain and thymic structures, and in behaviour and immune function, may be due, in part, to the differential exposure of males and females to gonadal steroid hormones during perinatal development (Hutchison and Steimer, 1984; Keefer and Holdregger,

1985; Grossman, 1984). Cells that express oestrogen receptors are likely to be closely involved in these phenomena.

Comparative studies on the distribution of oestrogen-receptive cells in vertebrate brains show that many steroid-sensitive brain regions are conserved in birds (Balthazart *et al*, 1989; Gahr *et al*, 1987), fish and reptiles (Kim *et al*, 1978) and rats (MacLusky *et al*, 1979; Vito and Fox, 1982; MacLusky *et al*, 1994). This finding suggests that there is an evolutionary advantage in the existence of differential brain development and the consequent differences in mating and maternal/offspring rearing behaviour. Experimental administration of steroid hormones, and pregnancy with its associated changes in hormone levels, affect the thymus (Olsen and Kovacs, 1996) and T-cell development within that organ, as well as B-cell lymphopoiesis in bone marrow (Medina and Kincade, 1994; Clarke and Kendall 1994; Rijhsinghani *et al*, 1996a).

1.2: Sex steroids and the brain

Oestrogens and testosterone exert several overall effects on rodent brain organisation during the early developmental period followed, in the adult, by activation of certain brain areas, such as the preoptic, by sex hormones. During the prenatal and early perinatal stage of development the relative difference between the sexes of the blood concentrations of these two hormones influences morphological differences in the brain (Garcia-Segura *et al*, 1986, McCormick *et al*, 1998). These alterations result in sex-related differences in male and female brains and on reproductive behaviour in the adult (reviewed by McCarthy, 1994). During the perinatal period, males are exposed to higher levels of oestrogen and testosterone than are females and this environmental difference results in sexual dimorphism of brain structure of the adult animals (Pang *et al*, 1978). Later, during development, the control of reproductive behaviour and gonadotrophin release is altered by this previous exposure (MacLusky *et al*, 1994).

Brain development differs between the sexes in, for example, synaptic density, axon outgrowth and synaptic organisation (reviewed by Pilgrim and Hutchison, 1994). Using *in vitro* studies with a PC 12 cell line (derived from rat pheochromocytoma), Lustig (1994) found that oestrogen and androgens induce inherent programmes in these cells. Androgens increased arborization and the receptive field of individual cells, thus increasing the likelihood of neuronal communication. Oestrogens induce the neuroarchitecture required for communication in the form of spines, synapses and gap-junction formation. The activities of androgens and oestrogens thus complement each other and hence modulate neural development and organisation in the various brain regions.

1.2.1: Sex differences in neuron numbers and cell density in the brain

In general, in those brain areas that are sexually dimorphic, neurons are larger in males than in females. In the preoptic area of the rat, neuron size and dendritic development are greater in males than in females (Gorski *et al*, 1980; Hammer and Jacobson, 1984). Male rats have a greater number of neurons and glial cells in the visual cortex than do females, although cell density is the same in both sexes (Reid and Juraska, 1992). Similar differences between the sexes are found in olfactory and limbic areas and in motor nuclei of the rat lumbar spinal cord (Breedlove, 1992). In contrast, there are some brain regions in which females have larger numbers of neurons. For example, females have higher numbers of dopaminergic neurons in the periventricular area of the hypothalamus (Simerley *et al*, 1985). Moreover, although males have larger numbers of neurons in the preoptic nucleus of the hypothalamus, the specific cells in this area that are immunoreactive to calcitonin gene-related peptide are more numerous in females than in males (Herbison, 1992). Female mice also have higher numbers of immunoreactive oxytocin cells *in vivo* (Hausler *et al*, 1990) and of prolactin cells *in vitro* (Beyer *et al*, 1992) in the hypothalamus. In the latter case these

differences are not dependent on the presence of sex steroids. These findings are summarised in Table 1.1.

Table 1.1: Differences in brain neuron number and size in rodents

| Brain area | Observation | Species | Male | Female | Reference |
|--------------------------------------|---|---------|------|--------|------------------------------|
| Preoptic area | neuron size and dendritic development | Rat | > | | Hammer and Jacobson, 1984 |
| Olfactory and limbic areas | neuron number | Rat | > | | Breedlove, 1992 |
| Visual cortex | neuron size and cell density | Rat | > | | Reid and Juraska, 1992 |
| Periventricular area of hypothalamus | dopaminergic neurons | Rat | | > | Simerley <i>et al</i> , 1985 |
| Hypothalamus preoptic area | cells reactive to calcitonin gene-related peptide | Rat | | > | Herbison, 1992 |
| Hypothalamus | oxytocin immunoreactive cells | Mouse | | > | Haussler <i>et al</i> , 1990 |
| Hypothalamus | prolactin cells | Mouse | | > | Beyer <i>et al</i> , 1992 |

(> = more in this sex than in the other).

1.2.2: Regulation of neuron number and size in the brain

Several mechanisms could be involved in the regulation of neuron numbers in developing mammals. There could be regulation of the production of neurons from precursor cells and of the migration of neuroblasts from deeper telencephalic cells in the cortex. There is evidence of continued cell proliferation in male brain in some groups of vertebrates (Goldman and Nottebohm, 1983; Taylor and Truman, 1992), but in mammals there is no evidence of this phenomenon.

While in many sexually dimorphic brain regions, neuron numbers are greater in one sex than in the other, average neuronal size, and hence dendritic branching possibilities, are greater in males in many vertebrate species. The preoptic area of early postnatal male rats shows this sex difference of greater neuron size (Hammer and Jacobson, 1984). Dopaminergic neurons in perinatal male rats show the same feature (Kolbinger *et al*, 1991). In females, however, some areas may show greater dendritic processes than in males. In female rats there is greater dendritic branching in the pre-frontal cortex than in males (Kolb and Stewart 1991). There is some evidence, in rats, that the cerebral cortex grows at a more uniform rate during early post-natal development in males than in females and this phenomenon could be due to movement of neurons, under the control of oestrogen, from deeper layers to the developing cortical area (Diamond, 1987).

Sex-specific regulation of neuronal death might be analogous to the death of T-cells, during their passage in the thymus, giving rise to differential shaping of the adult brain. Neuronal death during ontogenesis of the brain could be due

to an external stimulus as a trigger of pre-programmed cell death, or due to the removal of factors that would alter the micro-environment of the neuron, for example, by removal of a specific growth factor. Raf-1, a protooncogene product, is a protein kinase factor with a key influence on the *c-fos* gene (Rapp, 1991) that is involved in programmed cell death (Smeyne *et al*, 1993). The Raf-1 level in the female hypothalamus is double that found in males during development, where the levels are regulated by testosterone (Whorf and Tobet, 1992). It is possible that the presence of higher concentrations of testosterone in developing males protects them from the cell death that occurs in the hypothalamus of developing females. Oestrogen increases the transcription of mRNA for *c-fos* and for another protooncogene, *c-myc*, in non-neuronal cell lines (Loose-Mitchell *et al*, 1989), but also increases the survival of one oestrogen receptor-bearing cell line *in vitro* although not in another (Rasmussen *et al*, 1990). In male rats, therefore, androgens may have a positive effect on neuronal survival (Wright *et al*, 1988).

1.2.3: Sex steroids and axon development

It seems clear that testosterone and/or its metabolite oestradiol-17 β (oestradiol) are involved in observed differences in neuronal development of many species, though these effects differ between brain areas as well as between species (Toran-Allerand *et al*, 1983). The pattern for synaptic input is more complex. Different research groups have observed sex differences, but have made different observations on the ratio of synaptic density in different brain nuclei (Matsumoto and Arai, 1986; Chen *et al*, 1990).

Effects of sex steroids additional to those involving dendritic branching and synaptic density are those that may influence connectivity between neurons due to axonal outgrowth. If sex steroids promote axonal growth during development, axons of neurons sensitive to these steroids are likely to reach their target post-synaptic neurons earlier than 'rival' neurons, though the target cell itself may not be steroid-sensitive (Wolff and Missler, 1992). Both androgens and oestrogens promote neuritic extensions of cultured hippocampal cells (Brinton, 1993). Androgens and oestrogens are involved in the genomic regulation of the production of specific proteins associated with axonal outgrowth during development in rats (Rogers *et al*, 1991; Shughrue and Dorsa, 1993). As perinatal male rats are exposed to higher levels of these hormones, it would be expected that axon outgrowth would be uniformly higher in males than in females. As this is not the case (Simerley *et al*, 1985; de Vries *et al*, 1981), other factors may play additional roles in axon growth regulation.

1.2.4: Testosterone metabolism in the brain

The microenvironment of developing neurons is likely to depend upon the diverse metabolites of androgens present in the brain of perinatal male rats.

The best studied example of the metabolism of testosterone in the brain is the conversion of testosterone to oestradiol-17 β (oestradiol) within brain cells by the activity of the cytochrome P₄₅₀ enzyme, aromatase (Callard *et al*, 1978). The oestradiol made available to the brain by the catalytic action of this enzyme is crucial for the development of a 'male' brain. That oestrogen is required has been confirmed by recent studies using anti-sense nucleotides for the oestrogen receptor mRNA. Treatment of new-born male rats with these nucleotides results

in female-type differentiation of hypothalamus morphology and behaviour (McCarthy *et al*, 1993). The critical development phase coincides with the testosterone surge during the perinatal period in male rats (Meaney *et al*, 1985). There may be additional direct effects of testosterone on the development of male behaviour. The fate of testosterone once inside the cell can be:

- i) binding to the cytosolic androgen receptors;
- ii) conversion to dihydrotestosterone which binds to androgen receptors;
- iii) conversion to oestradiol through aromatase activity and subsequent binding to oestrogen receptors;
- iv) conversion to other androgen metabolites that bind to neither receptor and may become inactivated.

1.2.5: Role of aromatase in brain development

The involvement of the sex hormones in brain development and on the sex differences that have been observed appears to depend, in part, on the aromatization of testosterone to oestradiol in the brain (McEwen *et al*, 1979) (Figure 1.1). The enzyme involved is a complex of cytochrome P 450 aromatase complexed with NADPH-cytochrome-reductase.

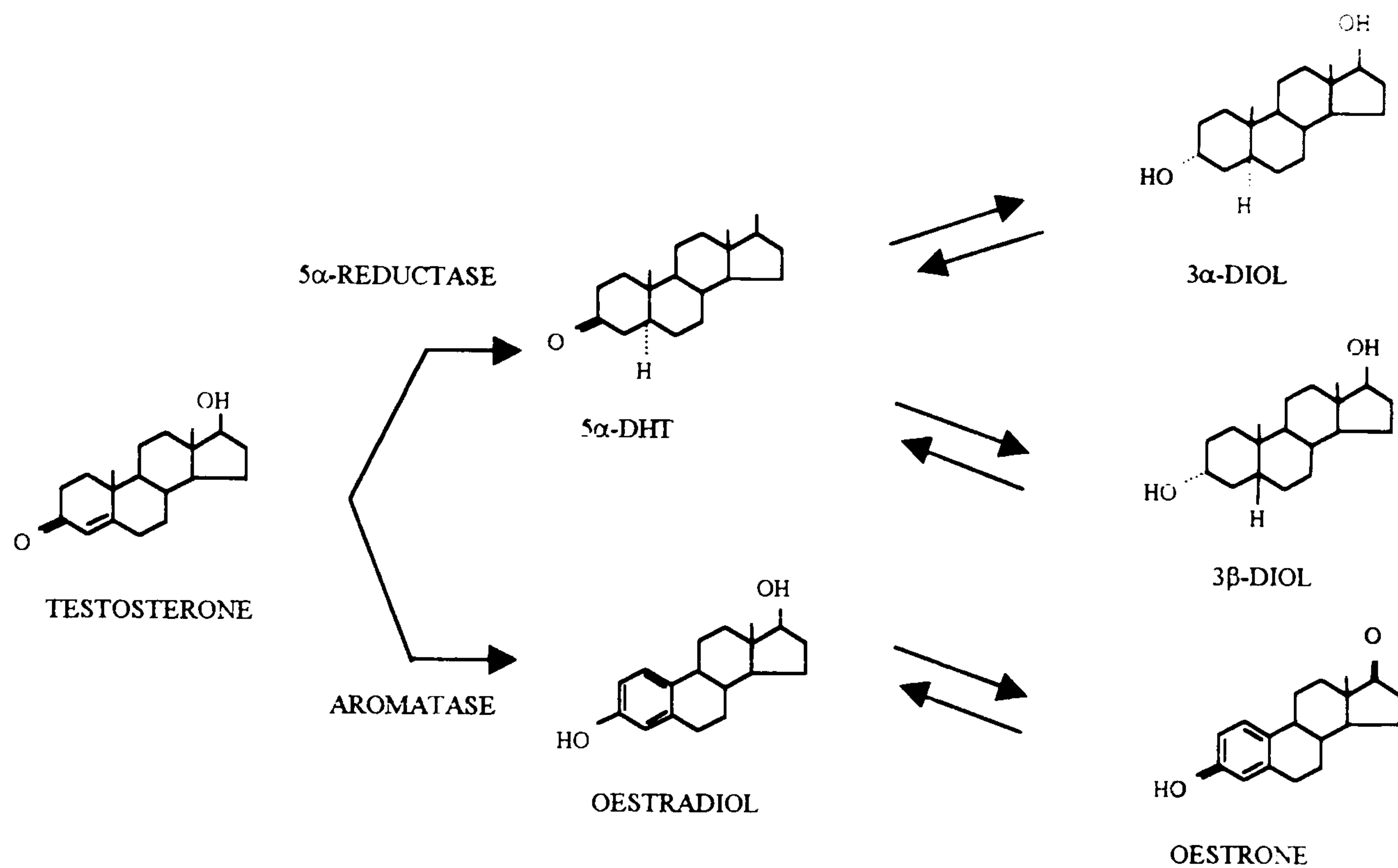


Figure 1.1: The 5 α -reductase and aromatase pathways. 5 α -DHT=5- α -dihydrotestosterone, 3 α -DIOL=5 α -androstan-3 α , 3 β -DIOL=5 α -androstan-3 β .

Two processes, masculinization and defeminization, occur during the perinatal period in mammals. Aromatase is active during brain development in limbic structures and in the hypothalamus, and is present in rat hypothalamic neurons and astroglia. Moreover, in rats, hypothalamus cells from males always have a higher aromatase activity than do female hypothalamus cells at embryonic day 15 in both cultured cells and in explant hypothalamus cultures. This sex difference is observed in the absence of androgens, although the cells had exposure previously to androgens earlier in development (Hutchison *et al*, 1995). Levels of the cytosolic receptor for androgens are similar in the hypothalamus, preoptic area, amygdala and septum of neonatal male and female rats. In the preoptic area of male rats, there are higher levels of aromatase activity and the higher concentrations of oestrogen in this area might explain the lower levels of nuclear androgen receptor (Beyer *et al*, 1993). There appears to be one form only of

aromatase with tissue-specific gene expression as the control point. Regulation of aromatase activity is likely to play a part in development (Meaney *et al.* 1985).

In general, oestrogen defeminizes and masculinizes the brain in developing males at different developmental stages. Oestrogens in mammals influence neurogenesis in the foetus, and synapse formation in the perinatal and adult rat. Testosterone is metabolized to 5 α -dihydrotestosterone and it is this form of the steroid that is required for differentiation of the external male genitalia (Woodman, 1997). 5 α -dihydrotestosterone is also found in developing brain areas, where it appears to have a role in defeminizing and/or masculinizing the developing brain.

While the aromatase system has been well defined in many avian species (Hutchison and Steimer, 1984), regulation of aromatase activity is less clearly defined in mammalian species. Testosterone appears to induce aromatase in mammals; however, testosterone may also be catabolized to biologically inactive forms and these forms may inhibit aromatase, thus regulating the concentration of testosterone available for conversion to oestrogens. Enzymes that are located in the same cells or brain nuclei as aromatase could also, by competing with aromatase for testosterone, limit the conversion of this hormone to oestrogen. Hutchison *et al* (1997) found sex differences in cultured mouse hypothalamus, though not in cultured cerebral cortex cells, with significantly higher aromatase activity in males at embryonic day 13 and 19 without apparent involvement of androgens at this early stage of development.

1.2.6: Other regulatory factors

Neurons and glia are not exclusively sensitive to sex steroids, but receive signals from other steroid hormones such as corticosteroids, neurosteroids and nerve growth factors. In some instances, oestrogens may influence neuron growth as a direct effect on growth-related genes such as those for microtubule-associated protein (Ferreira and Caceres, 1991). Moreover, the distribution of oestrogen receptors and of neurotrophin receptors in the brain overlaps (Toran-Allerand *et al*, 1992). The response to activation of both types of receptor could facilitate amplification of the activity of the hormones concerned. There is enhanced neuron differentiation by interactions of oestrogens with insulin-related peptides (Toran-Allerand, 1988). This effect could be a factor in the development of the sex differences in synaptic connectivity (Garcia-Segura *et al*, 1994). Steroid-sensitive and steroid-insensitive neurons could interact by releasing growth inhibitory factors or neurotrophic factors.

In addition to the effects of sex steroids and other factors originating from outside the brain, there may be direct effects on the development of neuron structure and brain organisation directed by genes on the X and Y chromosomes of neurons. Expression of regulatory genes carried on these chromosomes, and intrinsic to the neurons, could direct early neuronal development before sex steroids were present. In *Drosophila*, sex-specific development of abdominal neuroblasts is dependent on genes of the sex-determining hierarchy (Taylor and Truman, 1992). In mice, the *Sry* genes encode the testis-determining factor and expression of these genes occurs as early as the two-cell stage, long before gonad development occurs (Zwingman *et al*, 1993). The same *Sry* gene effect may

occur in cells of the nervous system that may also express this gene at an early stage of development. The effect could be significant as the gene product recognises the promoter section of the gene for aromatase.

Brain development in vertebrates is influenced by many factors, including sex hormones. Cell number, cell size, and synaptic connections are all regulated, at least in part, by the presence of sex steroids and their receptors. Enzymes regulate the metabolism of sex steroids in the brain and the effects of sex steroids appear to occur over several time periods, either directly or indirectly, on target cells.

Sex steroids have an important, though not exclusive, role to play in brain development in mammals and in the differences between the sexes that have been observed during brain differentiation. The different concentrations of sex steroids present in the brain of perinatal male and female rodents as well as the activity of aromatase and the conversion of testosterone to oestrogen in males appears to affect the fate of brain cells in several brain areas (Hutchison, 1993). If oestrogen does indeed influence the development of neurons, receptors for this hormone would be expected to be present in the cells that are affected.

1.3: Development of T-cells and the thymic environment

1.3.1: Thymic development of T-cells

In common with other progenitors of other mammalian leukocytes, pre-T-cells originate in the bone marrow. Pre-T-cells migrate to the thymus, the major site for T-cell development in mammals (Ford *et al*, 1966; Moore and Owen, 1967). The absence of an intact thymic microenvironment prevents the proper proliferation and differentiation necessary for building the peripheral T-

cell repertoire (Jenkinson and Owen, 1990). In the neonate mammal, the thymus represents a large tissue mass that continues to grow after birth until puberty, after which it involutes while remaining functionally active. The thymus consists of lobules divided into an outer cell-dense area (cortex) surrounding an inner region (medulla). There is little evidence of any extrathymic site for normal T-cell maturation, although oestrogen administration activates extrathymic T-cell differentiation in the liver (Okuyama *et al*, 1992). The main role of the thymus is to provide a microenvironment suitable for the development of precursor T-cells into potentially mature, appropriately functional, T-cells. This environment is provided by an extracellular matrix of fibroblasts in the thymus and by the thymic stroma, which consists of reticulo-epithelial cells derived from the pharyngeal region during development. Macrophages and dendritic cells of bone marrow origin also contribute to the structure and environment (Aguila *et al*, 1997). Precursor thymocytes enter the thymus devoid of the mature cell differentiation (CD) antigens that will be exposed later on their cell surfaces. It is during the period of residence of T-cells in this tissue that the markers necessary for future T-cell function are expressed on their cell surfaces in response to signals from non-thymocyte cells. Moreover, this period also influences cell positioning, cell proliferation, and gene rearrangement for T-cell receptor specificities. As they mature, T-cells express cell-surface markers and there is positive and negative selection of T-cells for proliferation and apoptosis (Miller, 1994).

Precursor cells that lack the surface markers, CD4 and CD8, travel through the cortex of the thymus to the cortico-medullary region guided by thymic epithelial cells in the cortex and medulla. They first express the CD3

polypeptide chains associated with the T-cell receptor (TCR) which assist thymocytes in binding to appropriate antigens. During this passage they also express the CD2 marker, which is involved in cell adhesion, as well as both the major T-cell surface marker antigens, CD4 and CD8. Immature CD4⁺ and CD8⁺ thymocytes are subject to positive and negative selection in the thymus. Those thymocytes bearing receptors for the recognition of foreign peptides complexed with appropriate self-antigens are allowed to mature, a process named positive selection (Anderson *et al*, 1994). Thymocytes with a high affinity for self major histocompatibility antigens (MHC) on thymic stroma could become auto-reactive and undergo apoptosis followed by phagocytosis by local macrophages or epithelial cells (Ashton-Rickhardt *et al*, 1994; Sebzda *et al*, 1994). This process is termed negative selection. Interactions between the positively selected cells and stromal cells lead to down-regulation of either their CD4 or CD8 expression (Anderson *et al*, 1994). Thus, it is during their passage in the thymus that T-cells express their characteristic patterns of CD markers and antigen receptors and functional self-MHC restriction develops (reviewed by Aspinall, 1997). Peripheral T-cells may also undergo apoptosis and there is evidence that the Fas molecule (APO-1 or CD95) is associated with this regulatory mechanism for deletion of potentially auto-reactive T-cell clones that may have escaped earlier apoptosis in the thymus (Musette *et al*, 1994). The pattern of CD markers and antigen-receptors expressed on the surface of mature T-cells are crucial to the development of an effective and appropriate immune response (Davey, 1989).

Cells that migrate from the thymus account for only a small proportion of those that arrive at that organ from the bone marrow, the majority dying within the thymus by apoptosis. After emergence from the thymus, T-cells circulate and express further CD antigens and migrate through the secondary lymph tissue for a limited time. Unless they meet and interact with their specific antigens, T-cells remain functionally inactive; interaction with correctly presented antigen leads to activation, clonal expansion, effector production/function and memory cell development (reviewed by Anderson *et al*, 1996).

1.3.2: The thymic environment

The thymus is a sizeable organ in young mammals. Within this organ, T-cell differentiation is controlled by the thymic microenvironment and in particular by the epithelial cells lying within the thymic cortex. Thymic epithelial cells secrete a large and heterogeneous group of hormones which include the thymosins (thymosin α 1, thymosin fraction 5, thymosin β 4), thymulin, thymopoietin, as well as some other less-well characterised peptide hormones. These hormones are crucial for T-cell differentiation and hence immune function (Kendall, 1991). There is also increasing evidence that thymic peptides are an important humoral link between the immune system and the neuroendocrine system. That growth hormone and prolactin are necessary for normal thymus mass and thymocyte number was an early finding in mice (Bartke, 1964). In rats, prolactin stimulates the release of thymulin from thymic epithelial cells (Gala *et al*, 1991), while suppression of prolactin secretion reduces the level of circulating thymulin (Dardenne and Savino, 1990). Growth hormone mRNA is present in the thymus of neonatal rats (Binder *et al*, 1994) and stimulates the release of

thymulin from murine thymic epithelial cells *in vitro* (Ban *et al*, 1991). Functional activity of receptors for growth hormone and prolactin are present on T-cells in rats (Gala, 1991; Dardenne and Bach, 1988) while the effects of these two hormones on thymic epithelium have been demonstrated in rats (Kelley *et al*, 1986), dogs (Goff *et al*, 1987), humans (Mocchegiani *et al*, 1990), and mice (Dardenne and Savino, 1990).

Lutenizing hormone-releasing hormone and thymus development may also be related. Treatment of neonatal female rats with luteinizing hormone-releasing hormone antagonist lowers thymocyte proliferation and thymus weight as well as inhibiting sexual maturation (Morale *et al*, 1991). While neuroendocrine hormones influence the thymus, communication is reciprocal and the thymus itself has a role in the regulation of hypothalamo-pituitary function. Surgical ablation of the thymus in new-born male and female mice (before 12 hours *post partum*) results in changes in the growth hormone and prolactin secreting cells of the pituitary. Several other endocrinopathies have been observed in congenitally athymic or thymus-deficient animals. Athymic mice also show low plasma concentrations of luteinizing hormone and follicle-stimulating hormone (Rouabhia *et al*, 1989).

1.3.3: Autoimmunity and sex differences

Do oestrogens influence development of the thymus? Several aspects of immune responsiveness differ between males and females in many species including humans (Weetman *et al*, 1981), mice (Weinstein *et al*, 1984) and rats (Wilder *et al*, 1982). In many mammalian species, females resist infection better than do males (Grossman, 1984; Ahmed *et al*, 1983), have higher background

immunoglobulin levels than males, (Butterworth *et al*, 1967) and stronger cell-mediated immunity as demonstrated most effectively by their more rapid rejection of allografts (Graff *et al*, 1969). Moreover females of several species are more susceptible to naturally occurring and experimentally induced autoimmune diseases than are males (Olsen and Kovacs, 1996). For example, there is a higher incidence in women of many autoimmune diseases such as rheumatoid arthritis, Graves disease and systemic lupus erythematosus (SLE); the incidence of SLE is 9-13 times higher in women than in men (Ahmed *et al*, 1985). Moreover, Sanchez-Guerrero *et al* (1995) have reported an increased risk of the development of systemic lupus erythematosus in postmenopausal women treated with oestrogen replacement therapy. In a spontaneous animal model of SLE, the F1 female progeny of New Zealand Black (NZW) and New Zealand White (NZW) mice develop the disease earlier than the males and have a shorter life, with 100 per cent mortality at ten months. Males have less than 10 percent mortality in this period (Theofilopoulos and Dixon, 1985). Pre-pubertal castration of the males of this strain results in disease patterns indistinguishable from those found in females and with 100 percent mortality at eleven months (Theofilopoulos and Dixon, 1985). MRL/MP-lpr/lpr mice also manifest a disease similar to SLE and females have a shorter life-span than males with 50 per cent mortality at five months (Theofilopoulos *et al*, 1981). Among experimentally-induced autoimmune diseases, autoimmune thyroiditis in normal PVG/c rats is more severe in females than in males (Ahmed *et al*, 1983) and male LEW/N rats are more resistant than are females to the development of induced polyarthritis in

response to injected peptidoglycan-polysaccharide fragments of streptococci (Allen *et al*, 1983).

Hormones of the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal axes play important roles in various animal models of autoimmunity such as systemic lupus erythematosus and in mice, in collagen-induced arthritis in mice and rats and in streptococcal cell wall, adjuvant and avridine arthritis models in rats. Autoimmunity, through hormones, is closely linked with gender, sex chromosomes and age. In animal models of autoimmunity, manipulation of sex-hormone levels can influence the course of the disease. In murine models of SLE, oestradiol is a potent accelerator of the disease on MRL mice (Carlsten *et al*, 1990). Female sex hormones are implicated in accelerated autoantibody production in (NZB/NZW)(F1) mice (Roubinian *et al*, 1977; 1978; Theofilopoulos and Dixon, 1985). Castration of male mice results in the female age pattern of disease onset. However, oestrogens suppress T-cell dependent pathology in collagen-induced arthritis in mice and rats. Testosterone and dihydrotestosterone treatment of female and castrated male lupus-prone mice inhibits the onset of the disease (Roubinian *et al*, 1977; 1978); conversely, these two sex hormones enhance collagen-induced arthritis in mice though not in rats (reviewed by Wilder, 1996). Moreover, studies with Lewis and Fischer F344 rats demonstrate that corticosteroids and their releasing factors are important in regulation of inflammation and the onset of autoimmune disease (Dhabhar *et al*, 1993). The hypothalamic-pituitary-adrenal axis is sexually dimorphic and is linked to the hypothalamic-pituitary gonadal axis, and oestrogens are associated with higher corticosteroid responses in females than in

males. These findings in animal models of autoimmunity have major relevance to greater understanding of the causes of development of autoimmune conditions in humans.

If oestrogens play a role in the development of the thymus, sex differences in oestrogen receptor concentrations in this tissue during the early postnatal period could account, in part, for some of the differences in autoimmunity that have been found in experimental animals and in humans.

1.4: Oestrogen receptor studies

1.4.1: Methodologies for receptor research: recent developments

Advances in molecular biology have, in recent years, led to the development of many new techniques for the detection, measurement and distribution determination of hormone receptors and their mRNAs in tissues, cells and cell homogenates.

Immunohistochemical techniques provide sensitive assays for the detection of protein (and other) antigens in frozen or fixed tissue sections or in viable cells. These techniques require an antibody, polyclonal or monoclonal, to link cellular antigen specifically to a stain that can be visualised by microscopy. The antibody can be conjugated to a fluorescent chromophore for illumination with ultraviolet light. In another refinement of the technique, enzyme-linked antibody is used to localize antigen and an insoluble coloured end-product of the enzyme catalysed reaction is precipitated for detection. Ferritin-labelled antibody probes can be used for locating antigens by electron microscopy. Immunogold probes are prepared by adsorbing a species-specific antibody to colloidal gold particles. Radioisotope-labelled antibody can also be used for the detection of

receptors by autoradiography. These methods tend to be semi-quantitative and the use of polyclonal antibodies may lead to false-positive reactions (Cuello AC, 1993). Western blot analysis permits the detection and separation of specific proteins in a tissue sample and, although it can quantify the tissue content of the protein, it does not measure the protein turnover.

In situ hybridisation techniques, like those of immunocytochemistry, are carried out on tissue slices but are used to detect specific DNA and RNA sequences using DNA and RNA complementary to the sequence under investigation. Oligonucleotide probes are now more routinely available than previously and can be labelled with radioactive isotopes or, more safely, using non-radioactive detection groups such as biotin. The technique is used chiefly to detect mRNA and to detect quantitative changes in gene expression after pharmacological interventions. The technique can be used in conjunction with Western blot analyses for protein to establish whether changes in mRNA concentrations may be associated with physiological changes in protein synthesis in the tissue under investigation. Northern blot hybridisation is another method that permits the determination of size and relative amount of mRNA in a preparation of total RNA. The technique can give semi-quantitative estimates of gene expression in various tissues (reviewed by Cowell *et al*, 1997).

A major step forward in the techniques of molecular biology was the development of the polymerase chain reaction by Kary Mullis in 1983. The technique amplifies DNA by using temperature sensitive Taq polymerase, a DNA polymerase in the cellular DNA replication reaction. Multiple rounds of enzymatic replication increase exponentially the amount of a specific gene

sequence within a few hours. Reverse transcriptase-polymerase chain reaction extends the technique to allow detection of mRNA. Initially, mRNA is transcribed to DNA, catalysed by reverse transcriptase. Random or specific primers for the DNA fragment are used to generate cDNA complementary to the RNA. The specific DNA fragments are selectively amplified in the polymerase step by using oligonucleotide primers flanking the target message. The specific amplified gene product (cDNA) is examined by agarose gel electrophoresis and verified by gene sequencing, Southern blot or restriction enzyme digest analysis. The sensitivity of the reverse transcription polymerase chain reaction is one thousand fold greater than earlier methods and very low concentrations of the mRNA in tissues can be detected by amplification of the mRNA present. The original two-step procedure has been replaced by a simpler method that combines reverse transcriptase and DNA polymerase activity. This one-step procedure minimises handling and reduces the risk of contamination (reviewed by O'Leary *et al*, 1997).

Receptors can also be detected in tissues by autoradiography using isotopes such as ^3H and ^{14}C . Animals can be treated with radiolabelled ligand and tissues removed for examination of ligand-labelled receptors. Alternatively, tissue sections removed from untreated animals are incubated with labelled ligand *in vitro*. The data can be quantified by recording the intensity of the image formed by the image of radioisotope decay on photographic emulsion.

Radioligand binding studies are used in order to quantify the concentration of receptors in intact cells or tissue homogenates. The advantage of this technique is that the binding characteristics of the receptor (affinity

constant of the receptor for specific ligands) can also be determined (see Section 2.2.4).

1.4.2: The oestrogen receptor

Steroid receptors such as the oestrogen receptor act under the influence of their lipophilic ligands, which cross the cell membrane readily, to regulate specific mRNA transcription. The receptor has a ligand-binding domain and a DNA-binding domain. The hormone-free receptor is associated with heat shock proteins (hsp), mainly hsp 90, which stabilises the receptor and prevents it binding to DNA. Upon binding of the ligand to receptor, the hsp dissociates and the ligand-receptor complex undergoes dimerisation (Pratt, 1993). The DNA binding site is exposed and the dimer is translocated to the nucleus where it binds to the oestrogen response elements in the promoter region of target genes to initiate and/or modulate gene transcription (Figure 1.2). However, steroids may diffuse directly into the nucleus and exert their effects through binding to nuclear receptors. Such activation of steroid receptors by the ligand is followed by receptor binding to steroid response elements on the DNA, stable complex-formation at the DNA enhancer site, and recruitment of transcription factors and RNA polymerase to initiate gene transcription (reviewed by Greenstein, 1986). Moreover, many steroids (neurosteroids) such as progestins and oestrogens, can have non-genomic effects (Grazzini *et al*, 1998). These effects include modulation of the γ -aminobutyric acid A receptor (GABA_A), lordosis behaviour in female rats, oocyte maturation, LHRH release from the hypothalamus, depolarization of neurons in several brain areas and changes in membrane fluidity (reviewed by Brann *et al*, 1995). The recent discovery of a novel form of the

oestrogen receptor (Kuiper *et al*, 1996), the oestrogen receptor-beta, to distinguish it from the oestrogen receptor-alpha, cloned from the uterus in 1986, has increased the complexity of possible oestrogen effects in different tissues (see also Chapter 4, Section 4.1).

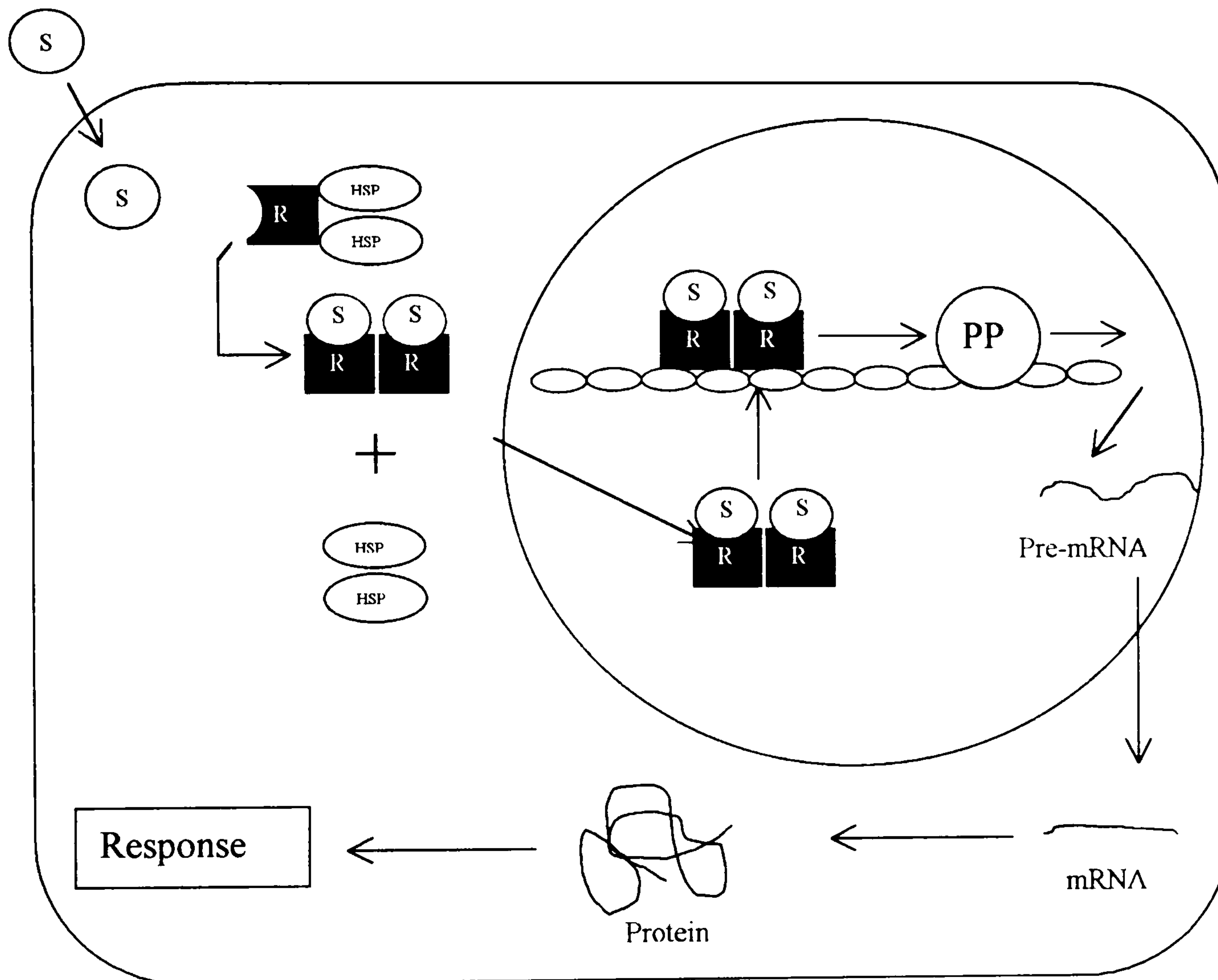


Figure 1.2: Model showing a theoretical mechanism of action of hypothetical steroid hormones. (S = steroid, R = receptor, HSP = heat shock protein, PP = polypeptide, mRNA = messenger RNA).

The observed differences between the sexes in brain development are associated with differential exposure to the sex hormones oestradiol and testosterone. Moreover the effects of these hormones can be blocked by

inhibition of aromatase activity in the brain. Oestrogen receptors are present in brains of perinatal mice (Keefer and Holdregger, 1985) and rats, where there are sex differences in their distribution and concentration in the hypothalamus (Kuhnemann *et al*, 1994; Don Carlos *et al*, 1995). Manipulation of sex hormone levels in the thymus can result in alterations in thymus size and can influence the course of autoimmune disease. Evidence of oestrogen effects on thymus size, and of sex differences in immune function, would suggest that the oestrogen receptor must be involved in the underlying and interacting mechanisms of immune responsiveness. Specific, high-affinity receptors for oestrogen are present in the thymus of rats (Grossman, 1984), mice (Kawashima *et al*, 1992) and humans (Nilsson *et al*, 1990). Grossman's work (1984) showed that the thymic oestrogen receptor has a high affinity for oestradiol, although receptor concentration in this tissue is one-hundred-fold lower than in the uterus. The receptor appears to be confined mainly to the reticulo-epithelial cells in this tissue, although oestrogen receptors have been detected also in thymocytes in humans (Weusten *et al*, 1986) and rats (Amir-Zaltzman *et al*, 1993).

What are the role of oestrogen and the binding characteristics of its receptor during early development of the thymus? As described earlier, there is good evidence of sex differences in the distribution of the oestrogen receptor in the brain and of its activity during development of that tissue. The activity of oestrogens in the uterus and brain, and the oestrogen receptor specificities and binding characteristics during development of these tissues is relatively well established. The thymus grows rapidly in the immature rat. If, as in brain and uterus, oestrogens exert their effects on the thymus through the oestrogen

receptor, an important question to address is that of the receptor characteristics during the early development of the thymus. Does the cytosolic oestrogen receptor in the thymus have the same specificity as in the uterus, the classic site for oestrogen activity? Do the concentrations and dissociation constants of the oestrogen receptor in the thymus differ between the sexes and at specific, early developmental stages in the rat? Does thymus weight differ between the sexes during early development? Does treatment with oestrogen antagonists or with aromatase inhibitors effect changes in the receptor binding or in thymus weight? Given the well known sex-related differences between males and females in the manifestation of autoimmune disease, it seemed relevant to seek to establish some of the properties of the oestrogen receptor and to try to find a means to manipulate its role, if any, in thymus development during this critical early period of differentiation.

The aims of this project were to characterise the cytosolic oestrogen receptor in the uterus, brain and thymus of male and female rats during the postnatal period from 5 to 30 days.

Examination of the specificity of the oestrogen receptor for oestradiol, as well as the affinity of oestrogen receptor for a number of inhibitors, would be necessary in order to establish whether or not the oestrogen receptor in the uterus, the classic tissue target for oestradiol, and thymus are identical. The specificity of oestrogenic responses depends upon the initial binding of oestradiol to the receptor. Much is known about the specificity of the receptor in the uterus but little is known of its specificity in the thymus. There could be differences between its affinity for oestradiol and for putative inhibitors of binding. These

differences, if present, could reflect receptor heterogeneity or the existence of tissue specific oestrogens. This study is described in Chapter 3.

The binding characteristics of the oestrogen receptor should be investigated in the uterus, brain and thymus of developing rats at an age when the thymus is still growing and when the levels of free circulating oestradiol are still low. There could be differences between the sexes and/or age groups in the expression of the receptor. Moreover differential exposure in the sexes to oestradiol could have developmental significance in the thymus as well as in the uterus and brain. This section of the project is reported in Chapter 4.

If there are any differences between the sexes and age groups in the expression of oestrogen receptor, treatment with oestrogen antagonists could alter this expression and oestrogen effects on thymus weight. If the thymic environment can be manipulated by drugs, it could shed significant light on the mechanisms of autoimmune disease development. This work is discussed in Chapter 5.

Each chapter has a short introduction, a brief section on the materials and methods specific to that study, results, and discussion.

Chapter 6 includes a final discussion of the project and the conclusion.

Chapter two

Materials and Methods

2.1: Materials

2.1.1: Steroids and non-steroidal compounds

| <u>Trivial name</u> | <u>Systematic name</u> |
|------------------------|---|
| Oestradiol-17 α | 3,17 α -Dihydroxy-1,3,5[10]-oestratriene |
| Oestradiol-17 β | 3,17 β -Dihydroxy-1,3,5[10]-oestratriene |
| Testosterone | 17 β -hydroxy-4-androsten-3-one |
| Progesterone | 4-Pregene-3,20-dione |
| Corticosterone | 4-Pregnene-11 β ,21-diol-3,20-dione |
| Oestriol | 3,16 α ,17 β -Trihydroxy-1,3,5[10]-oestratriene |
| Oestrone | 3-Hydroxy-1,3,5[10]-oestratrien-17-one |
| Diethylstilboestrol | trans-4,4-dihydroxy- α,β -diethylstilbene |
| Moxestrol | 1,3,5-oestratriene,3,17 β diol,11 β -methoxy,17 α - ethyne (R 2858) |
| 4-OHA | 4-Hydroxy-4-androstene-3,17-dione |
| Clomiphene citrate | 1-[<i>p</i> (β -diethylaminoethoxy)phenyl]-1,2- diphenylchloroethylene |

Tritiated oestradiol [2,4,6,7-³H-oestradiol] (specific activity 100 Ci/mmole) and (4-³H)-toluene reference standard were from Amersham International plc, UK; tritiated moxestrol (³H-moxestrol, specific activity 80-88 Ci/mmole) and unlabelled moxestrol were purchased from NEN Research Products, Germany.

2.1.2: Other Materials

Clomiphene, bovine serum albumin, (Sigma A7030) and Sigmacote were from Sigma Chemical Co., UK; Folin and Ciocalteu's phenol reagent, POPOP (1,4-di-2-(5-phenyloxazolyl)-benzene) and PPO (2,5-Diphenyloxazole) were from BDH Laboratory Supplies, UK; Sephadex G-50 (fine) and Sephadex LH-20 were from Pharmacia, UK; Diazepam Injection B.P. was from Phoenix Pharmaceuticals Ltd., UK; Hypnorm was from Janssen Pharmaceuticals Ltd., UK. All other chemicals and reagents were of 'Analar' grade obtained from BDH Laboratory Supplies, UK. Whatman No 1 filter paper was used throughout the experiments for gel filtration. Beekay Rat and Mouse Standard Diet was from Bantin and Kingman Universal, Hull, UK.

2.1.3: Glassware

The silica glass columns used for gel filtration and all glass test tubes were siliconized as follows. Clean, dry columns and test tubes were rinsed with Sigmacote, which forms a microscopically thin film of silicon on glass. Columns and tubes were drained of excess solution, allowed to dry overnight and finally rinsed with ethanol and distilled water.

2.1.4: Buffer solutions

The buffer solutions used for binding studies were prepared 24hr in advance, preserved with 0.2% (w/v) sodium azide and stored at 4°C. The buffer employed for estimating the free fraction of oestradiol in serum was prepared immediately before use. All buffers were made with deionised, double distilled water and the pH standardized at 7.4 at 20°C using a glass electrode and pH

meter. Mercaptoethanol was added to inhibit the oxidation of sulphhydryl groups in the cytosolic preparations from uterus, brain and thymus.

Buffer 1 (phosphate/sucrose/mercaptoethanol pH 7.4)

This buffer was used for preparing cytosolic receptors from uterus, thymus and brain. The composition was:

| | |
|----------------------------------|------------|
| Na ₂ HPO ₄ | 10mM |
| NaH ₂ PO ₄ | 10mM |
| sucrose | 250mM |
| sodium azide | 0.2% (w/v) |
| mercaptoethanol | 100 mM |

Buffer 2 (phosphate/sucrose pH 7.4)

Buffer 2 was used for determining the concentration of oestrogen binding sites in the various tissue cytosols and the dissociation constant (K_d) of the reaction between ligand and receptor. The composition was:

| | |
|----------------------------------|-------------|
| Na ₂ HPO ₄ | 10mM |
| NaH ₂ PO ₄ | 10mM |
| sucrose | 250mM |
| sodium azide | 0.2 % (w/v) |

Both buffers had previously been used for similar experiments in this laboratory.

Buffer 3 (phosphate pH 7.4)

This buffer was used for measuring the free fraction of oestradiol in serum. The composition was:

Na₂HPO₄ 150mM

NaH₂PO₄ 150mM

2.1.5: Scintillation fluid

Scintillation fluid, for estimation of radioactivity in the cytosolic and serum samples, was prepared by dissolving 6g PPO and 0.6g POPOP in 2L toluene. This fluid was used to convert the energy emitted by the β radioactivity in the ligand to energy emitted by the fluid as chemiluminescence. The counting efficiency of the counter, using the (4-³H)-toluene reference standard, was 48-50%.

2.1.6: Animals

For specificity studies (Chapter 3) and cytosol receptor studies undertaken on the binding of ³H-moxestrol in 5, 18 and 30-day-old Wistar rats (Chapter 4), male and female animals were obtained from a pathogen-free colony bred at St. Thomas's Hospital Medical Biological Services, London. For studies on the effects of treatment with oestradiol, clomiphene and 4-OHA on male and female Wistar rats (Chapter 5), animals were supplied by Bantin and Kingman Universal, Hull, UK. Animals from this latter group were allowed to settle for 24 hours before treatment or sacrifice and were housed 4-8 per cage, with or without their mother depending upon age. They were kept under conditions of

controlled lighting and heating (lights on, 08:00h-22:00h, lights off, 22:00h-08:00h; 21-22°C) and were allowed free access to Beekay Rat and Mouse Standard diet and tap water.

2.2: Methods

2.2.1: Anaesthesia

Hypnorm (0.315mg fentanyl citrate and 10 mg fluanisone/ml) and Diazepam (5mg Diazepam/ml) were used to anaesthetise rats prior to any treatment or sacrifice. Hypnorm (1 ml) and Diazepam (0.5 ml) were taken to 10 ml with distilled sterilised water. The anaesthetic was administered intramuscularly at a dose of 0.2 ml per 100 gm body weight. All procedures were carried as specified by Home Office regulations; Project Licence No.90/002004, Personal Licence No.70/004825.

2.2.2: Collection of blood and preparation of serum

At 5, 18 or 30 days of age, the animals were either decapitated (5 days) or anaesthetised (18 and 30 days) as described in Section 2.2.1. Trunk blood was collected from 5-day-old rats; blood from the other two age groups was collected by cardiac puncture using a heparinised needle. Blood was kept in vials on ice for one hour before centrifugation in an Eppendorf micro-centrifuge at 14,000 rpm for 10 minutes at 4°C. The serum was frozen in 1ml aliquots at -70°C. In the case of serum from 5-day-old rats, blood from two animals was pooled in order to obtain enough material for assay. Animals in the older age groups were sacrificed by cervical dislocation. The serum was assayed for the free fraction of oestradiol as described in Section 2.5.

2.2.3: Procedure for preparation of cytosols

The procedures used for measuring cytosolic oestrogen receptor were those described by Ginsburg *et al* (1974).

2.2.3.1: Dissection

The uterus was removed *via* a ventral incision and the two horns were removed by a cut just above the bifurcation at the cervix and another cut at the oviduct-utero junction. The uterus was trimmed free of adhering tissue. The thymus was removed *via* a thoracic incision. In order to remove the brain the skin of the head was cut along the mid-line dorsally and the skull-cap removed with scissors. The frontal lobes were raised, the exposed optic nerves were cut and the brain was lifted out and placed on an ice-cold Petri dish. The hypothalamus was dissected out using fine scissors. The area used extended from the optic chiasma rostrally to the mammillary bodies caudally and hypothalamic fissures laterally. The hypothalamus was lifted by the optic nerve and a dorsal cut was made 2 mm deep. Amygdala tissue was taken at the same time. Scissors were used to cut a slice 5 mm long and 1 mm deep. Cortex tissue was taken in the same way. The time taken for the complete dissection of all tissues from one rat was approximately 5 minutes. Excised tissues were placed immediately in the appropriate amount of ice-cold Buffer 1. The number of tissues pooled varied according to the weight of the tissues; the tissue:volume ratio is shown in Table 2.1.

Table 2.1: Wet weight to buffer volume for studies of oestrogen receptors in female and male Wistar rats.

| Tissue | Wet weight (mg/ml) |
|--------------|--------------------|
| thymus | 150 |
| uterus | 75 |
| cortex | 150 |
| hypothalamus | 150 |

2.2.3.2: Tissue homogenisation

Tissue preparation and procedures for the measurement of cytosolic oestrogen receptor employed the methods described by Ginsburg *et al* (1974).

Uterine tissues were minced with scissors and, because of their fibrous nature, were homogenized with two 5 second bursts using an Ultra-Turrex electrically driven mechanical homogenizer (BDH, UK). Thymus tissue was also minced with scissors and both brain and thymus tissues were homogenized manually using twenty strokes of a Jencons uniform ground-glass homogenizer and Teflon pestle at 0-4°C. The clearance between the pestle and the inner glass wall of the homogenizer was given by the manufacturer as 0.25mm. This clearance is required to avoid rupturing nuclei (Lovtrup-Rein and McEwen, 1966). Brain tissue was not minced first, but was homogenized manually in the same way as the thymus.

2.2.3.3: Centrifugation

A Beckman L8-M ultracentrifuge with fixed-angle aluminium rotor was used. The homogenate was transferred to chilled centrifuge tubes and placed in a pre-cooled rotor at 4°C. Centrifugation was carried out at 105,000g (42,000 rpm) for 1 hr at 4°C. This process separated and deposited cell particulate matter, cell debris, nuclei, mitochondria, lysosomes and microsomes and left a clear supernatant, which contained the cytosolic fraction of the cell. Uterine preparations had a variable layer of fat that was removed prior to storage. This procedure was essential in order to minimise the amount of non-specific low-affinity binding in this tissue. The supernatants were used as the cytosol preparations and were decanted into plastic tubes and frozen at -70°C. An aliquot of each cytosol was taken for later determination of the protein content (Lowry *et al*, 1951).

2.2.4: Assay of cytosols for steroid receptor-binding studies

When a large amount of steroid is mixed with a relatively small population of receptors for that steroid, equilibrium is rapidly established. Under these conditions, a constant amount of steroid is bound to the receptor and most of the steroid present is unbound or free.

Several techniques have been established for the separation and measurement of bound and free steroid. There are basically two types of technique. The amount of bound steroid can be measured without disturbing the equilibrium of the reaction or equilibrium conditions are not maintained as free steroid is removed from the system. Equilibrium dialysis is one example of the

former technique. This method was inappropriate for the present studies because the time taken to complete the dialysis could result in total loss of binding because of degradation of the receptors. Sucrose density-gradient centrifugation is another sensitive and well-proven method for separating specifically from non-specifically bound ligand. This method involves layering aliquots of incubate onto sucrose gradients followed by centrifugation at high speed and low temperature. Sucrose density-gradient centrifugation was unsuitable, though, for the measurement of large numbers of samples and the length of time of centrifugation (up to 24 hr) could cause dissociation of any complex present. Measurements of binding that disturb the equilibrium can be carried out using adsorbents such as dextran charcoal and this technique has been used to measure oestrogen binding in hypothalamus and pituitary (Ginsburg *et al*, 1974). However, this material adsorbs a small, but significant, proportion of the binding proteins as well and this amount has to be accounted for. Moreover, this method is not sensitive enough to detect oestradiol receptors in tissues where the concentration of these proteins is small. Another method that can be used for measuring oestrogen receptors is the enzyme immunoassay technique. Although it is sensitive for measuring the human oestrogen receptor and could be adapted to rat studies, it measures only receptor concentration and gives no idea about its binding affinity.

The high-affinity oestrogen binding-protein from cytosol of brain, uterus and thymus can be measured using the technique of gel filtration, a method which does not involve disturbing the equilibrium (Ginsburg *et al*, 1974). The gel used was the small pore lipophilic gel Sephadex LH-20. This method separates

molecules according to their size. Substances are eluted from the columns of Sephadex in order of decreasing molecular weight. Moreover, Sephadex LH-20 is lipophilic and hinders the elution of free oestradiol by adsorption. The technique has been used widely in studies involving small amounts of cytosol, it is sensitive, and is convenient for handling large numbers of small samples. An additional practical advantage is that, while most gels must be disposed of after each experiment, LH-20 can be regenerated by careful washing and rinsing with ethanol and distilled water and thus reused three times.

Gel filtration was chosen, therefore, as the most appropriate method for this study for four main reasons:

- (i) it had been used successfully in this laboratory for many years to detect and measure oestrogen-binding components in rat brain and uterus (Greenstein, 1978).
- (ii) the method accommodated the need for simultaneous examination of up to 12 different cytosols without the need for large amounts of tissue.
- (iii) Up to 72 cytosol samples could be handled at any one time.
- (iv) The low background value obtained in the void volume when ^3H -moxestrol was applied to the column was particularly important when working with low protein concentrations and contributed to the relatively high sensitivity of the method.

2.2.4.1: Preparation of steroids for binding studies

The tritiated steroids were supplied by the manufacturer, dissolved in toluene. The carrier was evaporated under a stream of nitrogen and

reconstituted to the required concentration in absolute ethanol. All steroids were kept at 4°C. Preparation of the steroids and of the cytosols with steroids for Scatchard analysis was carried out on ice.

Tubes 1-5 were prepared for use in the measurement of the total binding in the cytosol preparations and Tube E, for the measurement of the non-specific binding in the cytosol preparations. Buffer 2 (3.92ml) was placed in a siliconized glass tube labelled T1, and 1 ml of the same buffer was placed in each of 5 other tubes labelled T2-T5 and E. 80 µl 5×10^{-7} M 3 H-moxestrol was added to T1 (final concentration, 1×10^{-8} M) and mixed. 2ml of the contents of T1 were transferred to Tube E and 40µl 5×10^{-5} M unlabelled moxestrol in absolute ethanol added to the tube. 40µl absolute ethanol (less than 1% of the volume) was added to T1 and the contents of Tube 1 were serially two-fold diluted through Tubes 2-5 to give a range of final concentrations from 5×10^{-9} - 6.25×10^{-10} M.

2.2.4.2: Incubation of the cytosols

All prepared cytosols were assayed within two months of their preparation. Incubations were performed in 96-well polypropylene round-bottomed plates (Costar, Cambridge, Mass, USA) and sealed with Titerex Plate Sealers (ICN Biomedicals Ltd. UK). These trays had been tested and shown not to bind the steroids to their surface (Dhafer, 1992).

60µl cytosol was incubated with 60µl of the appropriate radioactive steroid from one of Tubes 1-5 or Tube E. Aliquots (60µl) of the various concentrations of steroid were also incubated with 60µl Buffer 2 in the same trays for estimation of the total radioactivity, in order to minimise errors due to

evaporation during the process. Incubations were carried out for 1 hr in a shaking water bath at 25°C, conditions that were determined to allow equilibrium to be established between the high-affinity receptor and the steroid (section 2.4.1). For assays in which moxestrol was employed, the final concentrations in the incubation were from $5.0 \times 10^{-9}\text{M}$ to $3.1 \times 10^{-10}\text{M}$ in the presence or absence of a 100-fold excess of unlabelled moxestrol (Figure 2.1).

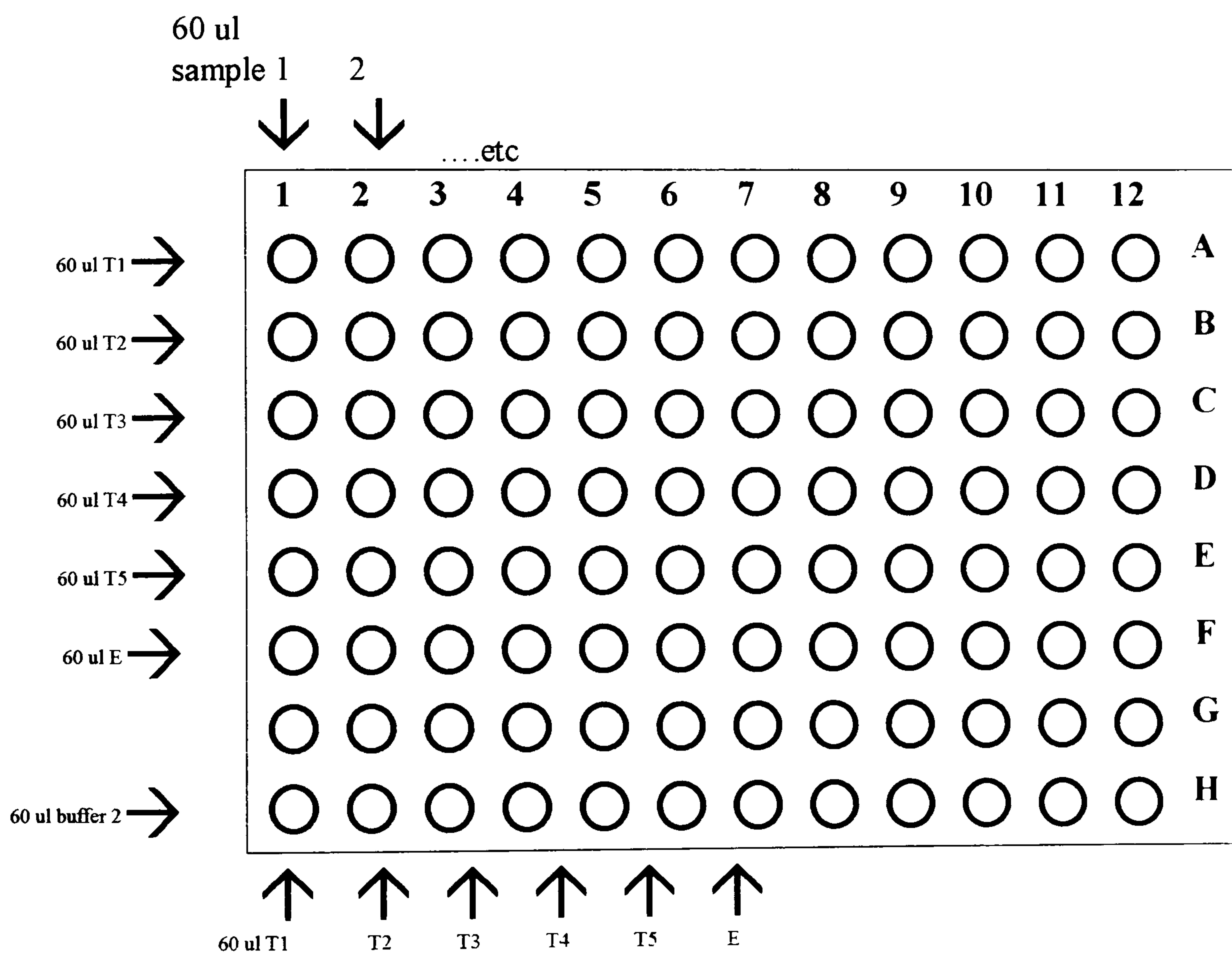


Figure 2.1: Schematic diagram illustrating the incubation of cytosolic samples with the different concentrations of moxestrol. Samples were placed in the trays in columns 1-12 (for 12 samples) and, in rows A-F, the different dilutions of the ligand were added. Row H was used to estimate the total counts per minute for each of the different dilutions of ligand.

In the case of ^3H -oestradiol- 17β , the five dilutions were from $3 \times 10^{-9}\text{M}$ to $1.88 \times 10^{-10}\text{M}$ in the presence and absence of a 100-fold excess of unlabelled oestradiol.

2.2.4.3: Preparation of Sephadex LH-20 columns

Silica glass columns 17 cm in length, 0.45 cm in internal diameter were used in this method. (Figure 2.2).

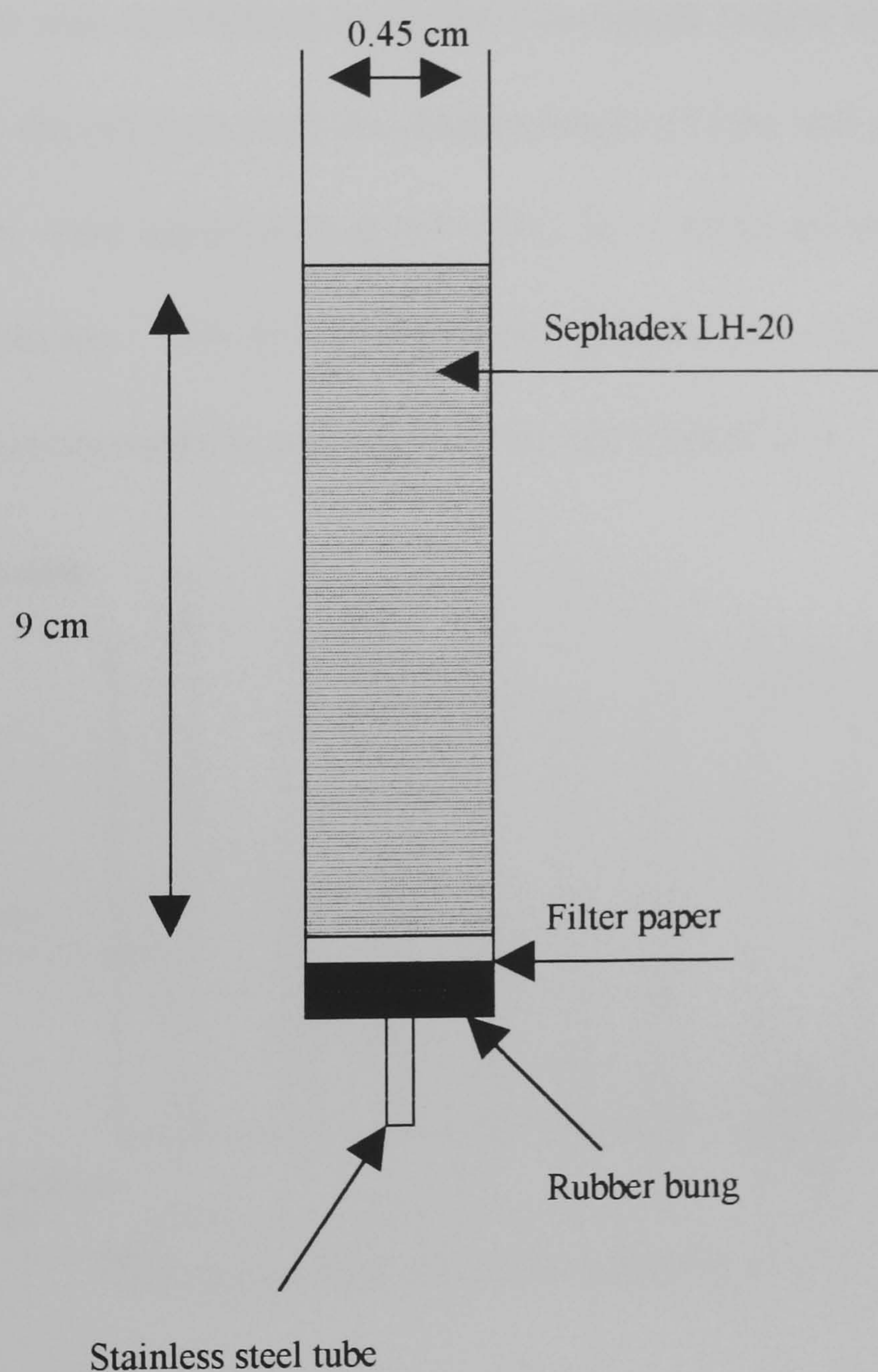


Figure 2.2: Schematic diagram showing the Sephadex column used in separating the bound and free moxestrol.

One end of each column was plugged with a small rubber bung through which was inserted a short piece of stainless steel tubing of internal diameter 1mm. Small (0.45 cm) circles of Whatman No 1 filter paper were passed down the glass columns, onto the inner surface of the rubber bung to support the bed of gel.

The columns were then mounted in a water bath designed to allow the simultaneous handling of six samples of each of twelve different cytosols. Sephadex LH-20 was equilibrated in Buffer 2 overnight before use. The swollen gel was run into the columns until the desired height (9 cm) was reached. During use, the columns were maintained at 0.5-2.0°C by a pump which circulated ice-water from a reservoir. This was necessary to minimise the dissociation of high-affinity binding components in contact with the gel (Figure 2.3).

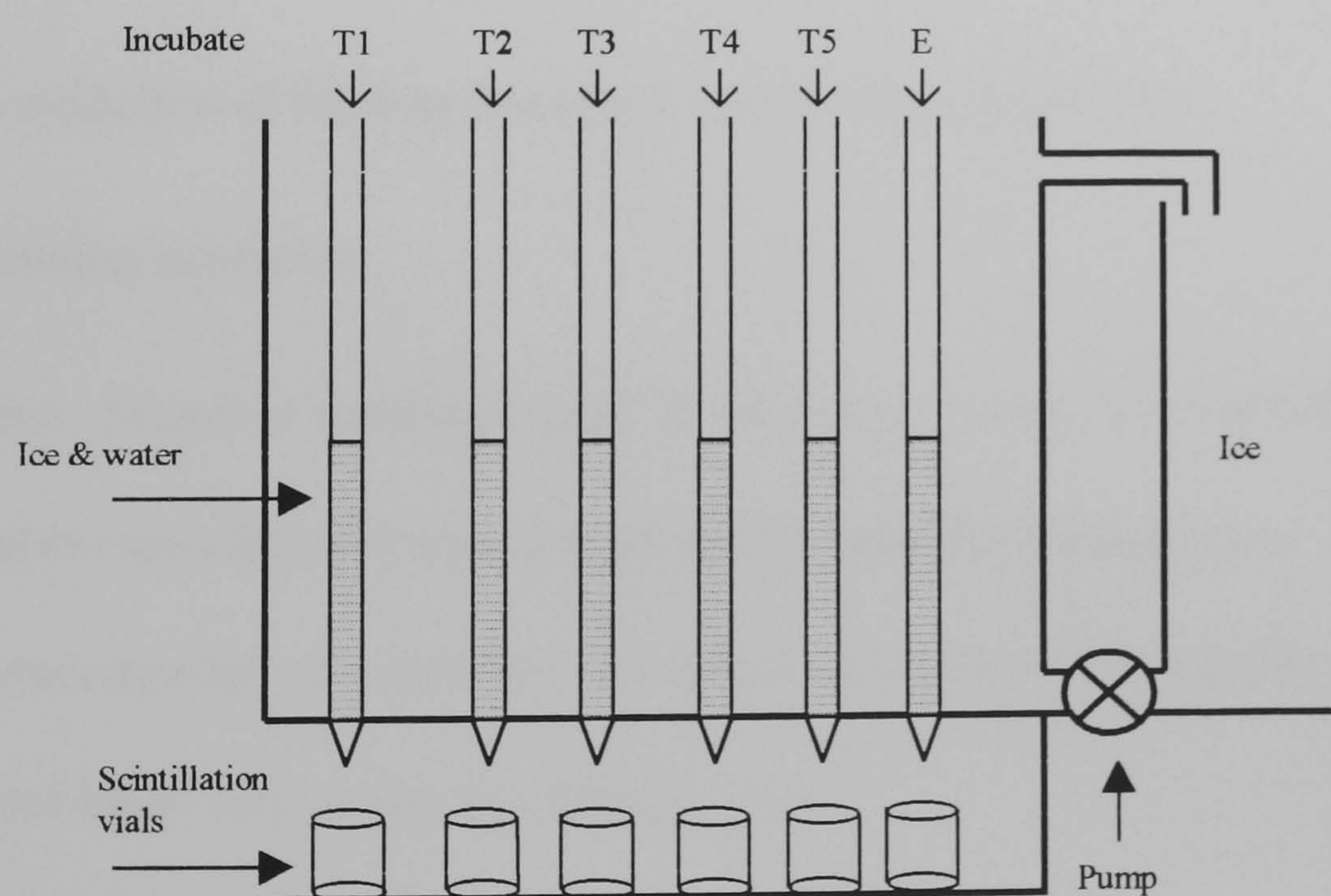


Figure 2.3: Schematic diagram showing the apparatus and the Sephadex columns used in the separation of saturated receptor molecules from the excess ligand in the column.

2.2.4.4: Separation of bound and free moxestrol

The samples were cooled following the 1 hr incubation. 100µl of the incubate was allowed to run into the column and was immediately washed into the column with 200µl of Buffer 2. The sample remained on the column for not more than 1hr to ensure that no dissociation occurred during the elution (section 2.4.2). At the end of this time, bound ³H-moxestrol was eluted into a scintillation vial with 700µl of Buffer 2, a volume that was sufficient to elute all bound hormone leaving the free hormone in the column (section 2.3.1).

2.2.4.5: Measurement of radioactivity.

Scintillation fluid (10ml) was added to the samples and the vials were kept in the dark overnight to allow extraction of the radioactivity. Radioactivity was measured using a Wallac 1409 scintillation counter.

2.2.5: Investigation of binding isotherms and binding parameters

2.2.5.1: Binding isotherms

When cytosol is incubated with ³H-moxestrol alone, the steroid binds to both saturable (specific) and non-saturable (non-specific) binding sites. Adding a 100-fold concentration of unlabelled moxestrol saturates all the specific sites and ³H-moxestrol binds only to the non-specific sites.

Upon increasing the concentration of ³H-moxestrol, the amount of specifically-bound hormone increases until saturation is achieved; however, when a 100-fold concentration of unlabelled moxestrol is added, saturation with the tritiated hormone will not be achieved because ³H-moxestrol is displaced from the limited number of specific binding sites.

The binding isotherm was established by plotting the total binding and the non-specific binding (unsaturable) against the total amount of ^3H -moxestrol added to the incubate. The non-specific binding produced a straight line with a positive slope. The total binding produced a logarithmic curve, which eventually reached a steady rate of increase parallel to the slope of the straight line describing the non-specific binding. By subtracting the non-specific binding from the total binding, the specific (saturable) binding curve was produced that increased initially and then produced a plateau. An example of an isotherm of specific binding is given in Figure 2.4.

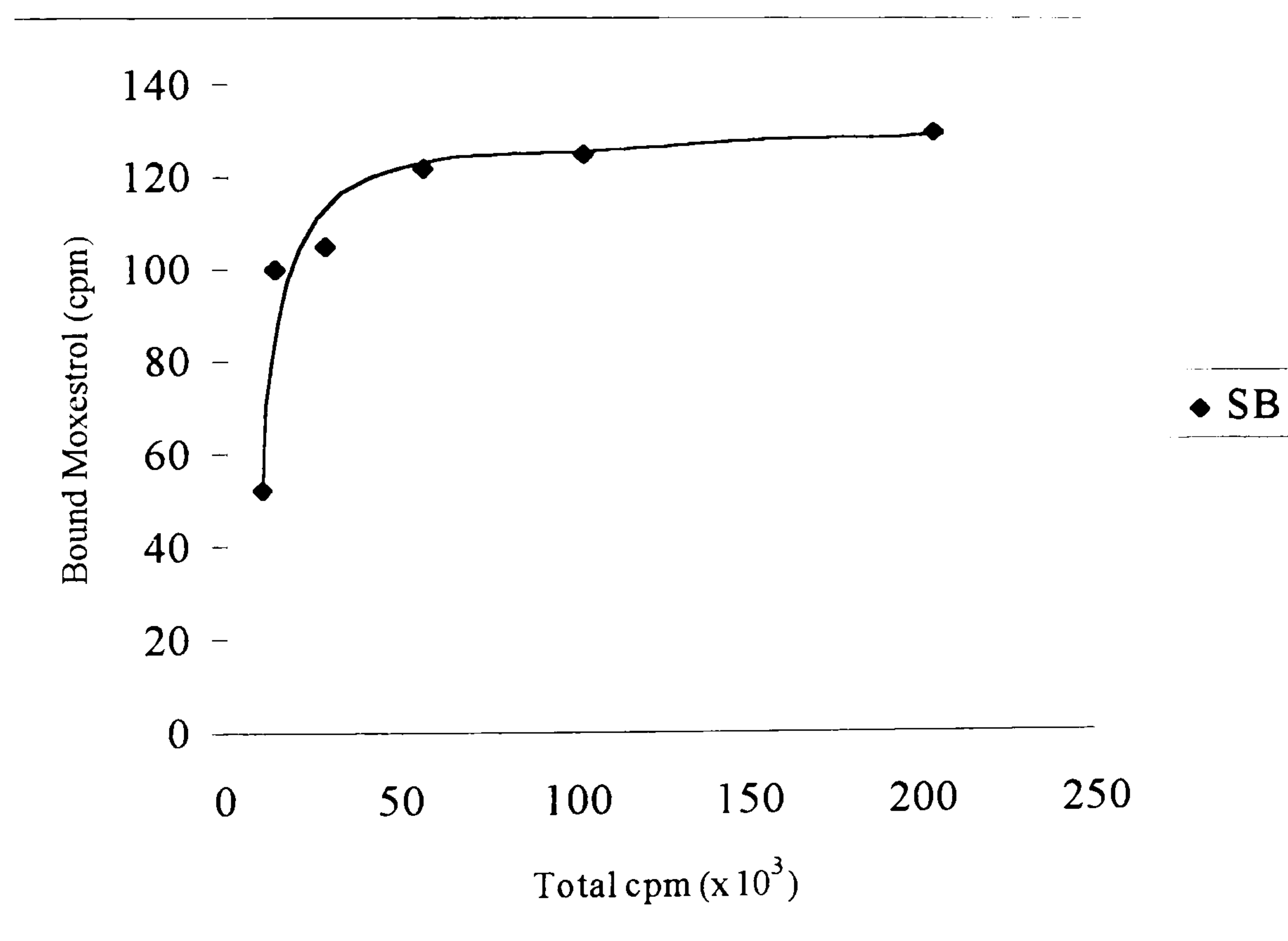


Figure 2.4: Isotherm (affinity) of the specific binding (SB) of moxestrol to oestrogen receptor in the thymus.

2.2.5.2: Determination of the factor to convert cpm moxestrol to molar concentration

If the amount of ^3H -moxestrol separated from 100 μl of incubate gave X cpm, then the amount of ^3H -moxestrol in the cytosol is 2X because the volume of cytosol is one-half the volume of incubate. The 100 μl of cytosol gives 2X cpm and 1L of cytosol gives 2×10^4 cpm. This figure is equivalent to:

$$\frac{2 \times 10^4 X}{\text{efficiency}} \text{dpm}$$

but since 1 Curie (Ci) gives 2.22×10^{12} dpm, then 1L of cytosol would contain:

$$\frac{2 \times 10^4 X}{\text{efficiency} \times 2.22 \times 10^{12}} \text{Ci}$$

and the number of moles of ^3H -moxestrol per litre of cytosol would be given by:

$$\frac{2 \times 10^4 X}{\text{efficiency} \times 2.22 \times 10^{12} \times \text{specific activity. (Ci / mole)}}$$

2.2.5.3: Transformation of binding isotherms to Scatchard plots

The parameters of the reaction between a ligand and a class (or classes) of binding sites are most readily determined from examining binding isotherm data. This information consists of measurements made at a fixed temperature of the amounts of bound ligand present under conditions of apparent equilibrium between a fixed concentration of binding sites and a range of concentrations of free ligand.

Such data, which is derived from the Law of Mass Action, were interpreted mathematically by Scatchard (1949). If each receptor binds a single ligand molecule the model for the ligand-receptor combination is given by:



Where,

L: Ligand

R: Receptor

k_{+1} The rate of the association reaction

k_{-1} The rate of the dissociation reaction

At equilibrium, the rate of the forward reaction (association) equals the rate of the backward reaction. The rate of the association reaction (V_1) is given by the equation:

$$V_1 = k_{+1}[L][R] \quad (2.2)$$

And the rate of the dissociation reaction (V_2) is given by the equation:

$$V_2 = k_{-1}[LR] \quad (2.3)$$

At equilibrium, $V_1 = V_2$, therefore:

$$K_{+1}[L][R] = k_{-1}[LR] \quad (2.4)$$

By rearranging the equation

k_{-1}/k_{+1} represents the molar equilibrium constant for the dissociation reaction:

$$\frac{[L][R]}{[LR]} = \frac{k_{-1}}{k_{+1}} = Kd \dots\dots\dots(2.5)$$

$$\frac{[L][R]}{[LR]} = Kd \dots\dots\dots(2.6)$$

Where

[L]: is the concentration of free ligand which equals the total ligand less [LR]

[R]: is the concentration of unoccupied receptor which equals the total concentration of receptor binding sites less the bound receptors [n-LR]

[LR]: is the concentration of ligand-bound receptor

k_{+1} the association rate constant,

k_{-1} the dissociation rate constant.

Taking reciprocals from equation 2. 6 and substituting [n-LR] for [R]

$$\frac{[LR]}{[L][n-LR]} = \frac{1}{Kd} \dots\dots\dots(2.7)$$

$$\frac{[LR]}{[L]} = \frac{[n-LR]}{Kd} \dots\dots\dots(2.8)$$

$$\frac{[LR]}{[L]} = n \frac{1}{Kd} - [LR] \frac{1}{Kd} \dots\dots\dots(2.9)$$

If [LR] = B, the concentration of the ligand-bound receptor

[L] = F, the concentration of the free ligand

$$\frac{B}{F} = n \frac{1}{Kd} - B \frac{1}{Kd} \dots\dots\dots 3.0$$

This equation represents the equation of a straight line of negative slope

$$Y = C - MX$$

where C is the intercept on the Y axis and M is the slope of the line.

The Scatchard equation predicts that, at a constant temperature, a plot of the ratio B/F versus B for binding of a ligand to a single class of non-interacting sites will be a straight line if the ligand molecule and binding sites are acting independently. The plot results in a straight line that has a slope of $-1/Kd$ and when extrapolated it will intercept the Y axis at n/Kd and the X axis at n (Figure 2.5).

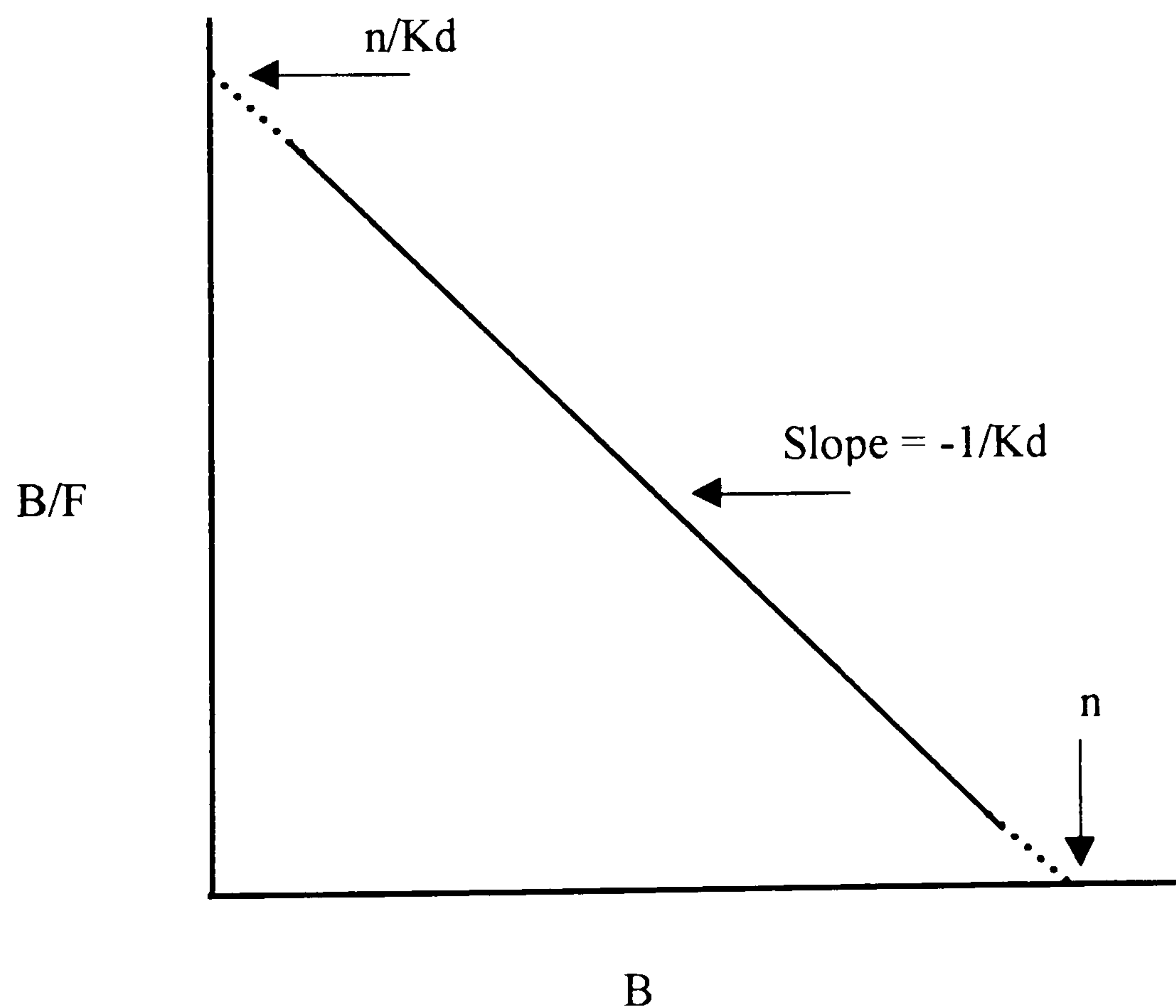


Figure 2.5: The Scatchard Plot which represents the equation $B/F = n/Kd - B/Kd$.

When such a linear relationship is shown it is indicative (though not conclusive evidence) of the presence of such independent behaviour of the reactants. This relationship may, indeed, not be independent. For example, allosteric effects may exist in which binding to one site on the macromolecule influences the probability of binding at other sites on the macromolecule. In such cases a linear relationship will not be shown between B/F and B . When the relationship is linear, then extrapolation to the Y axis yields the value of n/K_d and to the ordinate yields the value of n (or B_{\max}), the concentration of binding sites in the preparation, and nK_a , from which K_a , the equilibrium constant, can be calculated.

The slope of the line is a quantitative measure of the affinity of the binding sites for the ligand. The value of K_a describes the amount of bound complex which will form between any particular combination of binding sites and free ligand.

Certain assumptions must be made when using this model for this project:-

- (1) the ligand present is in an homogeneous form;
- (2) the ligand is univalent;
- (3) equilibrium is reached during the incubation period and bound and free ligand can be separated without disturbing this equilibrium;
- (4) the response shows a direct proportionality with the number of occupied receptors;
- (5) binding sites themselves do not interact;

(6) the reaction between ligand and receptor is simple and reversible.

If the data from the binding isotherm does not produce a straight line upon plotting B/F against B , then this indicates either:

- 1) the presence of more than one class of receptor, each of which binds specifically with the ligand at a different affinity or:
- 2) there is more than one binding site on the same allosteric molecule so that when the ligand binds to the first binding site it induces a configurational change, which results in a change the affinity of ligand at the other binding site.

2.2.5.4: Calculation of the molar dissociation constant (K_d) and the number of binding sites (n).

Each set of results of each sample was calculated by linear regression using an HP 48SX Scientific Expandable Calculator to produce the best-fit line for the Scatchard plot. The intercept of the line with the X axis gave the n value and the Y axis gave the nK_a value from which K_d was calculated by dividing n over the Y intercept. The programme is given in Appendix A.

The number of binding sites was expressed as fmoles per mg protein and the amount of protein was expressed as mg/ml of cytosol.

2.2.6: Protein Estimation

2.2.6.1: Reagents

Reagent A:

Na₂CO₃ 2 gm

NaOH 0.4gm

NaOH and Na₂CO₃ were dissolved in double distilled water and the volume made up to 100 ml.

Reagent B:

Sodium potassium tartrate 1 gm

CuSO₄.5H₂O 0.5 gm

The sodium potassium tartrate was dissolved in 80 ml double distilled water; the copper sulphate was added and the whole made up to 100 ml with double distilled water.

Reagent C:

Reagent A was mixed with Reagent B in the proportion 50:1

Reagent D:

Folin and Ciocalteu's reagent was diluted 1:1 with distilled water.

2.2.6.2: Protein standard:

Bovine serum albumin (BSA 100mg) was dissolved in 100 ml of double distilled water.

2.2.6.3: Procedure

Standard protein concentrations were prepared by adding 25 μ l, 50 μ l, 75 μ l, 100 μ l, 125 μ l, 150 μ l, and 200 μ l of 1 mg/ml BSA to 175 μ l, 150 μ l, 125 μ l, 100 μ l, 75 μ l, 50 μ l, and 0 μ l of distilled water. Samples (10 μ l) were each diluted with 190 μ l of distilled water. The blank was 200 μ l of distilled water.

3 ml of reagent C were added to each tube (sample, standard or blank) and, after mixing, the tubes were left at room temperature for 10 minutes. 300 μ l of reagent D were added to each tube, with rapid mixing, after which the solutions were left overnight at 4°C. The optical density was measured at 750 nm. The concentration of the protein in the samples was determined from a standard curve which was established by plotting the known standard protein concentrations against the optical density at 750nm (Figure 2.6).

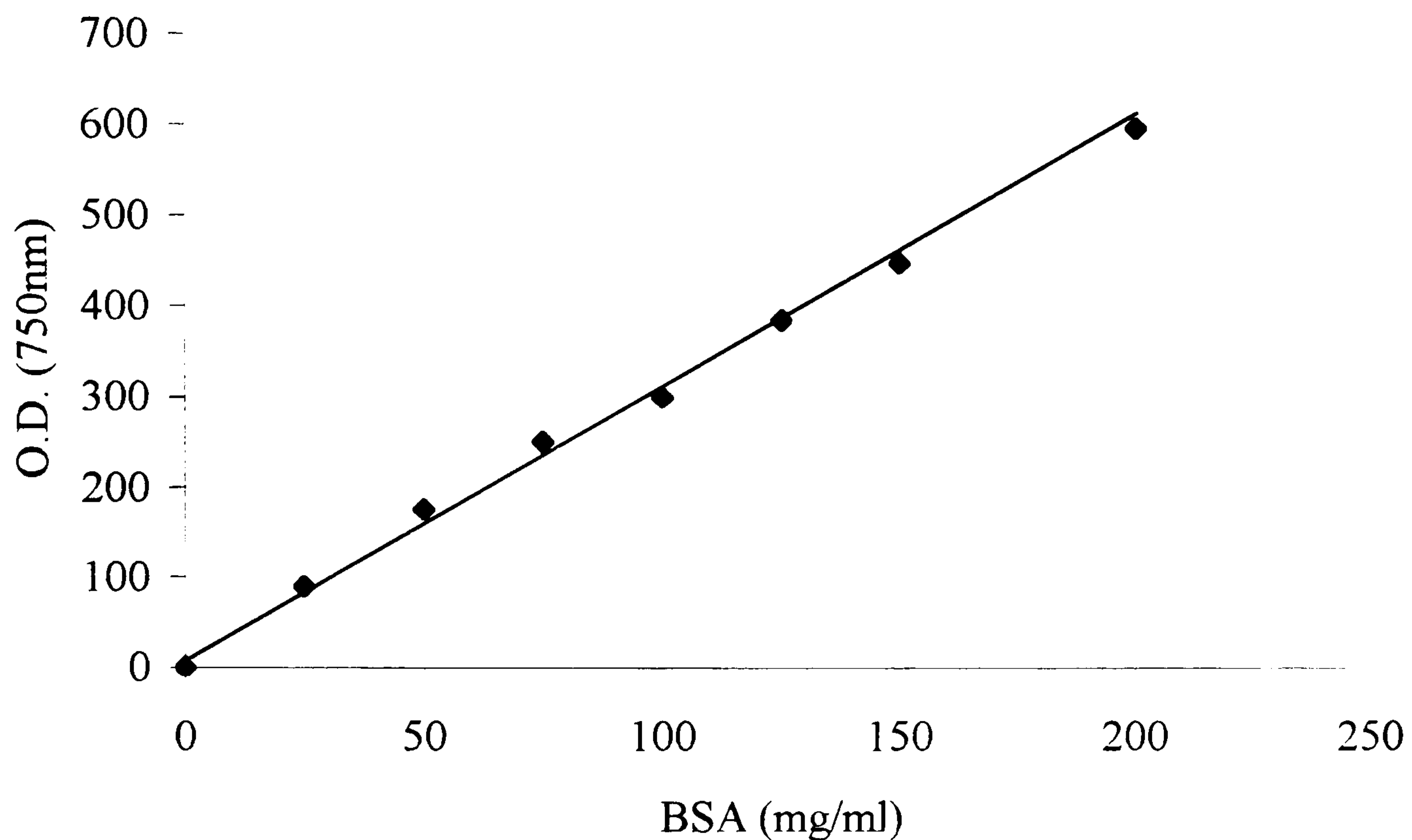


Figure 2.6: Standard curve for protein estimation.

2.3: Calibration of the Sephadex column

Experiments were performed in order to determine the void volume of the Sephadex column and in order to establish the rates of association and dissociation of the ^3H -moxestrol in the column.

2.3.1: Elution of specifically bound moxestrol

In order to establish the efficiency of the Sephadex columns for the separation of bound and free ligand it was essential to establish that the amount of buffer needed to elute the bound receptor was less than the amount needed to

elute the free hormone. The procedure for determination of the void volume was carried out as follows:

To 1 ml Buffer 2, 10 μ l 5x10⁻⁷M ³H-moxestrol were added. To 0.5 ml of this solution 5 μ l ethanol was added in order to measure the total binding (TB)(A, TB, Figure 2.7). In order to measure non-specific binding (NS), 5 μ l 5x10⁻⁵M unlabelled-moxestrol was added to the remaining 0.5 ml (B,NSB, Figure 2.7). To each of two siliconized glass test tubes 100 μ l of thymus cytosol was added with 100 μ l of either A or B and incubated for 1 hr at 30°C. At the end of this time, the tubes were placed on ice for 10 minutes and 100 μ l of each incubate was placed into a 0.45 x 9 cm LH-20 Sephadex column at 1-2°C. When the incubate had run into the column, 1.5ml Buffer 2 was added in order to elute the column. Fifteen 100 μ l fractions of eluate were collected into scintillation vials and 10ml of scintillation fluid was added to each vial, which were kept in the dark overnight before the radioactivity was counted. Most of the radioactivity appeared before the 9th fraction with a peak at the 6th fraction and background level was reached by the 9th fraction. A typical elution profile is shown in Figure 2.7.

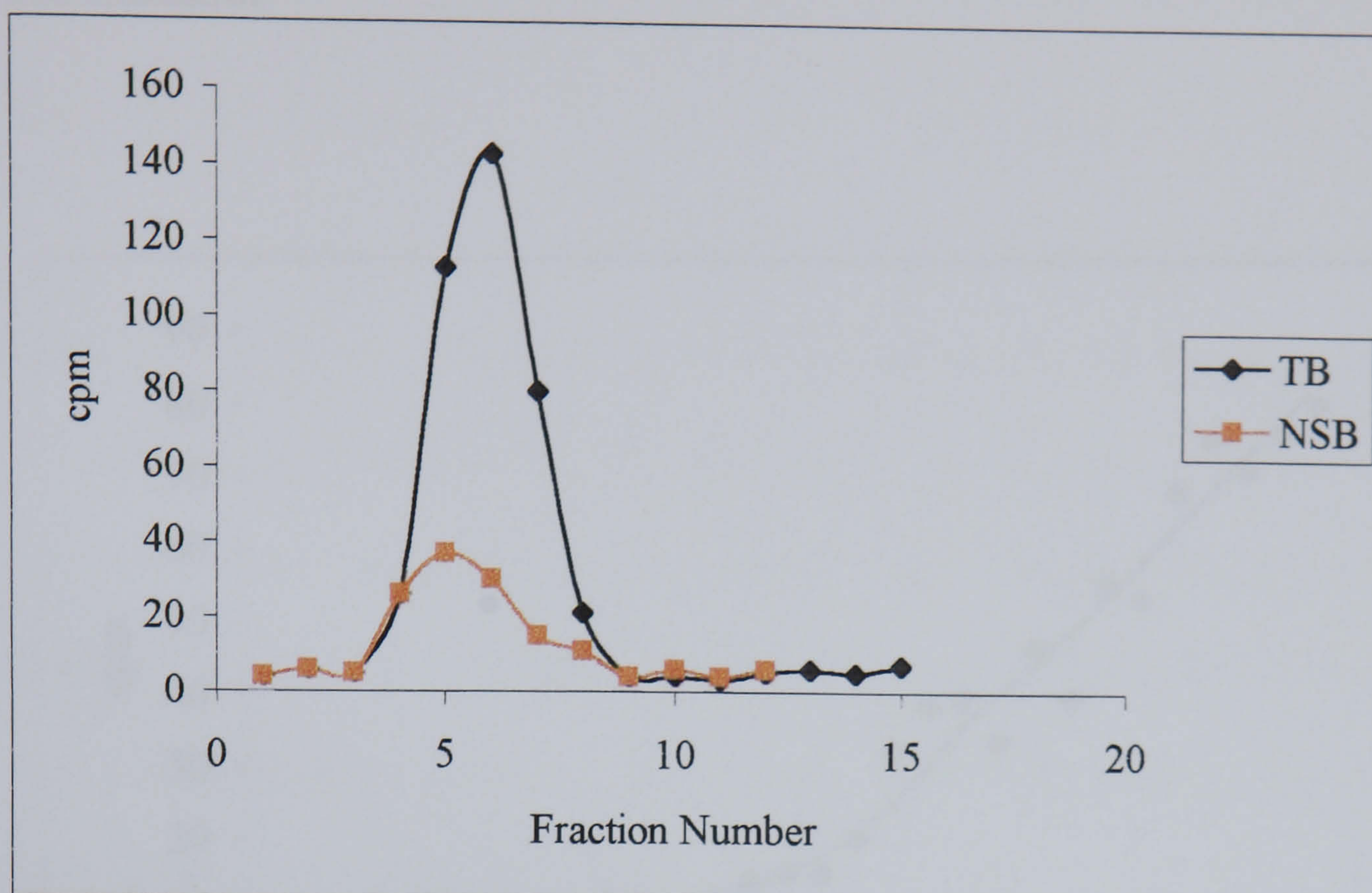


Figure 2.7: Elution of bound moxestrol. (TB = total bound, NSB = non-specific bound).

2.3.2: Elution of free moxestrol

To 1 ml Buffer 2, 10 μ l 5 \times 10⁻⁷ M ³H-moxestrol was added. 100 μ l of this preparation were put into a 0.45 x 9cm LH-20 column at 1-2°C. The column was eluted with 3 ml of Buffer 2 and 30 100 μ l fractions of the eluate were collected into scintillation vials. Scintillation fluid (10 ml) was added to each vial and the vials were kept in the dark overnight and the radioactivity was counted.

The radioactivity in the eluate was within the background range of the counter at fraction number 10 when all the bound tracer would have been eluted. However, free moxestrol was eluted from fractions 15-30 (Figure 2.8). These

experiments established that the column technology clearly separated bound and free moxestrol.

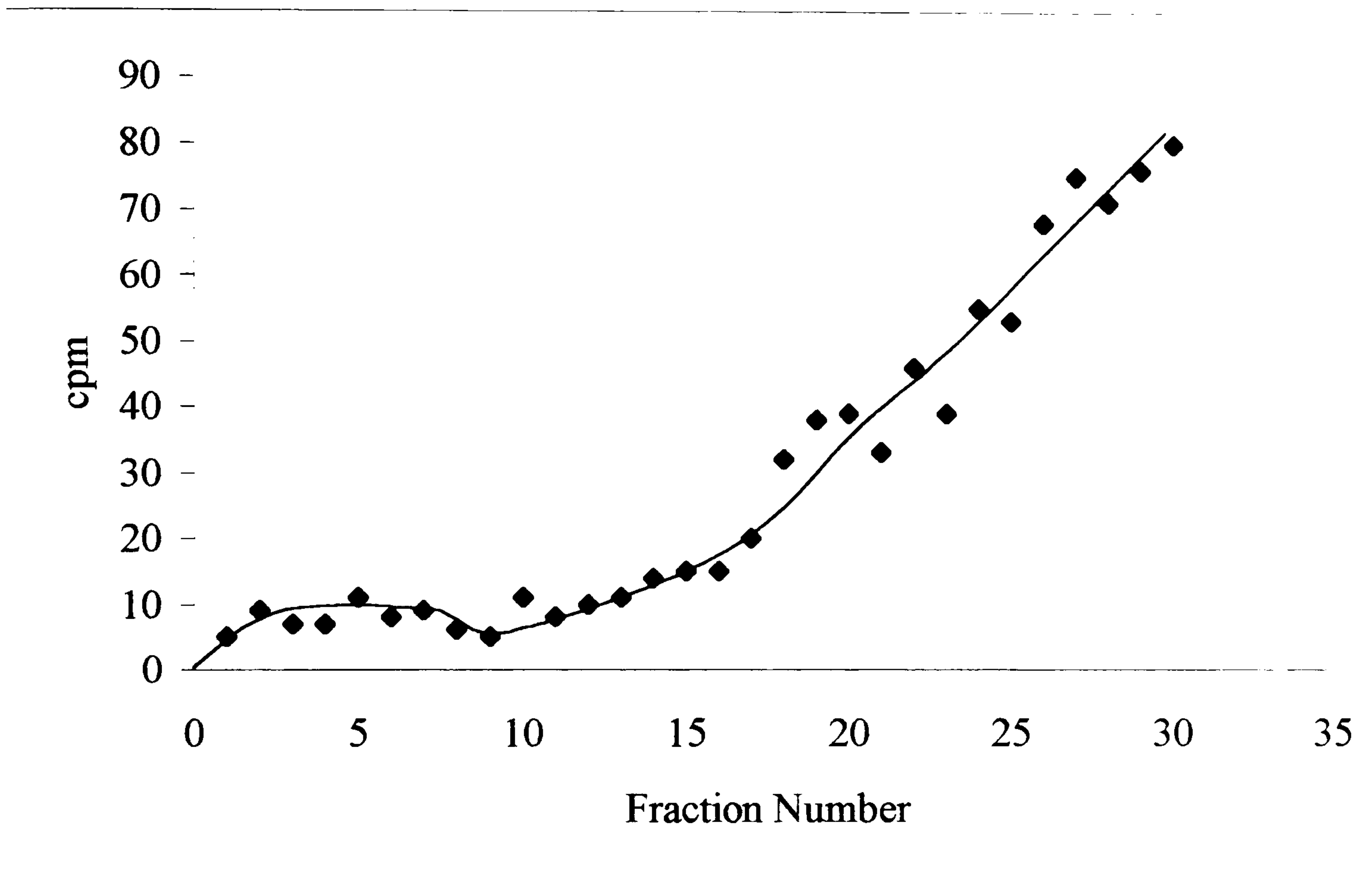


Figure 2.8: Elution curve of the free hormone from fractions eluted after the ligand-bound hormone had been eluted.

2.4: Determination of association and association rates of the binding of moxestrol to the oestrogen receptor.

2.4.1: Association study

Association studies were carried out in order to establish the time needed for equilibrium to be established in the incubation. Cytosol preparation (1ml) was placed in each of two silconized glass tubes, A and B. 15.4 ml 5×10^{-7} ^3H -moxestrol and 15.4 ml ethanol were added to tube A in order to measure total binding. To tube B 15.4 ml $5 \times 10^{-7}\text{M}$ ^3H -moxestrol and 15.4 ml 5×10^{-5} unlabelled moxestrol were added in order to estimate the non-specific binding. The tubes were incubated at 30°C for 10, 20, 30, 40, 50, 60, 70, 80, 90, and 120 minutes. At the end of each incubation period, 100 μl of the incubate was placed on the 45 x 9cm Sephadex LH-20 column at $1-2^\circ\text{C}$ and 200 μl Buffer 2 was added. After 30 minutes, the column was eluted with 800 μl of the same buffer and the eluate collected into a scintillation vial. When all the samples had been collected, 10 ml scintillation fluid was added to each vial. The radioactivity was counted after overnight storage in the dark.

Specific binding to oestrogen receptor reached a plateau after 40 minutes incubation and lasted for at least 2 hr. Observations were made three times each in the uterus, thymus, and hypothalamus (Figure 2.9).

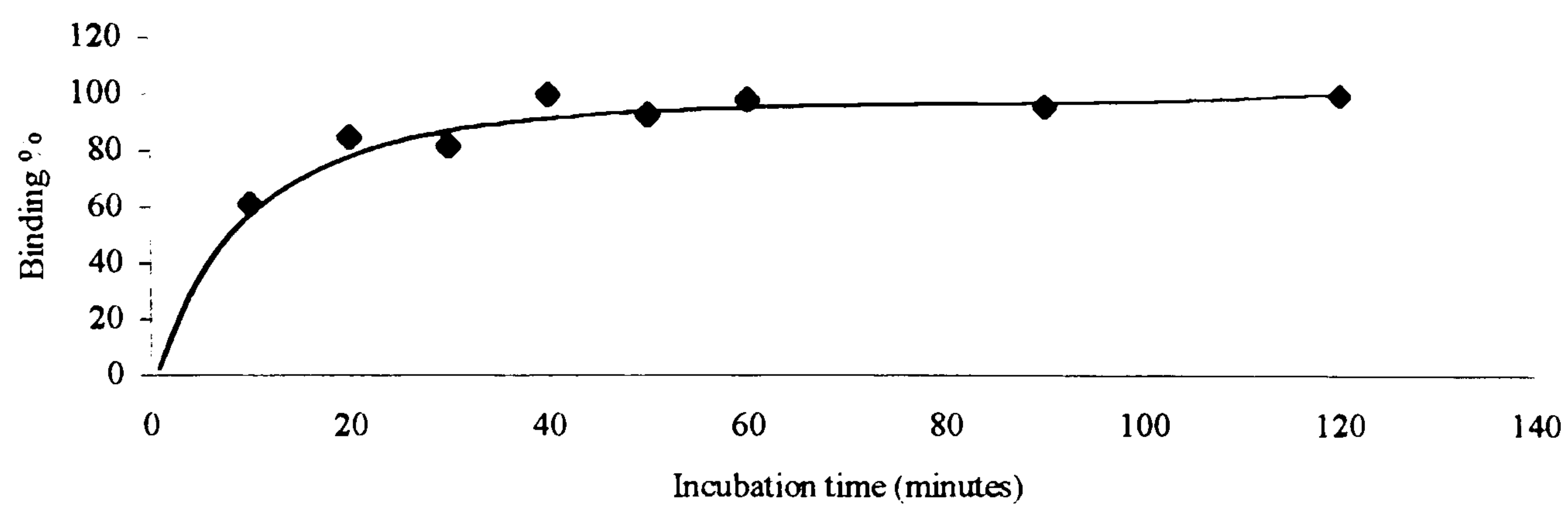


Figure 2.9a Uterus

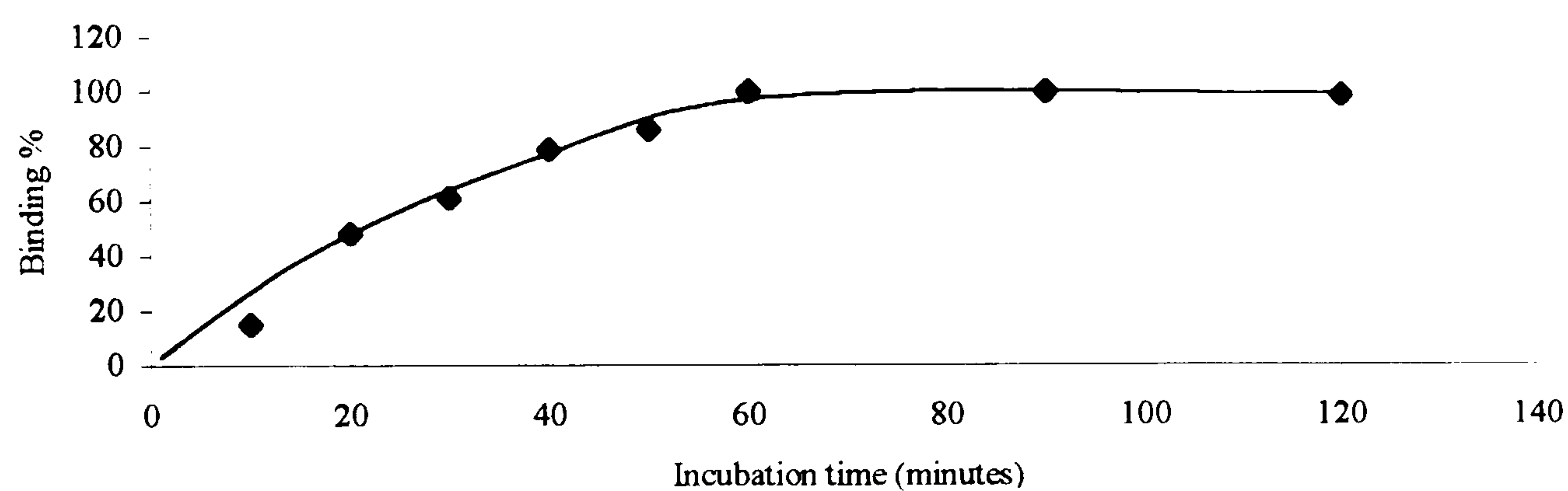


Figure 2.9b Thymus

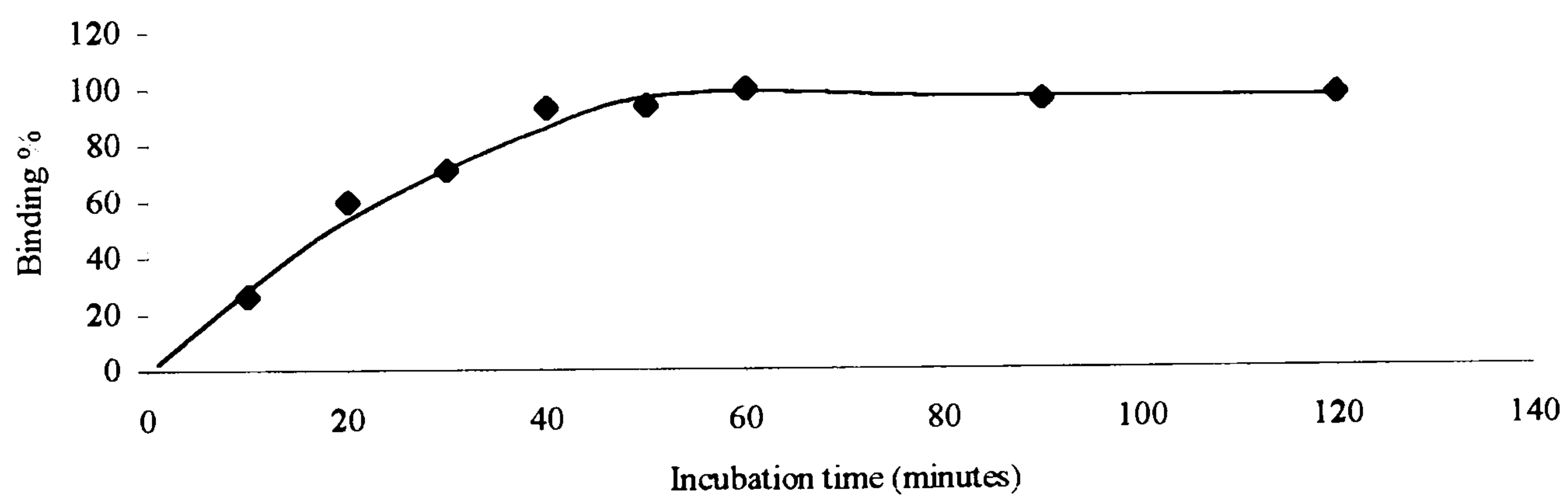


Figure 2.9c Hypothalamus

Figure 2.9: Typical association curves for cytosolic specific binding in uterus, thymus and hypothalamus.

2.4.2. Dissociation studies

Dissociation studies were also performed. Conditions for saturable binding of the oestrogen receptor with moxestrol required incubation of the samples at 30°C. The Sephadex columns used for the Scatchard analysis were kept at 1-2°C. In order to establish that the eluate collected from the columns contained all the receptor molecules saturated with the labelled ligand, the time period that the preparation could stay in the column before dissociation of ligand and receptor was investigated.

To each of two siliconized glass tubes were added 650 µl of the cytosol preparation. To one of the two tubes (A) was added 10µl 5x10⁻⁷M ³H-moxestrol and 10µl ethanol in order to measure total binding. To the other tube (B) was added 10µl 5x10⁻⁷ M ³H-moxestrol and 10µl 5x10⁻⁵M unlabelled moxestrol to measure non-specific binding. The tubes were incubated for 1 hr at 30°C. 100µl of the incubate from either tube A or tube B was placed in each of 8 Sephadex columns and washed in with 200µl Buffer 2. These samples were left on the column for 5, 15, 30, or 60 minutes before being eluted with 800 µl Buffer 1 into scintillation vials. Scintillation fluid (10ml) was added to each volume of eluate and the radioactivity was counted after overnight extraction in the dark.

The amount of specific binding remained constant over the 1 hr period examined (Figure 2.10) and three observations for each were made for uterus, thymus and hypothalamus. The animals used for the association and dissociation studies were 30 days old and no binding was detectable in the cortex of this age group.

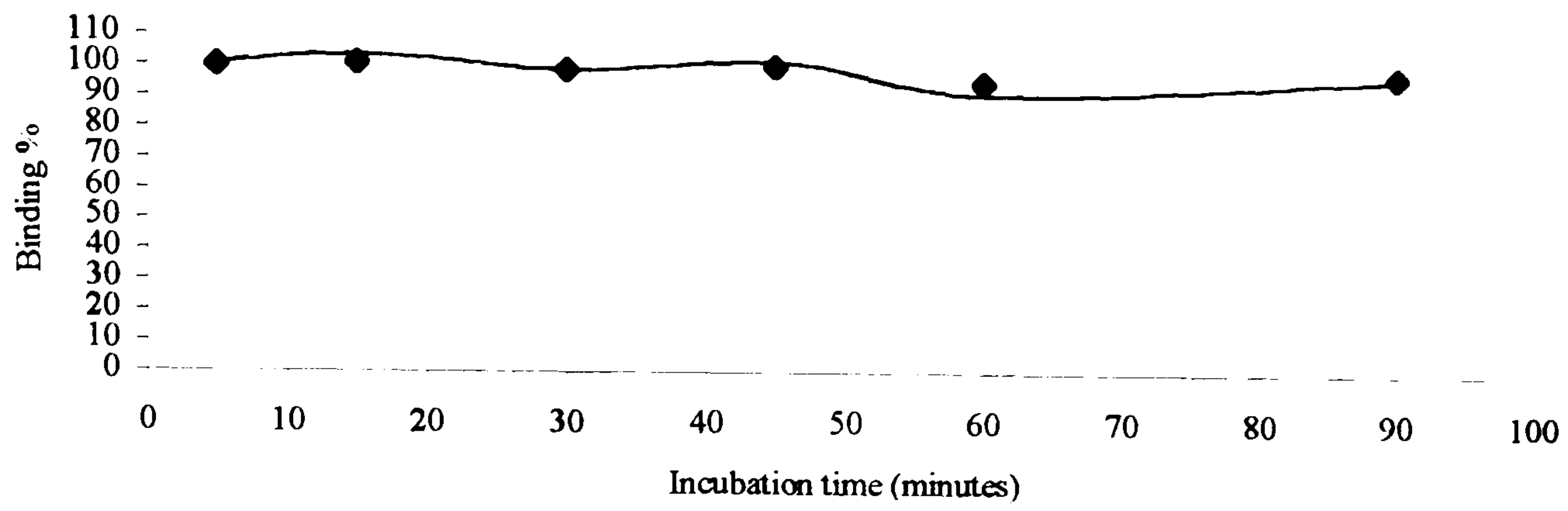


Figure 2.10a Uterus

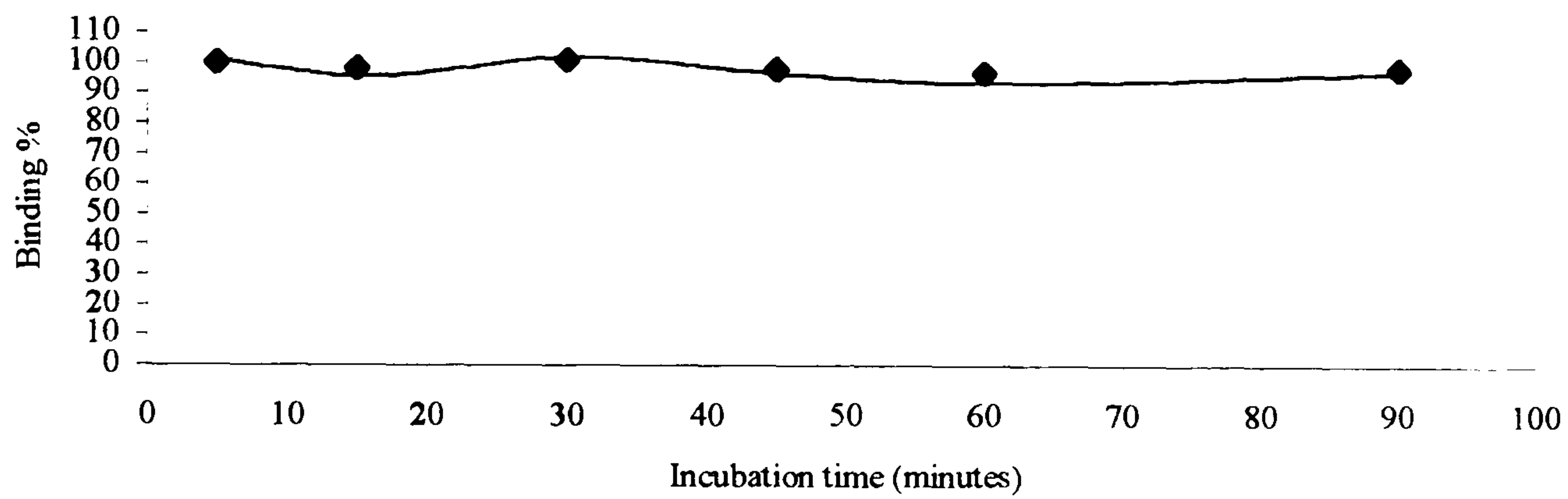


Figure 2.10b Thymus

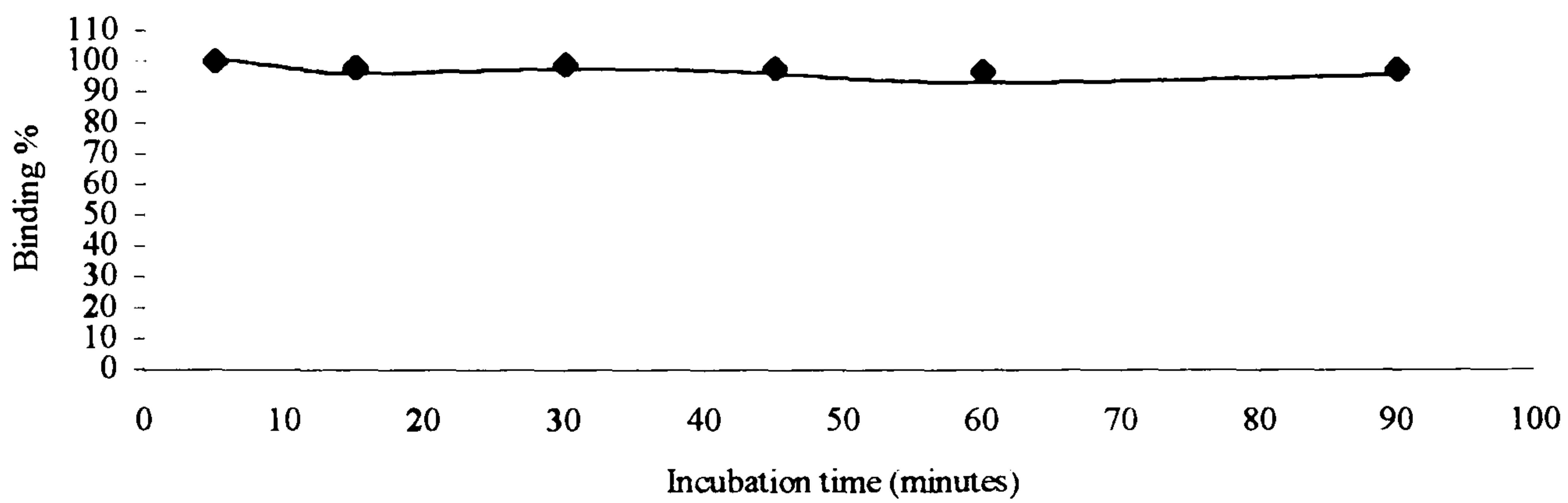


Figure 2.10c Hypothalamus

Figure 2.10: Typical dissociation curves of cytosolic specific binding in the uterus, thymus and hypothalamus.

These results established that no significant dissociation occurred during the period that the eluted hormone-bound receptor remained in the column.

2.5: Measurement of the free fraction of oestradiol in serum

The method used that of Greenstein *et al* (1977)

2.5.1: Incubation of the serum

Serum (0.8 ml) was incubated with 5 μ l (5×10^{-7} M) 3 H-oestradiol for 1 hr at 37°C.

2.5.2: Collection of fractions of serum

Sephadex G50 (fine) was suspended in Buffer 3 (previously degassed by evacuation) and allowed to equilibrate for 1hr at room temperature. Columns of the gel (0.45cm x 7cm) were prepared. The volume of 3 drops of Buffer 3 eluted from each column was measured. This volume was used to estimate the total radioactivity in 3 drops of eluate when separation of albumin-bound 3 H-oestradiol and the free hormone occurred by gel filtration.

The serum sample was placed on the column (maintained at 37°C) and allowed to permeate the gel. The column was eluted with Buffer 3 from a Mariott flask to maintain constant pressure. For each sample, 30 three-drop samples were collected into scintillation vials. 10 ml scintillation fluid were added and the samples were kept in the dark overnight before counting the radioactivity.

The steroid-bound protein eluted in fractions 4-9, and the free hormone appeared in fractions 15-22 (Figure 2.11).

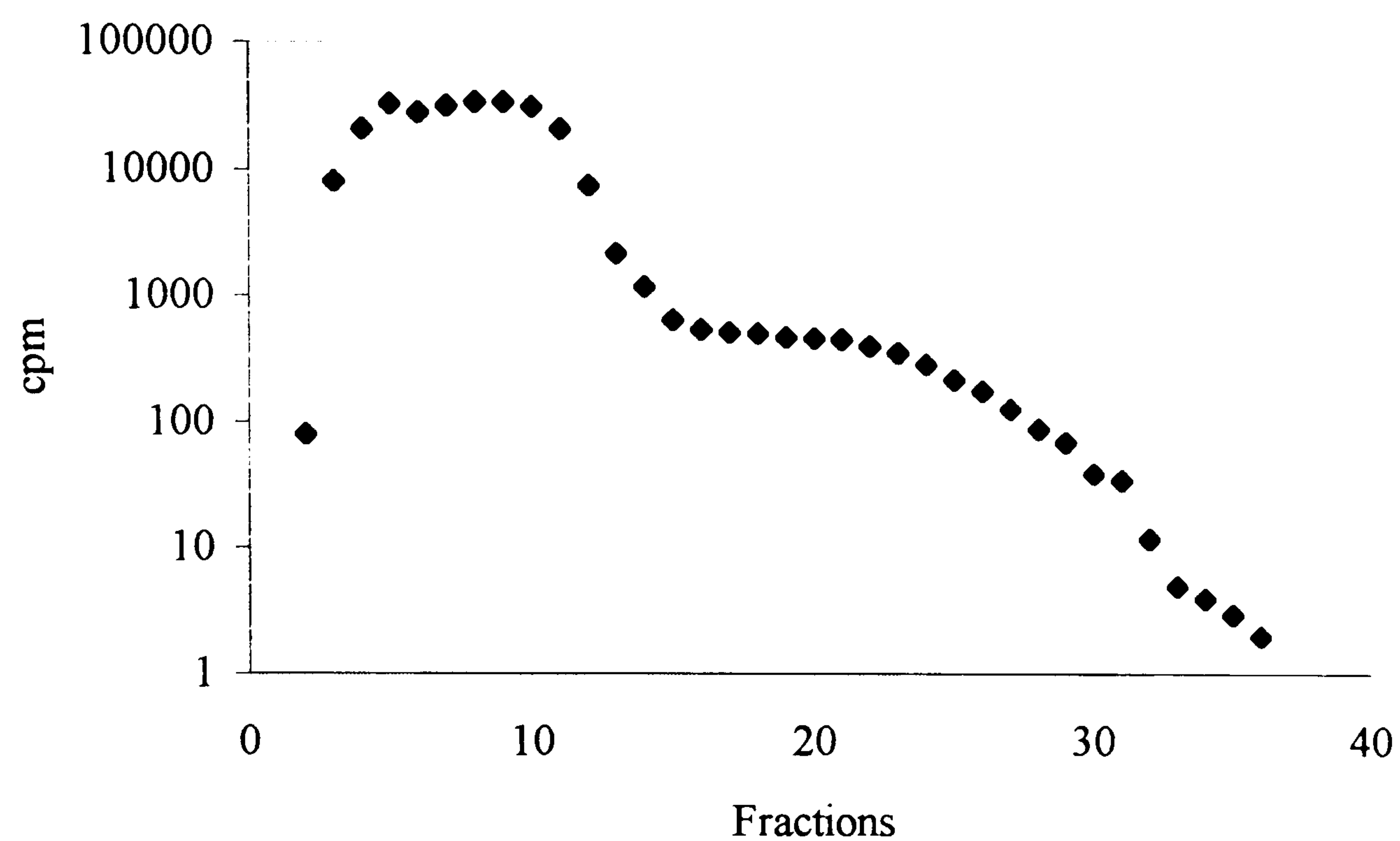


Figure 2.11 The elution profile of alpha-fetoprotein-bound ^3H -oestradiol and free ^3H -oestradiol in serum.

The ratio of the free hormone to the bound hormone was determined from this elution profile by dividing the counts per minute of the second plateau (free hormone, fractions 15-22) by the counts per minute of the first plateau (alpha-fetoprotein-bound hormone, fractions 4-9).

2.6: Statistical Analyses

Statistical analyses of the results obtained in all the experiments were undertaken using Sigmaplot. Initially, tests were run to show that variances of individual groups were equal before Two-way Analysis of Variance. Where the variances of individual groups were not equal, ANOVA on Ranks and Student-Newman-Keuls tests were used.

Chapter three

**Specificity of oestrogen-binding protein for its ligand in uterus
and thymus of females Wistar rats at 30 days**

3.1: Introduction

Oestradiol, in common with other steroid hormones, exerts its effects on its target tissues through a primary association reaction with a specific intracellular receptor. The hormone-receptor complex binds to specific oestrogen-responsive elements to initiate transcription of the target genes (reviewed by Greenstein, 1978). In the uterus, the classic site for oestrogen activity, many studies have shown that this hormone-binding protein is a high-affinity, low capacity receptor that is highly specific for its ligand, oestradiol. (Jensen *et al*, 1968).

Several studies have established the specificity of the oestrogen receptor in uterus and in brain in many species (Korenman 1968; 1969; 1970; de Sombre *et al*, 1971; Sholl and Kim, 1989; Handa *et al*, 1991). The specificity of the receptor in these tissues has been tested against a range of steroidal and non-steroidal compounds in order to establish whether there may be heterogeneity of oestrogen receptor in different tissues or tissue-specific oestrogens or anti-oestrogens (Ginsburg *et al*, 1977). Similar work has not been carried out, thus far, on the specificity of oestrogen receptor in the thymus of immature rats, although many studies have confirmed the presence of a high-affinity, low capacity oestrogen-binding protein in thymus from humans (Nilsson *et al*, 1984), rats, mice and cattle (Grossman *et al*, 1979a). Numerous observations suggest that this organ is responsive to oestradiol (Stimson, 1980; Grossman *et al*, 1982; Pearce *et al*, 1983). The specificity of the receptor for the different competitors examined was assessed as the ability (potency) of the competitor to alter the apparent affinity the receptor for oestradiol. The greater the apparent lowering

of the affinity constant of the receptor for oestradiol, the greater the specificity of the receptor for the competitor in question. If, for example, the receptor had no affinity for the putative inhibitor/competitor, the apparent affinity of the receptor for oestradiol would not be altered in the presence of the compound in question.

It is well known that the female of various mammalian species has enhanced immunological reactivity relative to the male (Rhodes *et al*, 1969; Inman, 1978; Masi and Kaslow, 1978). Moreover, the reticuloepithelial stroma cells in the thymus of rats produce immunoregulatory factors whose synthesis is influenced by oestrogens, progesterone, corticosteroids and androgens (Stimson *et al*, 1980). Oestradiol administration causes thymic regression in male and female rats, an effect that can be blocked by inhibition of aromatase (Greenstein *et al*, 1988b, 1992b). As the thymus is responsive to steroid hormones, this study was undertaken in order to determine whether the oestrogen-binding protein in the thymus shows the same or similar specificities as the oestrogen receptor in uterus of immature female Wistar rats.

3.2: Materials and Methods

3.2.1: Animals

Immature female Wistar rats, aged 27 days, were purchased from Bantin and Kingman and kept 5 per cage (56 x 38 x 20 cm) in the Biological Research Services Facility in the Rayne Institute, St.Thomas's Hospital. They were kept under conditions of controlled lighting and temperature, (lights on, 08:00h-22:00h; lights off, 22:00h-08:00h; 19-20°C) and were allowed free access to BK rodent diet which is steroid free, and to tap water. The concentration of oestrogens in tap water was 1.47pmole (0.004 ppm) (Thames Water Authority).

This concentration was below the level that would be likely to have an effect on the animals in these studies (Beigel *et al*, 1998).

3.2.2: Preparation of the cytosols

When the rats reached 30 days, they were anaesthetised and sacrificed by cervical dislocation, in accordance with United Kingdom Home Office regulations and as described in Sections 2.1 and 2.2. Uteri and thymi were dissected as described in Section 2.2.3. Individual tissues were placed in 1.0 and 1.5 ml, respectively, of ice-cold Buffer 1. Tissues were not further separated into subtypes (e.g. thymocytes and thymic epithelial cells). The tissues were homogenized as described in Section 2.2.3 and the resulting homogenate was centrifuged at 105,000g (42,000 rpm) in a Beckman L8-M ultracentrifuge using a fixed angle aluminium rotor for 1 hr at 4°C. The supernatant, which contained the soluble cell proteins, was decanted into plastic tubes and frozen at -70°C prior to assay which occurred within two months of preparation. Nuclei remained intact during the separation (tested by microscopy) but were not collected.

3.2.3: Preparation of non-radioactive steroids for competition studies

Stock solutions of the steroids were made up in absolute ethanol in the following concentrations (Table 3.1).

Table 3.1: Concentrations of stock solutions of the steroids used in competition studies.

| Compound | Concentration (M) |
|------------------------------------|---------------------|
| Oestradiol-17 β (Oestradiol) | 1×10^{-9} |
| Oestradiol-17 α | 10×10^{-6} |
| Oestrone | 10×10^{-6} |
| Oestriol | 10×10^{-6} |
| Diethylstilboestrol | 20×10^{-6} |
| Testosterone | 20×10^{-6} |
| Corticosterone | 20×10^{-6} |
| Progesterone | 20×10^{-6} |

3.2.4: Measurement of the binding of oestradiol in the presence of competitors

Cytosols were incubated with ^3H -oestradiol in the presence and the absence of a 100-fold excess of unlabelled oestradiol. Cytosol (0.1ml) was incubated with 0.1 ml of Buffer 2 (Section 2.1.4) together with one of a range of final concentrations of ^3H -oestradiol (0.03 - $1.0 \times 10^{-9}\text{M}$) 1hr at 25°C . Parallel

incubations were carried out in the presence of a 100-fold excess of unlabelled oestradiol. In order to keep the ethanol content of the incubations equal, ethanol was substituted for unlabelled oestradiol in those incubations which contained only the tritiated steroid.

In order to measure the binding of unlabelled competitors in the high-affinity oestradiol binding system, parallel incubations were carried out in the presence of competitors in final concentrations listed in Table 3.2. These concentrations were chosen because previous work by Ginsburg *et al* (1977) had established that these concentrations produced only partial inhibition of the high-affinity binding of ³H-E2 to any uterus or brain cytosol fraction at concentrations up to 10⁻⁶ M (Table 3.2).

Incubations were carried out in a shaking water bath at 25°C for 1 hr. Separation of bound and free ³H-E2 was achieved using gel filtration as described in Section 2.2.4. In this case, however, because tritiated oestradiol was used, 6 cm columns of Sephadex LH 20 were used (Ginsburg *et al*, 1974) and only 5 ml scintillation fluid was added to the vials before overnight extraction and counting.

Table 3.2: Final concentrations in the incubations of steroids used in competition studies.

| Compound | Concentration (M) |
|------------------------|----------------------|
| Oestradiol-17 β | 5×10^{-10} |
| Oestradiol-17 α | 2.5×10^{-8} |
| Oestrone | 2.5×10^{-8} |
| Oestriol | 2.5×10^{-8} |
| Diethylstilboestrol | 1×10^{-10} |
| Testosterone | 1×10^{-7} |
| Corticosterone | 1×10^{-7} |
| Progesterone | 1×10^{-7} |

3.2.5: Construction of binding isotherms for [^3H]-oestradiol in the absence and in the presence of other steroids.

The Edsall and Wyman (1958) equation states that for competitive interaction between a ligand and an inhibitor at a single class of binding site,

$$\frac{1}{1 + K_i I} = nK_i \dots\dots\dots(1)$$

where B = the concentration of ligand / macromolecule complex
 K_a = the equilibrium association constant of the reaction

F = the concentration of free ligand

K_i = the equilibrium association constant of the
receptor-inhibitor

I = the concentration of free inhibitor

By rearrangement, an expression similar in form to the Scatchard equation can be obtained:

$$\frac{B}{F} = \frac{K_a (n - B)}{1 + K_i I} \dots\dots\dots(2)$$

Comparison of this expression with the Scatchard equation :

$$\frac{B}{F} = K_a (n - B) \dots\dots\dots(3)$$

shows that the sole effect of a fixed concentration of competitive binding inhibitor on the binding of a ligand to a single class of non-interacting binding sites will be to decrease the slope of the plot by a factor of $\frac{1}{1 + K_i I}$. The rectilinear nature of the plot and the intercept where $B/F=0$, at the binding capacity of the system, will not be affected. If, however, binding inhibition is due to non-competitive interaction, the Scatchard plot in the presence of the inhibitor will be curvilinear as $\frac{K_a}{1 + K_i I}$ will vary with ligand concentration.

At least four experiments were carried out with each inhibitor in which Scatchard plots were constructed from five values of bound and free oestradiol in the presence and in the absence of fixed concentrations of the inhibitors. In each case, the compounds inhibited the binding of oestradiol and the Scatchard plot was shifted by a linear reflection about the intercept on the abscissa according to the prediction for competitive interaction.

The dissociation constant for the inhibitor (K_{di}) can be calculated if K_a and n are known together with values of F and B in the presence of inhibitor from the following equation derived from the Edsall and Wyman equation:

$$K_{di} = \frac{1}{nK_a \frac{F}{B} - (K_a F + 1)} \dots\dots\dots(4)$$

The intercept on the ordinate, nK_i , is related to nK_a by the same factor therefore,

$$\frac{n K_a}{1 + K_i I} = nK_i \dots\dots\dots(5)$$

This plot represents the two equations:

a) $B/F = K_a (n - B)$

b) $B/F = K_i (n - B)$

and the relationship between these two given is given above in equation 5.

3.2.6: Statistical analyses.

Regression analysis was used to obtain a measure of the slope and the coefficient of correlation for each Scatchard plot obtained. Plots for which the correlation coefficient was less than 0.75 were not used in the results. Statistical analyses of the results were undertaken, using Sigmaplot, testing that there were no significant differences in the intercept (n) on the x axes of the data obtained.

3.3: Results

Tissues from immature female rats were used for this study since, in these animals, the concentration of circulating oestrogen that might interfere in the experiments was low and the thymus was large. Moreover, atrophy of this tissue, which is associated with increasing levels of steroids in older animals, has not begun and circulating oestrogen-binding protein, alphafetoprotein, which would interfere in the binding reaction, has disappeared from the bloodstream by this age (Raynaud *et al*, 1971; Greenstein *et al*, 1992).

At the concentration of each putative competitor used in this study, Ginsburg *et al* (1977) showed that only partial inhibition of the high-affinity binding of [³H]-oestradiol occurred in cytosol incubate at a concentration of 1.0×10^{-9} M. Isotherms were constructed for the binding of [³H]-oestradiol to the high-affinity sites in uterus and in thymus tissue cytosols in the presence and absence of these chosen concentrations of the compounds.

The Scatchard analysis obtained from both thymus and uterus yielded straight lines, a result that is consistent with the presence of only one class of binding site. Figure 3.1a and b are representative plots from which it can be seen that the inhibitor depressed the slope of the Scatchard plots. Nevertheless, the line passed through the abscissa at a point that was not apparently different from that obtained in the absence of the inhibitor. This point is the binding capacity (n) and gives a measure of the concentration of bound receptors at full binding capacity of the system. The result (no change in value of n, alteration of Kd) provides evidence that the tested steroid was showing inhibition that was

competitive in nature. All of the steroids tested exhibited competitive antagonism to binding of tritiated oestradiol.

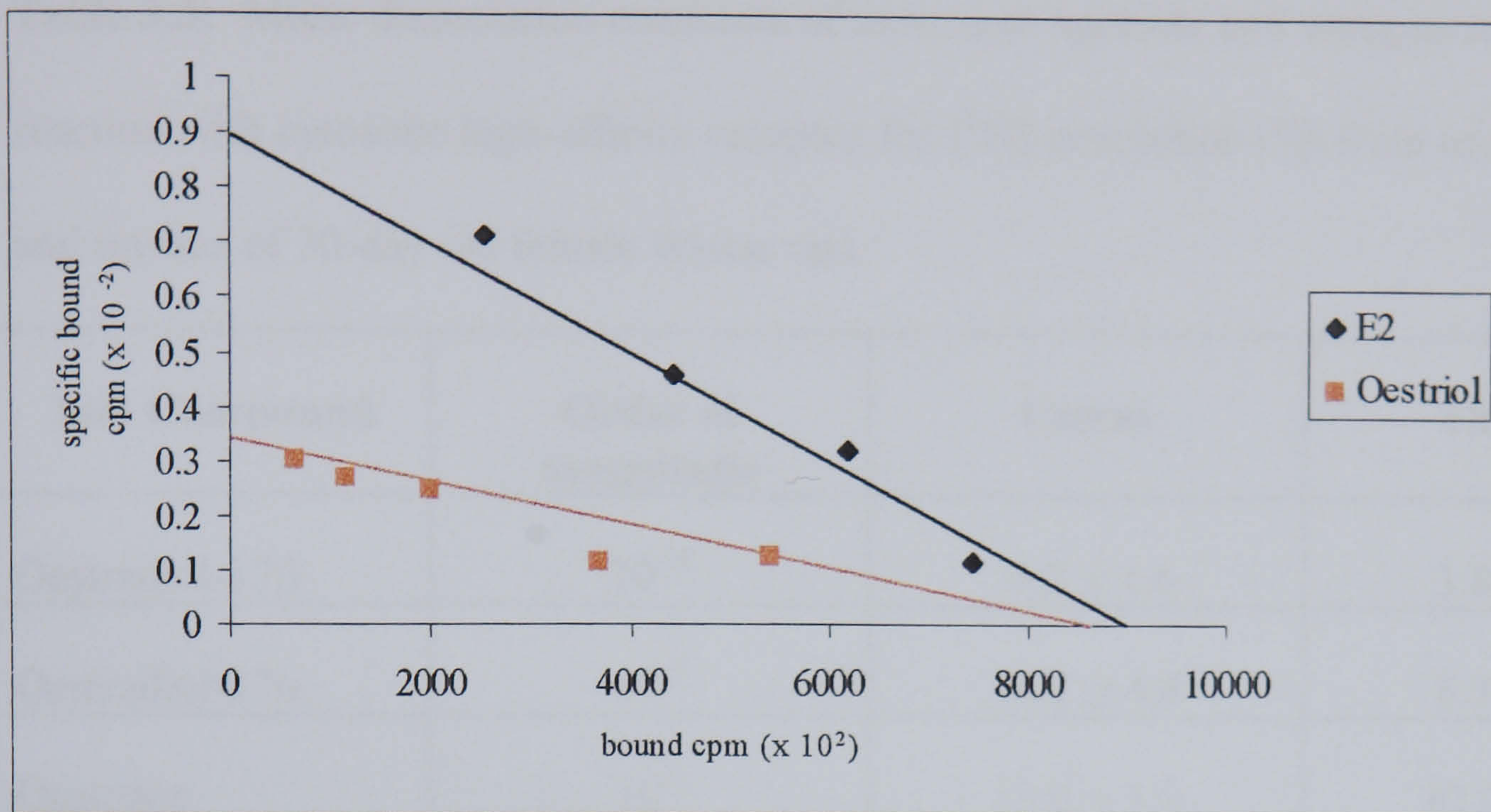


Figure 3.1 a: Inhibition of ^3H -oestradiol binding by oestriol (2.5×10^{-8} M) in female Wistar rat uterus cytosols.

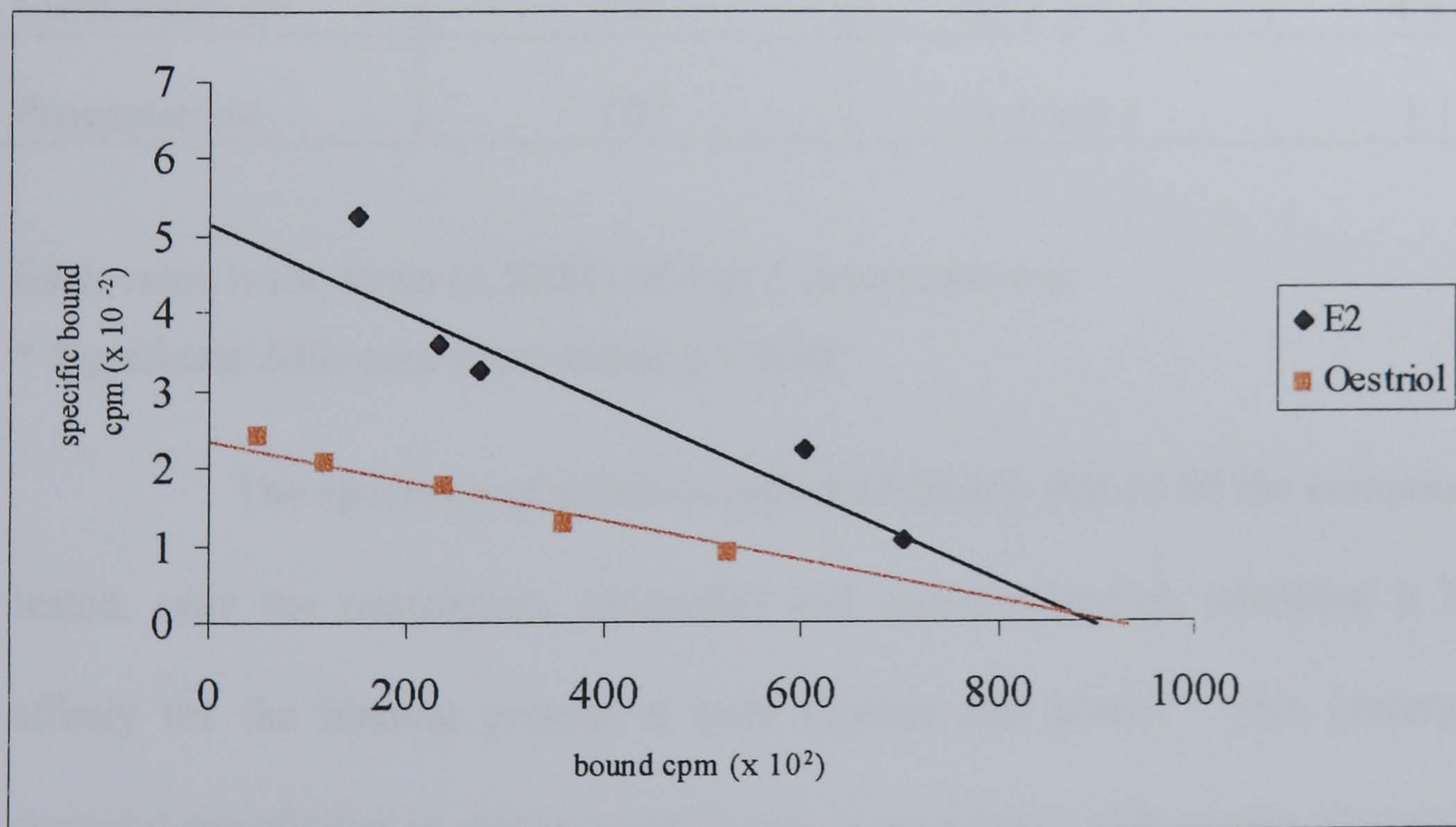


Figure 3.1 b: Inhibition of ^3H -oestradiol binding by oestriol (2.5×10^{-8} M) in female Wistar rat thymus cytosols.

The mean value of the K_d values obtained using thymus and uterus cytosols are shown in Table 3.3.

Table 3.3: Molar dissociation constants of oestrogen agonists and antagonists in reaction with cytosolic high-affinity receptor for [³H]-oestradiol-17β from uterus and thymus of 30-day old female Wistar rats.

| Test Compound | Order of magnitude | Uterus | Thymus |
|---------------------|--------------------|------------|------------|
| Oestradiol-17β | 10 ⁻¹⁰ | 4.2 ± 1.6 | 3.8 ± 1.7 |
| Oestradiol-17α | 10 ⁻⁹ | 12.2 ± 4.4 | 8.3 ± 1.2 |
| Oestrone | 10 ⁻⁹ | 13.0 ± 3.0 | 12.0 ± 4.3 |
| Oestriol | 10 ⁻⁹ | 14.3 ± 3.3 | 8.1 ± 1.2 |
| Diethylstilboestrol | 10 ⁻¹⁰ | 2.7 ± 1.1 | 0.5 ± 0.1 |
| Testosterone | 10 ⁻⁸ | 5.5 ± 1.6 | 4.4 ± 2.2 |
| Corticosterone | 10 ⁻⁸ | 33.3 ± 1.1 | 4.4 ± 1.9* |
| Progesterone | 10 ⁻⁷ | 1.5 ± 0.2 | 1.1 ± 0.7 |

Each value is the mean (± SEM) of 4 or 5 determinations.

* Significant difference from uterus p < 0.05.

The spectrum of affinities indicated clearly that of all the compounds tested, only the oestrogens, oestradiol and diethylstilbestrol, exhibited a high affinity for the binding protein in both thymus and uterus. This pattern of steroidal specificities in uterus cytosols was in agreement with results obtained by others (Ginsburg *et al*, 1977). The dissociation constants of the compounds for the receptor ranged from 10⁻¹⁰M to 10⁻⁷M in uterus and in thymus. In both tissues, the order of dissociation constants, from lowest to highest was,

oestradiol \equiv diethylstilbestrol (thymus and uterus), $<$ oestradiol-17 α \equiv oestrone \equiv oestriol (thymus and uterus), $<$ corticosterone (thymus), $<$ corticosterone (uterus), $<$ progesterone \equiv testosterone (thymus and uterus). In both thymus and uterus, the dissociation constant for oestradiol and diethylstilbestrol showed similar values of the order of 10^{-10} M which is consistent with known circulating concentrations of the hormone (Brown-Grant *et al*, 1970). The dissociation constants of oestrone, oestriol, and oestradiol-17 α , which has a hydroxyl group at position 17 oriented in the opposite plane from that of oestradiol, were of the order of 10^{-9} M. This value was the same for both tissues and was significantly higher than those for oestradiol and diethylstilbestrol. The dissociation constants for testosterone and progesterone were of the order of 10^{-8} M and 10^{-7} M and appeared higher in uterus and thymus than those of the other compounds. However, these values were significantly different ($p < 0.05$) only from those of oestradiol and diethylstilbestrol in both tissues. The affinity of corticosterone for the thymus receptor was significantly higher ($p < 0.05$), ($K_{di} = 33.3 \times 10^{-8}$ M), than the value obtained for the uterus ($K_{di} = 4.4 \times 10^{-8}$ M). The differences in the relative affinities of the receptor for the compounds tested could reflect differences in the expression of the two oestrogen receptor sub-types, alpha and beta, in these two tissues

3.4: Discussion

The receptor for the compounds tested in this study was oestrogen-selective in the thymus and in the uterus and appeared to be similar though not necessarily identical in both tissues. The results obtained were consistent with the possibility that the thymus does possess high-affinity oestrogen receptors

although this study could not distinguish between alpha and beta subtypes of the receptor in the thymus or in the uterus. The molar dissociation constant for oestradiol in the thymus was of the order of 10^{-10} M which was compatible with the known physiological concentrations of circulating hormone (Brown-Grant *et al*, 1970). This value was similar to oestradiol binding in adult male (Brodie *et al*, 1980) and adult female rat thymus (Morgan and Grossman, 1985). Nilsson *et al*, (1984), characterising the receptor in human tissue, showed that the Kd for oestrogen in the thymus and uterus are similar and also of the order of 10^{-10} M. In contrast to the results in rats in this present study, they found that in human thymus, diethylstilbestrol has a lower affinity for the oestrogen receptor than does oestradiol, although the affinity in uterus of these two compounds is similar. Studies of the specificity of the oestrogen-binding protein in the thymus have not previously been undertaken in the immature rat, but the pattern of steroidal specificities in uterus cytosols was in broad agreement with results obtained by others in rats (Ginsburg *et al*, 1977) and New Zealand B/W F1 mice (Athreya *et al*, 1989).

Similarities were found in uterus and thymus in the rank orders (that is to say, the potency of the compound to cause a decrease in the apparent Kd of the receptor for oestradiol) of the affinities of the steroids studied. The molar dissociation constant for oestradiol in the uterus was $4.2 \pm 1.6 \times 10^{-10}$ M; the value for thymus was $3.8 \pm 1.7 \times 10^{-10}$ M. The values found were also in agreement with earlier reports of specificities of receptor binding in uterus (Ginsburg *et al*, 1977) and in the thymus of male rats for oestradiol (Brodie *et al*, 1980).

From this evidence it could be argued that circulating oestradiol of gonadal origin may act directly on the thymus. The dissociation constant for the binding reaction was in good agreement with the values reported by others for oestrogen receptors (Grossman, *et al* 1979a) and was sufficiently low to exclude the possibility that an alphafetoprotein-like molecule was involved (Radanyi *et al*, 1977). That oestrogen can stimulate the thymus to release a humoral factor is confirmed because this effect is abolished in oestradiol-treated, thymectomized rats (Brodie *et al*, 1980). Moreover, cytosolic oestradiol receptors are localised in the thymic medulla, a site commonly associated with the synthesis of immunoregulatory hormones (Grossman *et al*, 1979b; Grossman *et al*, 1982). The target for oestrogen action could be the reticuloepithelial cells of the thymus. Plasma concentrations of oestradiol in the physiological range inhibit thymus development in immature rats, alter thymocyte responsiveness to mitogens (Gilbody *et al*, 1992), and inhibit thymus regeneration after orchidectomy of ageing male rats (Greenstein and Fitzpatrick, 1988a). The effects of oestradiol are probably complex since it may actually cause proliferation of certain cortical elements of the thymus (Glucksman and Cherry, 1968).

The results of this study showed that the thymus and uterus had similar spectra of affinities for all of the compounds tested with the surprising exception of corticosterone. For this compound, the thymus cytosolic receptor had an affinity which was ten-fold higher than that of the uterus. Possible experimental artefacts should be considered in seeking to explain the observed difference between the binding constant of the receptor for corticosterone in the uterus and thymus. It is possible that endogenous inhibitors were present in uterus cytosols

that interfered with the binding reaction of corticosterone in this tissue. This source of artifact seems unlikely, however, because the effect should have been present in all estimates for uterine binding and should have affected all the estimates of uterus K_{di} in a similar way. The same argument can be used to discount the influence of errors in the estimation of unbound oestradiol for the basis of the calculation of K_{di} rests on the assumption that equilibrium between receptor and inhibitor had been reached within the period known to be adequate for the attainment of oestradiol-receptor equilibrium. This period had been established in preliminary experiments (Section 2.4.1).

There may be tissue-specific factors in thymus or in uterus that modify the affinity of corticosterone for the receptor in each of these tissues. Thus, the apparent specificity of the oestrogen receptor would differ slightly in the two tissues. This phenomenon is not unknown in other receptor systems. For example, alterations to androgen receptor affinity caused by mutations have been found in human prostate patients with complete or partial androgen insensitivity syndrome (Lubaka *et al*, 1989) and the affinity of androgens for their receptors differs between brain and prostate (Barley *et al*, 1975).

The result that the thymus receptor exhibited a higher affinity for corticosterone, the steroid for which the only significant difference was found, than did the uterus receptor cannot be explained at present. The method of preparation of the cytosols may have contributed to the difference, although this cause seems unlikely since the same buffer was used for both tissues, and both tissues were processed in parallel during the same assay procedures. It is also possible that the receptor may not be the same in the two tissues. There may also

be heterogeneity of oestrogen receptors since a variant oestrogen receptor mRNA has been described in human mammary tumours (Garcia *et al*, 1988) and a mutated oestrogen receptor has been reported in a murine transformed Leydig cell line (Hirose *et al*, 1991). The discovery of a second oestrogen receptor (ER β) (Kuiper *et al*, 1996) could also have implications for the observed differences in corticosterone affinity in the two tissues. These differences may reflect different structures for the receptors, and different responses to the ligand. Differential oestrogen receptor subtype distribution in reproductive, brain and immune tissues could account for the ability of oestrogens to exert tissue specific effects. In addition to distribution ratio differences between oestrogen receptor subtypes, there could be differences in the oestrogen response elements in the DNA that might respond differently to the alpha or beta homodimers or heterodimers of the oestrogen receptors. The mediation of gene transcription via the AP1 enhancer site might also differ depending upon receptor sub-type (Paech *et al*, 1997). Paech *et al* showed that the oestrogen receptor-alpha (ER α) and oestrogen receptor-beta (ER β) subtypes signal in opposite ways when complexed with natural oestradiol at an AP1 site. The oestradiol-bound alpha receptor activated transcription while the beta receptor had the opposite effect and inhibited transcription. Moreover, tamoxiphen, an anti-oestrogen, was a potent activator with the beta receptor at the AP1 site. Further experiments using Western and Northern blot techniques might clarify whether there are indeed differences in the molecular structure of the oestrogen receptor in the thymus that could account for the observed differences in the affinity of the cytosolic

oestrogen receptor in this tissue compared with the values found for uterus and those reported for brain tissues.

The relatively high affinity of the thymus receptor for corticosterone may be significant in view of the well known immunosuppressive actions of the glucocorticoids. Determination of the development of autoimmune and inflammatory conditions such as systemic lupus erythematosus and rheumatoid arthritis depend, in part, upon interactions between the immune system and the hypothalamo-pituitary-adrenal axis. The response to acute stress, cognitive or non-cognitive, is the release of corticosterone from the adrenals in the rat (cortisol in humans) and these steroids are potent immunosuppressive hormones. (reviewed by Harbuz *et al*, 1997). The Lewis strain of rat is susceptible to experimental allergic encephalomyelitis after challenge with guinea pig basic myelin protein. This strain shows defects which include a lower response to stress in the hypothalamo-pituitary-adrenal axis. In this animal model of autoimmune disease, the progress of the condition can be stopped by treatment with corticosterone. Evidence from studies of Lewis rats suggests that autoimmune disease may result from a hyporesponsive hypothalamo-pituitary-adrenal axis (Derijk and Sternberg, 1994). Adrenalectomy can also increase the severity of induced disease in autoimmune-prone rats (Macphee *et al*, 1989). Thus stress, by increasing glucocorticoids in circulation, maintains immune homeostasis in the face of infection when an immune response has been generated by this stressor. Glucocorticoid activity in the brain is associated with modulation of protein synthesis (Mileusnic *et al*, 1986) and this finding could be significant for alterations in the thymus in response to stress.

Reductions in basal (early morning) serum cortisol levels in humans with rheumatoid arthritis, reductions in adrenal function in patients with multiple sclerosis, and glucocorticoid resistance in patients with systemic lupus erythematosus have been observed. These findings suggest that the hypothalamo-pituitary-adrenal axis may be compromised in a variety of autoimmune disorders (Harbuz *et al*, 1997). Dehydroepiandrosterone may also have a physiological role in regulating the immune response. This adrenal steroid may oppose effects of glucocorticoids although the mechanism of such a role has not been elucidated.

Communication between the hypothalamo-pituitary-adrenal axis and the hypothalamo-pituitary-gonadal axis also modulates the expression of autoimmune disease. The effects of sex steroids are inhibited by glucocorticoids; conversely sex steroids modulate the hypothalamo-pituitary-adrenal axis. Paradoxically, the hypothalamo-pituitary-adrenal axis is more active in females (human) than in males and should predispose females to lower rather than higher incidence of autoimmune conditions. Testosterone may have a protective effect in males; oestrogens have effects that vary between different diseases. Oestrogen in some cases increases B-cell mediated immunity but decreases cell-mediated immune responses. Thus, oestradiol suppresses experimental arthritis in animal models of the disease but increases the severity of systemic lupus erythematosus. Cells of the immune system express genes for stress peptides but locally expressed corticotrophin releasing hormone appears to have the opposite effect to the centrally released hormone in that the locally released hormone has an enhancing effect on the immune response (reviewed by Harbuz *et al*, 1997).

There is, moreover, evidence that stress-induced centrally released corticotrophin releasing hormone activates the sympathetic nervous system which influences immune activity in the periphery independently of glucocorticoids themselves (reviewed by Downing and Kendall, 1997). The mechanisms for this regulation include neurotransmitter synthesis and release, expression of receptors for these compounds and effects of their activity such as alterations to cell proliferation and activity of cells of the immune system. Evidence thus far on the role of autonomic system modulation of the immune response suggests that sympathetic activation depresses T-cell immunity but enhances antibody production. Further studies, using molecular biological techniques such as Western blot (for mRNA differences for oestrogen receptor sub-types) or Northern blot (for molecular weights of oestrogen receptor sub-types) might identify the presence of ER α and ER β receptors in the thymus and enable the relative concentrations of the sub-types, if they are present, to be detected.

In summary, the thymus, a primary lymphoid tissue, may *via* interactions of glucocorticoids, sex hormones and sympathetic innervation respond and possibly develop differently in males and females. The finding of the present study, that oestrogen receptor affinity for corticosterone was one order of magnitude higher in the thymus than in uterus, could provide additional mechanistic explanations of autoimmune susceptibility in individuals in whom the stress response axis might be altered. This alteration could result in altered ratios of oestrogen receptor sub-types in individuals. There could be differential expression of receptor subtypes between the sexes in thymus although there is no evidence at present of such a difference. The expression of different sub-types of

oestrogen receptors could be modulated by corticosterone itself or by alterations of innervation of the thymus itself either genetically programmed and/or induced by stress during development.

Although there may be structural differences between the cytosolic oestrogen receptor in uterus, brain and thymus, there may also be differences during ontogeny in cytosolic oestrogen receptor characteristics in the tissues and/or in their response to oestradiol or to agonists and antagonists of this hormone. Further studies of these tissues were undertaken in order to investigate oestrogen receptor concentrations and binding constants in developing rats as well as responses to oestrogen agonists and antagonists in uterus, thymus and brain.

Chapter four

The development of oestrogen receptors in uterus, brain and thymus in female and male Wistar rats from 5 to 30 days

4.1: Introduction

4.1.1: Steroid metabolism in the brain

A variety of steroid metabolic pathways appear to co-exist in the brain. Active and inactive metabolites of corticosterone, oestradiol and testosterone are formed; there may be competing pathways; and stress and social stimuli also affect the activity of sex steroids in this tissue. The pathway for steroid synthesis from acetate, cholesterol, and pregnenolone is common to the adrenal cortex, testis and ovary. Hence, neuroactive steroids may originate from these organs (Gore-Langton and Armstrong, 1994). Steroids may also be synthesized *de novo* in the brain from cholesterol or derive from the metabolism of steroid hormones present in the blood. Moreover, steroids may also be available from various nutritional or environmental sources.

Dehydroepiandrosterone (DHEA) is produced in the adrenal cortex from the pregnenolone precursor and, in some species, this hormone may be secreted in amounts that exceed cortisol and corticosterone (Goulding and Flower, 1997). However, DHEA, pregnenolone, and their sulphates are also synthesized locally in the central nervous system and have therefore been termed neurosteroids.

The mammalian brain contains 5 α -reductase, 3- α -hydroxysteroid dehydrogenase and aromatase, enzymes involved in the conversions of testosterone, progesterone and corticosterone metabolism.

5 α -reductase occurs as two isoforms and both forms reduce androgens, progestagens and corticosteroids although the affinities of the two isoforms for the substrates differ. The type 1 isoenzyme form of 5 α -reductase is more abundant in the brain than the type 2 form. There is some

evidence that 5 α -reduced metabolites formed as a result of 5 α -reductase activity are necessary for the process of myelination in developing rats at three weeks (reviewed by Celotti *et al*, 1997). 5 α -reductase is widely distributed in various brain areas including the hypothalamus. It is present in glial and ependymal cells of different areas, in oligodendrocytes and, in perinatal rats, in germinal zones and differentiating fields although no sex differences in its expression have been identified thus far. The formation of dihydrotestosterone, the 5 α -reduced metabolite of testosterone, is high in white matter structures in the brain and appears to have a role in the development of functions associated with modulation of GABA-operated chloride channels. 5 α -reductase in the spinal cord may be associated, through the formation of dihydrotestosterone with motoneuron growth (Matsumoto *et al*, 1988) and in masculinization of the sexually dimorphic spinal nucleus of the bulbocavernosus in the rat lumbar spinal cord (Goldstain and Sengelaub, 1992). Its association with the development of secondary male sex characteristics is well characterised and it also acts synergistically with oestradiol in the induction of male sexual behaviour in castrated male rats. Moreover, administration of dihydrotestosterone to female rats can cause organisational changes in brain and in behaviour (Celotti *et al*, 1997).

The conversion of 5 α -reduced steroids to the 3 α -hydroxylated derivatives is catalysed by 3 α -hydroxysteroid dehydrogenase in astrocytes. The products of these reactions (dihydrotestosterone to 3 α -hydroxysteroid, dihydroprogesterone to tetrahydroprogesterone, dihydrodeoxycorticosterone to tetrahydrodeoxycorticosterone) may be active by non-genomic interactions at the

plasma membrane in regulation of the amount of intracellular 5 α -reduced hormones available for genomic interactions (Melcangi *et al*, 1990).

Pregnenolone sulphate interacts with the γ -aminobutyric acid receptor (GABA_A, the ionotropic GABA receptor), as do steroids such as corticosterone, tetrahydroprogesterone, and tetrahydrodeoxycorticosterone. Pregnenolone sulphate and corticosterone interact with the GABA_A receptor in a mixed GABA-agonist/ antagonist manner and this interaction is inhibited by the neurosteroid, DHEA sulphate (Majewska and Schwartz, 1987). There is much evidence that GABAergic steroids modify the functioning of central GABA_A receptors *in vivo* and may participate in the physiological control of central nervous system excitability (reviewed by Alonso and Lopez-Coviella, 1998).

The precursors of GABA-enhancing steroids, 5 α -pregnan-3 α -ol-2-one (THP) and tetrahydrodeoxycorticosterone (THDOC) are the ovarian and adrenal hormones, progesterone and corticosterone respectively. Conversion of the precursors of THP and THDOC takes place in the ovaries and adrenal cortex (respectively) through activity of 5- α -reductase and 3- α -oxidoreductase. These reactions also occur in the central nervous system, primarily in glial cells (reviewed by Celotti *et al*, 1997).

Effects of the neurosteroids, progesterone, corticosterone and their reduced metabolites may include potentiation of GABAergic inhibition in neurons involved in the lordotic response of female rats (Schwartz-Giblin and Pfaff, 1987). Pregnenolone sulphate may alter male sexual behaviour through its GABA-antagonistic properties (Baulieu *et al*, 1997).

Neurosteroid concentrations vary in the brain according to environmental and behavioural circumstances such as stress sex recognition or aggressiveness. A physiological function of neurosteroids in the nervous system has been suggested by the association of pregnenolone with memory formation in the hippocampus of ageing rats; progesterone, synthesized in Schwann cells in the peripheral nervous system may have a role in myelin repair (Mayo *et al*, 1993).

The enzymatic activity necessary for progesterone synthesis in the brain has been found in the hypothalamus, hippocampus, olfactory bulb, striatum, septum, cerebellum and cortex (Martini *et al*, 1993) mainly in glial cells (Jung-Testas *et al*, 1989) and oligodendrocytes (Akwa *et al*, 1993) but also in neurons (Arnold and Breedlove, 1985).

Aromatase is expressed in a wide variety of tissues in males and females including the uterus, brain and thymus. Activity of this enzyme converts testosterone to oestradiol. The regional distribution of the enzyme has been investigated in rats by methods that include immunohistochemistry, enzymatic activity assays and *in situ* hybridization techniques (Roselli and Resko, 1993; Lauber and Lichtensteiger, 1994). While 5-alpha-reductase is widely distributed in the brain, aromatase is expressed chiefly in brain areas concerned with reproductive function, that is to say in neurons of the hypothalamus, preoptic, limbic, and amygdala. The role of 5alpha-reductase and dihydrotestosterone in the brain remain controversial; those of aromatase, testosterone and oestradiol are relatively well established (reviewed by Celotti *et al* 1997).

The characterisation and distribution of aromatase in the brain and the significance of its role in brain differentiation are relatively well understood.

Aromatase catalyses the conversion of testosterone to oestradiol. The secretion of testicular testosterone peaks in male rats during the late pre-natal and early post-natal period (up to 10 days after birth). Blood testosterone concentrations then fall to low levels characteristic of the pre-pubertal period in males and as puberty is reached, the concentration in blood of testosterone and other androgens rise rapidly to adult levels (Balthazart, 1997).

In perinatal rats, testosterone appears to be necessary for the differentiation of aromatase-containing neurons, that is to say for masculinization of the neural anatomy necessary for the differentiation of oestrogen-synthesising neurons in the male brain. Androgens also enhance the growth of aromatase-containing neurons in the brain. Testosterone, converted to oestradiol by the activity of aromatase, defeminizes brain areas such as the hypothalamus. That oestradiol is necessary for this process is demonstrated by the finding that defeminization of the brain in androgen-receptor deficient mutant mice is induced by administration of oestradiol. Early exposure to oestradiol and testosterone in males appears to lead to desensitisation in males to later (adult) exposure to oestradiol. In female rats, in the perinatal and early post-natal period, very low concentrations of testosterone and available oestradiol are present in the blood and in their absence, a female brain develops (reviewed by Pilgrim and Hutchison, 1994). Castration of perinatal male rats results in the development of a feminized brain and a female pattern of response to oestradiol and progesterone in later adult mating behaviour. Conversely, females given testosterone perinatally develop into androgenized females. The concentration of free oestradiol in the blood of perinatal rats is low because the maternally derived

hormone is bound to alphafetoprotein. In perinatal and early postnatal (up to 15 days) females, therefore, only very low doses of oestradiol are available for brain and other oestradiol-sensitive tissues at this stage. In male, testicular testosterone is present in high concentrations in the perinatal and early postnatal period and its aromatization to oestradiol in the brain is a key event in the development of this tissue. In other tissues such as the thymus, aromatization may contribute to differential development in males and females of this primary organ of the immune system.

Whether neurosteroid and/or non-genomic sex steroid activity exert any effects on the establishment of sex differences during brain development remains to be established. Effects of neurosteroids on glial cell growth and differentiation have been observed in the central and peripheral nervous system; in particular, in the role of pregnenolone in memory and in progesterone-enhanced growth of myelin basic protein-synthesising oligodendrocytes (Baulieu, 1997). Possible non-genomic effects could include alterations by steroid hormones on cell membrane fluidity, binding of steroids to membrane-bound receptors, regulation of GABAA receptors on plasma membranes and activation of steroid receptors by factors such as dopamine (Brann *et al*, 1995).

4.1.2: Steroid receptors

The biological effects of steroid hormones are mediated by specific receptors. At least two types of steroid receptor have been identified in cells responsive to steroids. Genomic (classical) activity is mediated by nuclear receptors specific for the effect; non-genomic (non-classical) effects are mediated by steroid binding of membrane-bound receptors and are characterised

by rapid effects of short duration. The boundaries between effects of the two types of receptor are not sharp and overlapping effects due to interactions of the two types of receptor are known (reviewed by Tuohimaa *et al*, 1996).

4.1.2.1: Intracellular steroid receptors

The classical oestrogen receptor belongs to a superfamily of protein transcription factors whose activity depends upon the binding of specific ligand and interaction with nucleotide sequences known as hormone-response elements on target genes. This type of response generally results in long-lasting effects (Arnold and Gorski, 1984). Members of the superfamily bind various ligands including vitamin D, retinoic acid, thyroid hormones, as well as steroid hormones such glucocorticoids, and the sex hormones. Transcriptional activation is mediated by the AF-1 region in the N-terminal domain of the receptor and may be regulated by epidermal growth factor and insulin-like growth factor; the structure of AF-1 varies considerably between receptors. Steroid binding, in the ligand-binding AF-2 domain, is highly conserved in the superfamily. Steroids bind to a common hydrophobic fold in a ligand-binding fold of the protein structure of the receptor. Steroid binding causes a conformational change of a C-terminal helix in the receptor and creates an interacting surface to which co-activators can bind (reviewed by Parker, 1998).

DNA binding is associated with two zinc fingers in the AF-1 domain. Steroids bind as homodimers (although binding of heterodimers of the alpha- and beta-oestrogen receptors is known) to steroid response elements on the DNA. The first zinc finger determines the specificity of the DNA binding. Two types of steroid response element have been identified; both are palindromic half-sites

made up of 6 base-pair half sites with a 3 base-pair spacer. The oestrogen response element is the more conserved of the two types of element; the second type is common to the glucocorticoid, progesterone, androgen, and mineralocorticoid response elements. A third type of response element has been identified for thyroid hormone, retinoic acid and vitamin D. The second zinc finger is associated with receptor dimerization. Heat shock proteins (hsp), such as hsp 90 bind to specific sequences in the AF-1 domain (see Figure 1.2). Between the AF-1 and AF-2 domain lies a hinge region that permits rotation of the receptor. The N-terminal of the AF-1 region appears to be more active in gene transcription than the AF-2 region (Ing and O'Malley, 1995). Ligand binding and phosphorylation-induced conformational changes of the steroid receptor trigger transcriptional activation/repression. Moreover, proteolysis of ligand-bound receptor and new synthesis of ligand-free steroid receptor appear to be involved in termination of transcription activation and/or repression (reviewed by Tuohimaa *et al*, 1996).

In some circumstances, intranuclear steroid receptors can be activated in the absence of ligand binding. Dopamine, through physiological activation of the membrane neurotransmitter receptor can mediate the activation of progesterone receptor (Power *et al*, 1991). Moreover, there is some evidence that the nuclear oestrogen receptor may bind to specific DNA sequences in the absence of oestradiol binding to the receptor (Gorski *et al*, 1993).

More recently described, are membrane receptors for steroids whose activation by ligand may be coupled to membrane ion channels or second messenger systems which result in rapid and transitory changes in neuron

excitability (Brann *et al*, 1995). For example, possible mechanisms for non-classical steroid-induced effects include changes in membrane fluidity, and hormone actions on the plasma membrane on GABA_A receptors and activation of a cascade of second messenger systems.

There are, moreover, interactions between the responses of the two types of receptor, genomic and non-genomic since activation of intracellular steroid receptors may alter conductance and membrane receptor activation may also regulate gene expression through regulation of transcription factor signal transduction (reviewed by Alonso and Lopez-Coviella, 1998).

4.1.2.2: Membrane-bound steroid receptors

Membrane receptors for progesterone are found in hypothalamus where hormone binding to the receptor is effective in causing the release of luteinizing-hormone-releasing hormone (Ke and Ramirez, 1987) and in dopamine release by the corpus striatum where it can have stimulatory or inhibitory effects (Dluzen and Ramirez, 1991). Moreover, there are sex differences in that male rats have lower specific binding for progesterone in the hypothalamus and central nervous system than do females (reviewed by Alonso and Lopez-Coviella, 1998). Oestrogens can also have rapid non-genomic effects on hypothalamus where the inhibitory effect of oestradiol on luteinizing hormone release from the pituitary may be due to non-genomic control of the release luteinizing hormone releasing hormone by the hypothalamus. Oestradiol also has rapid (and therefore membrane receptor-mediated) effects on the spontaneous and induced firing rate of hypothalamic neurons during the oestrus cycle, on changes in the membrane internalisation process, on pituitary prolactin release, and on striatal and

dopamine and hypothalamic norepinephrine release in the hypothalamus. In other areas of the central nervous system, oestradiol causes depolarisation in neurons in the hippocampus and cerebellum, areas associated with memory and learning (Vanderwolf and Cain, 1994).

Membrane binding of testosterone is less well characterised than that of oestradiol but membrane receptors for testosterone have been identified in the olfactory bulb of adult male rats where its role may be in the detection of chemical cues from females and/or in memory processes of an olfactory nature (reviewed by Ramirez and Zheng, 1996).

In general, steroid-induced actions can take place very rapidly (seconds or minutes) or over longer periods (hours or days). Permanent effects mediated by steroids are attributed to intracellular receptor activity; conversely, short-term effects are generally ascribed to activation of membrane-bound hormone receptors. The timing, perinatal and prepubertal versus adult, of receptor activation seems to be an important determinant of whether permanent or transitory changes in the brain result from activation of intracellular or membrane receptors in target areas of responsive tissues (reviewed by Alonso and Lopez-Coviella, 1997).

4.1.2.3: Alpha and beta oestrogen receptors

The actions of oestradiol were thought to be mediated by a single oestrogen receptor until the recent cloning of a novel oestrogen receptor, oestrogen receptor-beta ($ER\beta$) in prostate tissue (Kuiper *et al*, 1996). The oestrogen receptor exists as two sub-types, oestrogen receptor-alpha ($ER\alpha$) and the beta receptor, $ER\beta$. $ER\beta$ is highly homologous to $ER\alpha$ (95 percent) in the

DNA-binding domain (AF-1) but 55 percent homologous in the C-terminal ligand-binding domain (AF-2). The two sub-types differ in slightly in their affinity for oestrogenic substances or antagonists. The order for the ER α is: diethylstilbestrol > hexestrol > dinestrol > 4-OH-tamoxiphen > 17beta-oestradiol > coumestrol, > ICI-164384 > estrone, > 17alpha-oestradiol > nafoxidine > moxestrol > clomiphene > oestriol, > 4-OH-oestradiol > tamoxiphen, > 2-OH-oestradiol, > 5androstene-3beta, 17beta-diol, genistein. The order for the ER β is: dinestrol > 4-OH-tamoxiphen > DES > hexestrol > coumestrol, > ICI-164384 > 17beta-oestradiol > oestrone, genistein > oestriol > nafoxidine, 5androstene-3beta, 17beta-diol > 17alpha-oestradiol, clomiphene, 2-OH-oestradiol > 4-OH-oestradiol, tamoxiphen, moxestrol.

The tissue distribution and/or relative level of ER α and ER β expression differs markedly. There is moderate to high expression of ER α in uterus, testis, pituitary, ovary, kidney, epididymis and adrenal. Expression of ER β is moderate to high in prostate, ovary, lung, bladder, brain, uterus and testis. Saturation ligand binding analysis of the two sub-types with 16alpha-iodo-17beta-oestradiol shows a single binding site with high affinity ($K_d = 0.1\text{nM}$ for ER α ; $K_d = 0.4\text{nM}$ for ER β) (Kuiper *et al*, 1997). ER α and ER β are differentially expressed in discrete sub-regions of the amygdala in females rats. The sub-types differ in their response to oestradiol treatment which results in decreased ER α mRNA expression in the arcuate ventromedial hypothalamus but increased expression of ER β mRNA in the arcuate (Osterlund *et al*, 1998). Further studies confirm the presence of ER α and ER β mRNA throughout the rostral-caudal extent of the brain and spinal cord. Some areas, such as the olfactory bulb in females, neurons

of the cerebellum, and some nuclei of the hypothalamus express only ER β . In contrast, ER α mRNA only is found in the ventromedial hypothalamus. Neurons of other areas including the bed nucleus of the stria terminalis and cortical amygdala express both forms of the oestrogen receptor. While neurons of the cerebral cortex and hippocampus also express mRNA for both sup-types of the oestrogen receptor, ER β is much more highly expressed than ER α (Shughrue *et al*, 1997).

The distribution of ER α and ER β differs in the ovary of the rat. ER α is highly expressed in stromal cells while ER β is concentrated in granulosa cells of developing follicles. In the uterus, ER α is abundantly expressed in stromal and epithelial cells of the endometrium. In male rats, ER β but not ER α expressing-cells are found in prostate and Sertoli cells (Shughrue *et al* , 1998a).

The ER β has been identified in mice (Shughrue *et al*, 1998b) and monkeys (Register *et al*, 1998) as well as in humans and rats. Moreover, studies using ER α knock-out mice, deficient specifically for ER α but not ER β confirm that ER β is localised in many brain areas, including those associated with learning and memory (Shughrue *et al*, 1998b).

These new findings, of differential specificity and tissue distribution of the two forms of the oestrogen receptor, may provide new insights in oestrogen action during the development of the brain, reproductive tissues and in other tissues such as the thymus.

4.1.3: Oestrogen receptors in the brain

Oestrogen receptors are present in rat brain and they are concentrated in regions that are known to be responsive to oestradiol. These areas include the preoptic area, hypothalamus, cerebral cortex and amygdala (MacLusky *et al*, 1987; Yokosuka *et al*, 1995). Aromatase, which catalyses the conversion of testosterone to oestradiol, is also present in the preoptic area, hypothalamus, and hippocampus (MacLusky *et al*, 1994).

The preoptic area of the brain is associated with many sexually differentiated reproductive functions (Arnold and Gorski, 1984; Meisal and Sachs, 1994). During development of the limbic-hypothalamic circuit, chemical and morphological differences in males and females appear to depend upon differential exposure to sex steroids during the prenatal period (Micevych and Ulibarri, 1992). Circulating testosterone in prenatal and postnatal male rats is aromatized to oestrogen in the brain where it effects its defeminizing properties through activation of oestrogen receptors (Greenstein, 1978; MacLusky *et al*, 1987). These effects include sexual dimorphism of specific neural areas and sexual behaviour in adult mammals such as the rat (Fox *et al*, 1978; MacLusky and Naftolin, 1981). In the preoptic area of the brain, several binding studies have not detected sex-related differences in levels of cytosolic oestrogen-binding receptors (Fox *et al*, 1978; McLusky *et al*, 1979). Autoradiographic studies similarly found no sex difference in the number of oestrogen-concentrating cells in the preoptic area of neonatal rats (Friedman *et al*, 1983a; Sheridan *et al*, 1974). On the other hand, the levels of mRNA for the oestrogen receptor in this area co-vary with levels of oestrogen receptor and are lower in male rats at birth

than in females. Moreover, higher concentrations of oestrogen receptors in the preoptic area of female rats were found in other studies (Kuhnemann *et al.*, 1994). At this age males have higher levels than do females of circulating testosterone, which is available for aromatization to oestrogen (Don Carlos *et al.*, 1996).

There is an apparent paradox in the role of oestrogen in the development of a defeminized brain in males. It may be that oestrogen acts in synergy with androgens in this developmental process and 'primes' the brain in males for testosterone-dependent structure formation and for the later characteristic male response to oestradiol. At puberty, the concentration of cytosolic oestrogen receptors in the preoptic area in female rats decreases at the same time as levels of nuclear oestrogen receptor and cytosolic progesterone receptor increase. This is the period of increased levels of circulating oestrogen produced in the developing ovaries and is associated with vaginal opening and the first pre-ovulatory gonadotrophin surge that heralds the start of reproductive activity (Woodman, 1997).

Early studies on the differences between levels of oestrogen receptor in the hypothalamus and pituitary of immature male and female rats suggested that there were no differences in nuclear binding between the anterior pituitary of males and females. In some studies, the hypothalamus of the female shows a limited ability (compared with the uterus) to bind oestradiol, but the male hypothalamus has no capacity to bind this hormone (Clark *et al.*, 1972). Kuhnemann *et al.* (1994) used *in vitro* autoradiographic analysis of oestrogen binding in the rat brain between ages 2 -10 days. They found sex differences in

the number of oestrogen receptors present in the periventricular preoptic area, medial preoptic area and bed nucleus of the stria terminalis, though not in the nucleus or ventromedial nucleus of the hypothalamus nor in the cortical or medial nucleus of the amygdala. Sex differences in the density of oestrogen receptors have also been identified in rat hypothalamus using oestrogen receptor-immunochemical methods (Yokosuka *et al*, 1995).

Sexually dimorphic brain functions include the hypothalamic activity of the inhibitory neurotransmitter, γ -aminobutyric acid (GABA), although sex steroids have no direct effect on the cell numbers or on GABA uptake by GABAergic neurons. Efferent or afferent connections with other dimorphic neurons as well as neurosteroid interactions may be responsible for the observed sex dimorphism in this group of cells (Lieb *et al*, 1994). Timing of the critical period for the effects of androgens and their metabolites on sexually dimorphic development appears to be related to developmental differences in the ability of cells to bind these hormones.

The function of elevated oestrogen receptor concentrations in the developing cortex is not fully understood. Oestrogen concentrations in this area of the brain of developing rats reach a maximum during the first post-natal week and this period coincides with rapid growth and development of neurones. Administration of oestrogen during this period has long been known to enhance the process of brain myelination in cortex (Curry and Hein, 1966) and this finding supports a physiological role for oestrogen in brain development. *In vitro* studies in dissociated cortex cultures (Uchibori and Kawashima, 1985) and in cortical explant cultures (Toran-Allerand *et al*, 1988) have also demonstrated that

oestrogen enhances neurite outgrowth, either directly or by interaction with other growth factors. Recent studies have detected oestrogen receptors in auditory, somatosensory and visual cortices of neonatal rats with a peak at post-natal day 3, although there were no sex differences (Yokosuka *et al*, 1995). This study gave similar results to the autoradiographic studies by Shughrue *et al* (1990), which showed high concentrations of oestrogen receptors in mouse cortex with a peak at ten days after birth. In other brain areas, such as the hippocampus, development differs between males and females and is regulated by the binding of oestradiol to the oestrogen receptor (Diamond *et al*, 1982; O'Keefe and Handa, 1990).

4.14: Oestrogen receptors in the thymus

There is good evidence that, in many species, oestrogen receptors are present in the thymus. The dissociation constant for oestradiol in these tissues is of the order of 10^{-10} - 10^{-9} M. Moreover, the receptor is similar to that in the uterus in its specificity for oestradiol and in its dissociation constant in humans (Nilsson *et al*, 1984, Weusten *et al*, 1986), rats (Morgan and Grossman, 1985) and mice (Aubrey-Thompson, 1981) (Section 1.4).

It is mainly cells of the reticuloepithelial cell population within the thymus that express receptors for oestrogen in rats (Imanishi *et al*, 1980). Using monoclonal antibodies to the oestrogen receptor and the progesterone receptor, and polyclonal antibodies to thymulin, Kawashima *et al* (1991) identified the reticuloepithelial cells and Hassall's corpuscles as the sex steroid-sensitive and thymulin-producing cells in the medulla of rat thymus. Furthermore, oestrogen receptors are present in the nucleus and in the cytoplasm of this group of cells.

They propose that sex steroids exert their effects on developing thymocytes through reticuloepithelial cells whose response to oestrogen is the production and release of thymulin as a regulator of T-cell differentiation.

Oestradiol and dexamethasone *in vivo* alter the relative numbers of T-cells at their different developmental stages in thymus. While the overall effects of these compounds on the relative changes are similar in the shorter term, the oestradiol effects take longer (by 48 hours) to appear. This finding suggests that dexamethasone may act directly on the thymocytes while the oestradiol effects are mediated via the reticuloepithelial cell population (Screpanti *et al*, 1989). Oestrogen receptors are present in thymocytes and oestrogen receptor mRNA is also found in the cytosol of these cells in mice, although in lower concentrations than in reticuloepithelial cells (Kawashima *et al*, 1992).

Thymocyte sub-population ratios are altered by treatment with oestradiol (Novotny *et al*, 1983) with a significant increase in mature T-cells of the helper type (CD4+). This finding is confirmed by work that shows that oestradiol causes a decrease in the number and proportion of intermediate T-cells of the CD4+/CD8+ population, with a proportional increase in the percentage of the less mature CD4-/CD8- T-cells, and to a lesser extent the mature CD4+ and CD8+ subsets (Screpanti *et al*, 1989). It is possible that CD4+CD8+ T-cells express higher concentrations of oestrogen receptors than the less mature T-cells and are more severely affected by oestrogen treatment. Perhaps, treatment with oestradiol interferes with the progressive maturation of immature T-cells over the period of treatment; oestradiol might also promote the maturation of T-cells from the CD4+CD8+ to the CD4+ or CD8+ populations. Oestrogen treatment blocks

T-cell development in the thymus of Balb/c mice although oestrogen deprivation by oophorectomy does not enhance the process (Rijhsinghani *et al*, 1996b). There is much evidence, therefore, that oestradiol causes changes in the thymus and that oestrogen receptors are present in thymocytes as well as in the reticuloepithelial cells in this tissue.

Several observations suggest that immunological dimorphism develops during sexual maturation. In male hamsters, depression of immunity occurs when testosterone levels are increasing at puberty (Blazovec and Orsini, 1976); in human females there is a significant increase in the circulating levels of immunoglobulin G as oestrogen levels start to rise (Stoica *et al*, 1978; Weetman *et al*, 1981). This is a period when juvenile arthritis, more common in females than in males, also develops (Aarow *et al*, 1985). Such dimorphism may be established early during the development of T-cells in the thymus and may be a result of the difference in the thymic microenvironment due to the different effects of the male and female sex hormones. Gonadectomy results in different effects on the thymus in 30-day and 75-day-old male and female rats (Leposavic *et al*, 1996). Differences between the sexes are found in the size and total thymocyte yield of the thymus regardless of age or treatment, while gonadectomy at 30 days (though not at 75 days) results in increases in thymic weight in both sexes. At 30 days, the females in the control group have a higher percentage of CD4-/CD8- thymocytes than do gonadectomised females, while the opposite effect is found in males. Gonadectomy at this age, however, results in an increase in both sexes of the CD4+CD8- subset. The difference in response to gonadectomy between the sexes at 30 days suggests that these two subsets of T-

cells may differ in their age-related sensitivities to local sex hormone action. Alternatively, altered sex hormone availability after gonadectomy could effect changes in the reticuloepithelial cells in the thymus and on their release of thymic hormones.

If differences in immune responsiveness exist between males and females and these differences are due to the different hormone environments in males and females, the role of sex hormone receptors must be crucial in the development of this phenomenon. Studies in humans, guinea pigs, mice and rats, confirm the presence of oestrogen receptors in the thymus (Nilsson *et al*, 1990; Screpanti *et al*, 1982, Kawashima *et al*, 1992).

Concentrations of available free oestradiol are low in perinatal and neonatal rats, due to the presence in the blood of immature rats of a protein called alphafetoprotein that binds oestrogens (see Figure 2.11). This binding of oestradiol by alphafetoprotein is reflected in the low level of the free fraction of the hormone and is of biological significance in brain and uterus development. Alphafetoprotein is secreted by the foetal liver, decays slowly after birth and is undetectable after 28 days (Nunez *et al*, 1971; Greenstein *et al*, 1978; Greenstein, 1992). Testosterone levels are high in male rats during the early post-natal period (Weisz and Ward, 1980) and could be aromatised in the thymus as well as in the brain to oestradiol. Differences in the male and female brain as well as in the thymus may be due, in some part, to differences in the microenvironment of these tissues during the early post-natal period.

The present study was undertaken in order to investigate differences in the oestrogen receptors in brain and thymus of male and female Wistar rats

between the ages of 5 and 30 days. Levels of circulating oestradiol are known to be low at the start of this period of development, but start to rise by 22 days (Greenstein, 1992). Nevertheless, the free fraction of oestradiol in serum was also measured in order to confirm its value in these animals.

4.2: Materials and Methods

4.2.1: Animals

Male and female Wistar rats were purchased from Bantin and Kingman, as before, and were allowed to settle for 48 hours before sacrifice. The age groups studied were 5 days, 18 days, and 30 days. Animals to be sacrificed at 5 and 18 days were kept with their litter mates and mother in one cage (56 x 38 x 20 cm); those rats to be used at 30 days had been weaned (at 21 days) before delivery from the supplier and were housed 5 per cage (56 x 38 x 20 cm). All animals were housed in the Biological Services Facility in the Rayne Institute, St. Thomas's Hospital under conditions of controlled lighting and temperature (lights on 08:00h-20:00h; lights off 20:00h-08:00h; 19-20°C) and were allowed free access to BK rodent diet which is steroid free, and to tap water. These ages were chosen as the thymus was still growing, the brain was undergoing development, and the production of oestradiol in the ovaries, while increasing, was still well below adult levels (Greenstein, 1978).

4.2.2: Chemicals

Chemicals used for this study are given in Section 2.1.1-2.1.5.

4.2.3: Methods

4.2.3.1: Serum was prepared by the method given in Section 2.2.2.

4.2.3.2: Cytosols from brain, thymus and uterus were prepared by the method given in Section 2.2.3.

4.2.3.3: Steroids for binding studies were prepared by the method given in Section 2.2.4.

4.2.3.4: ^3H -oestradiol-17 β (^3H -E2) and ^3H -moxestrol were each prepared at a concentration of $5 \times 10^{-7}\text{M}$ and stored at 4°C .

4.2.3.5: Unlabelled moxestrol and unlabelled oestradiol-17 β were prepared in the same way as their tritiated counterparts at a concentration of $5 \times 10^{-5}\text{M}$ and stored at 4°C .

4.2.4: Assay of cytosols from the brain, thymus and uterus for steroid-receptor binding activity.

4.2.4.1: Incubation of the cytosol.

Cytosols, prepared from the three different age groups of male and female Wistar rats, were stored at -70°C and were assayed within two months of their preparation by the methods described in Section 2.2.4. Briefly, $60\mu\text{l}$ cytosol was incubated with $60\mu\text{l}$ ^3H -moxestrol in final concentrations in the incubate ranging from $2.5 \times 10^{-9}\text{M}$ to $3.1 \times 10^{-10}\text{M}$ in the presence or absence of a 100-fold excess of unlabelled moxestrol. These incubations were performed in 96-well polypropylene round-bottomed plates, which were sealed and placed in an agitating water bath for 1hr at 25°C . Aliquots of the total of tritiated and unlabelled moxestrol ($60\mu\text{l}$) were also incubated in order to establish the total

steroid added to each of the incubation samples. The samples were cooled to 2°C before separation of bound and free moxestrol was carried out.

4.2.4.2: Separation of bound and free moxestrol

Columns of Sephadex LH20 were prepared as described in Section 2.2.4.3. The columns were filled to 9 cm with Sephadex LH-20 and were cooled for 1 hr in a waterbath maintained at 0.5-2.0°C. When the sample had cooled to 2.0°C after incubation, 100 µl was applied to each column and washed immediately with 200µl of Buffer 2. The samples remained on the columns for 1hr, the time established (see Section 2.4) for equilibration of the bound and free steroid to take place. Each column was eluted with 700µl Buffer 2 and the equivalent volume of eluate was collected into scintillation vials. Up to 94 columns (12 samples) could be operated at one time. Scintillation fluid (10ml) was added to each vial and, following overnight extraction, the radioactivity was counted as before.

4.2.4.3: Construction of Scatchard Plots

Scatchard analysis (Section 2.2.5.4) on the data was using the computer programme given in Appendix A. Regression analysis was used to obtain a measure of the slope and the coefficient of correlation for each Scatchard plot obtained.

4.2.5: Measurement of the free fraction of oestradiol in serum of female and male Wistar rats at 5,18 and 30 days

Measurement of the free-fraction of oestradiol in serum was done by the method of Greenstein *et al* (1987) (Section 2.5). Graphs were constructed using semi-logarithmic graph paper in order to estimate the albumin-bound

³H-oestradiol eluted (between fractions 4 and 9) and the free ³H-oestradiol eluted (between fractions 15 and 21). The free fraction was then estimated from these data.

4.2.6: Statistical Analyses

Statistical analyses of the results obtained in this set of experiments were undertaken as described in Section 2.6. The analysis tested for significant differences in body and tissue weight, binding affinity of the oestrogen receptor for ³H-moxestrol and for the concentration of oestrogen receptors between the sexes and the three age groups.

4.3: Results

4.3.1: Body weights of female and male Wistar rats at 5, 18 and 30 days.

The body weights of female and male Wistar rats in the different age groups are shown in Figure 4.1. Between the ages of 5, 18 and 30 days, the body and tissue weights of male and female Wistar rats increased. There were no differences in body weight between males and females at 5 days or at 18 days ($p > 0.05$). By 30 days, however, male rats were heavier than female rats of the same age group ($p < 0.05$).

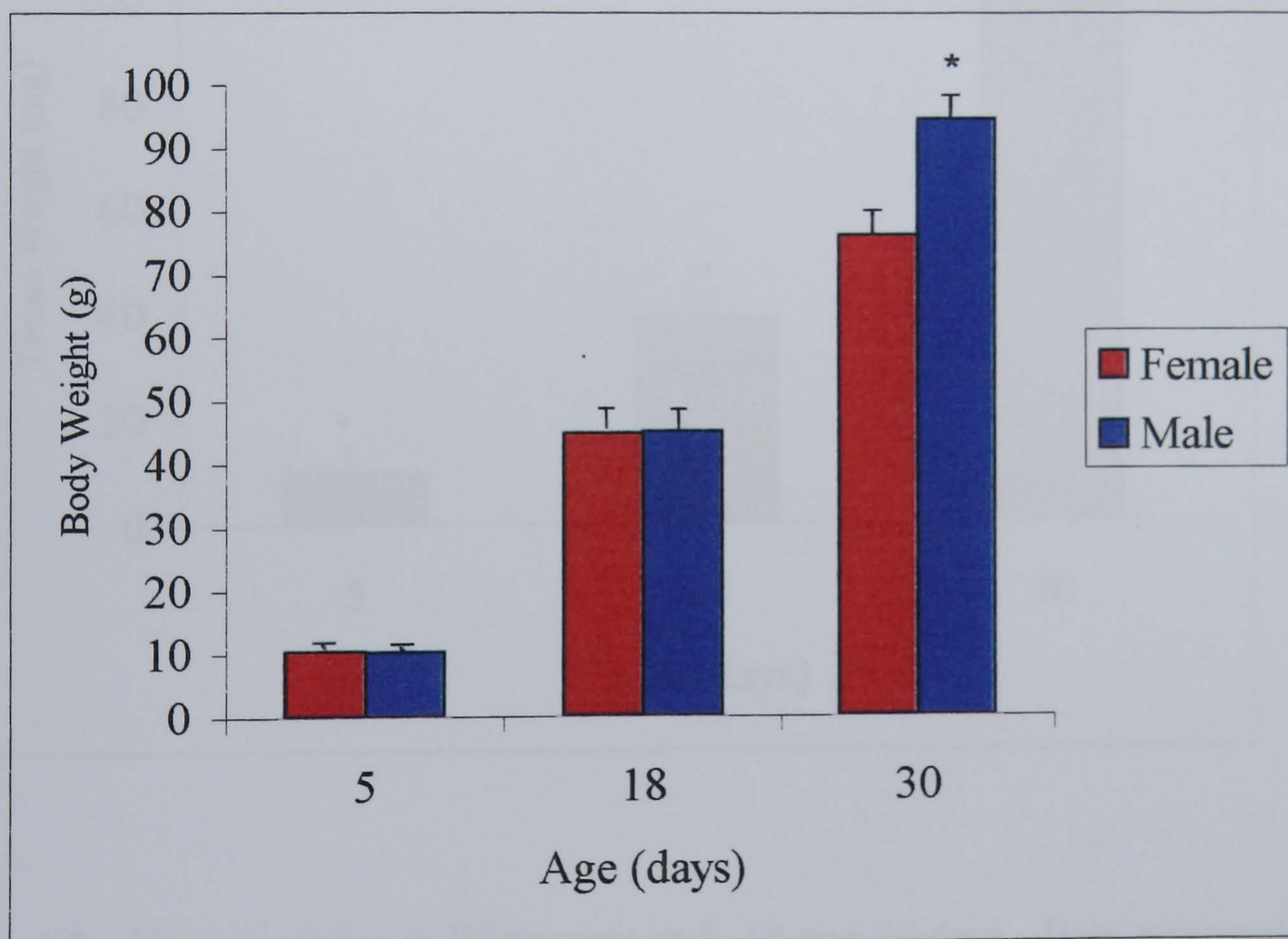


Figure 4.1: Body weights (g) of female and male Wistar rats at 5, 18 and 30 days. Bars represent the mean \pm SEM of 9-10 observations/group. (*Males differ from females of the same age group, $p < 0.05$, Two-way ANOVA.)

4.3.2: Tissue weights of female and male Wistar rats at 5, 18 and 30 days.

4.3.2.1: Uterus

The weight of the uterus in the three different age groups is shown in Figure 4.2. The weight of the uterus increased between 5, 18, and 30 days ($p < 0.05$). Between 5 and 30 days the rise appeared to be exponential.

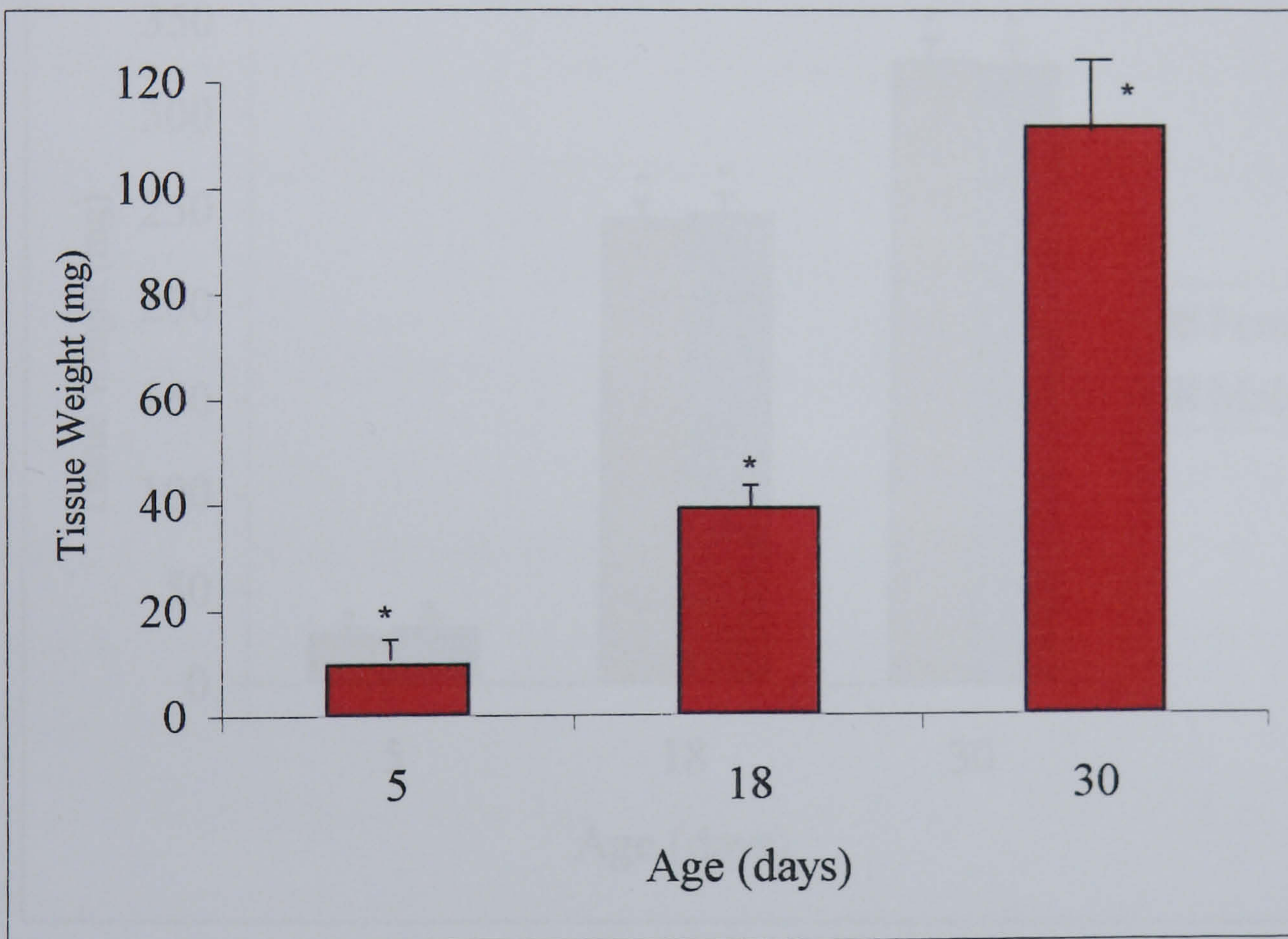


Figure 4.2: Uterus weights in Wistar rats at 5, 18 and 30 days. Bars represent the mean \pm SEM of 9-10 observations/group. (*Differs from the females of the other groups, $p < 0.05$; Kruskal-Wallis One-Way ANOVA on Ranks, Dunn's Multiple All Pairwise Comparison).

4.3.2.2: Thymus

Thymus weights of female and male Wistar rats are shown in Figure 4.3. The thymus in both sexes was heavier at 18 days and at 30 days than at each earlier age ($p < 0.05$ in each case). There was no sex difference in the weight of this tissue between males and females within any of the age groups ($p > 0.05$).

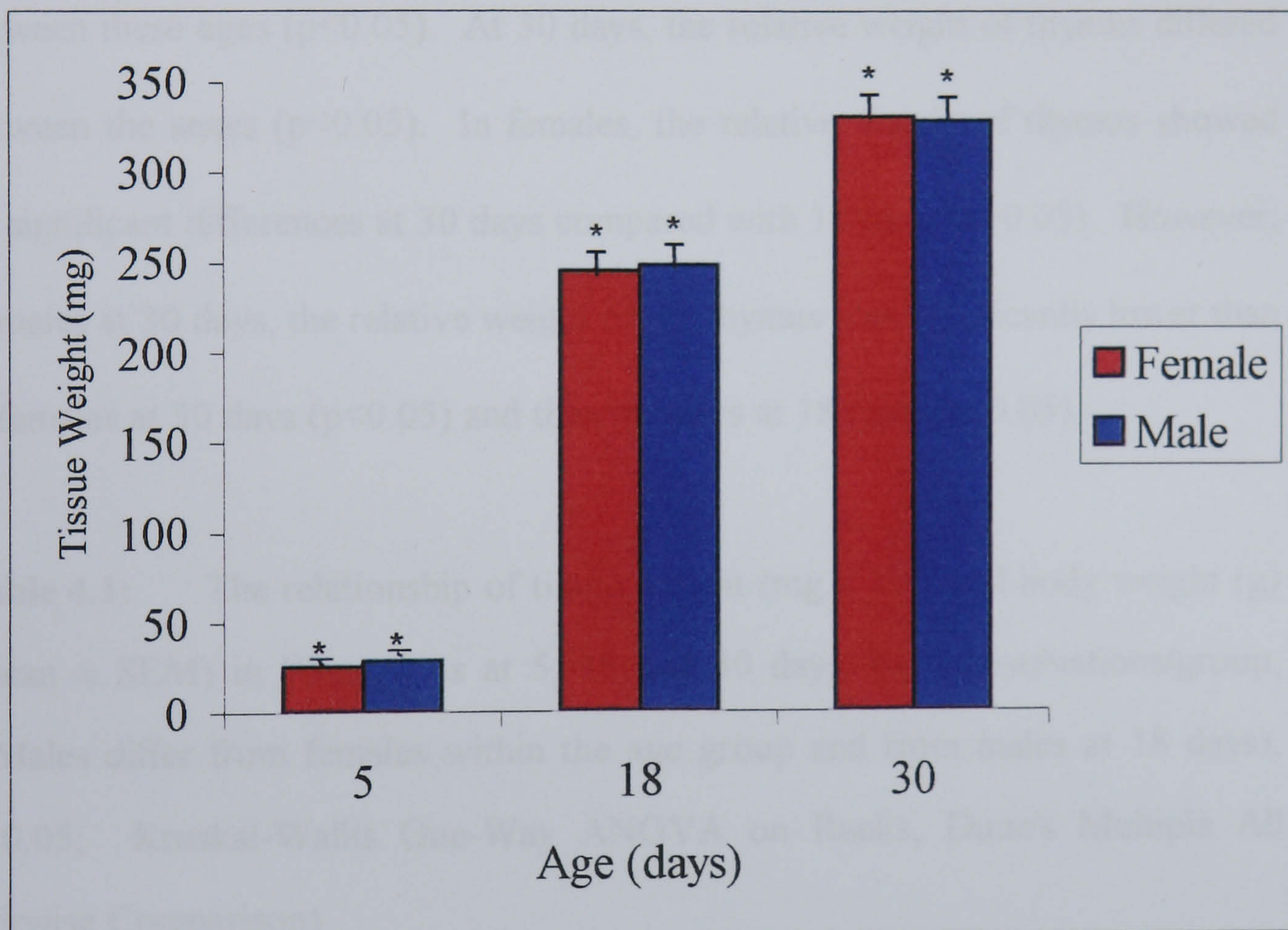


Figure 4.3: Thymus weight in female and male Wistar rats at 5, 18 and 30 days. Bars represent the mean \pm SEM of 9-10 observations/group. (*Differs from other age groups, $p < 0.05$; Kruskal-Wallis One-Way ANOVA on Ranks, Dunn's Multiple All Pairwise Comparison).

4.3.3 The tissue-to-body weight ratio in uterus and thymus in female and male Wistar rats at 5,18 and 30 days.

The ratio of tissue weight to body weight is shown in Table 4.1. The ratio for the uterus did not alter between 5 and 18 days ($p>0.05$). By 30 days, however, it had increased significantly over the value found in the two younger age groups ($p<0.05$). The thymus weight-to-body weight ratio was similar in males and females at 5 and 18 days ($p>0.05$), but increased in both sexes between these ages ($p<0.05$). At 30 days, the relative weight of thymus differed between the sexes ($p<0.05$). In females, the relative weight of thymus showed no significant differences at 30 days compared with 18 days ($p>0.05$). However, in males at 30 days, the relative weight of the thymus was significantly lower than in females at 30 days ($p<0.05$) and than in males at 18 days ($p<0.05$).

Table 4.1: The relationship of tissue weight ($\text{mg} \times 10^4$) and body weight (g) (mean \pm SEM) in Wistar rats at 5, 18 and 30 days. 6-10 observations/group; (*Males differ from females within the age group and from males at 18 days), $p<0.05$; Kruskal-Wallis One-Way ANOVA on Ranks, Dunn's Multiple All Pairwise Comparison).

| Sex | Tissue | 5 days | 18 days | 30 days |
|--------|--------|----------------|----------------|-----------------|
| Female | Uterus | 9.4 \pm 0.9 | 8.7 \pm 1.0 | 14.6 \pm 2.3 |
| Female | Thymus | 24.9 \pm 1.6 | 54.8 \pm 2.3 | 43.4 \pm 1.5 |
| Male | Thymus | 28.1 \pm 1.7 | 55.2 \pm 3.0 | 34.6 \pm 1.2* |

4.3.4: Determination of the concentration of cytosolic oestrogen receptors in uterus, hypothalamus, cortex and thymus

4.3.4.1: Uterus

The concentration of oestrogen receptors (E2R) in the uterus of Wistar rats is shown in Figure 4.4. The concentration of oestrogen receptors in the uterus approximately doubled between each of the age groups and was significantly different in all three age groups ($p < 0.05$).

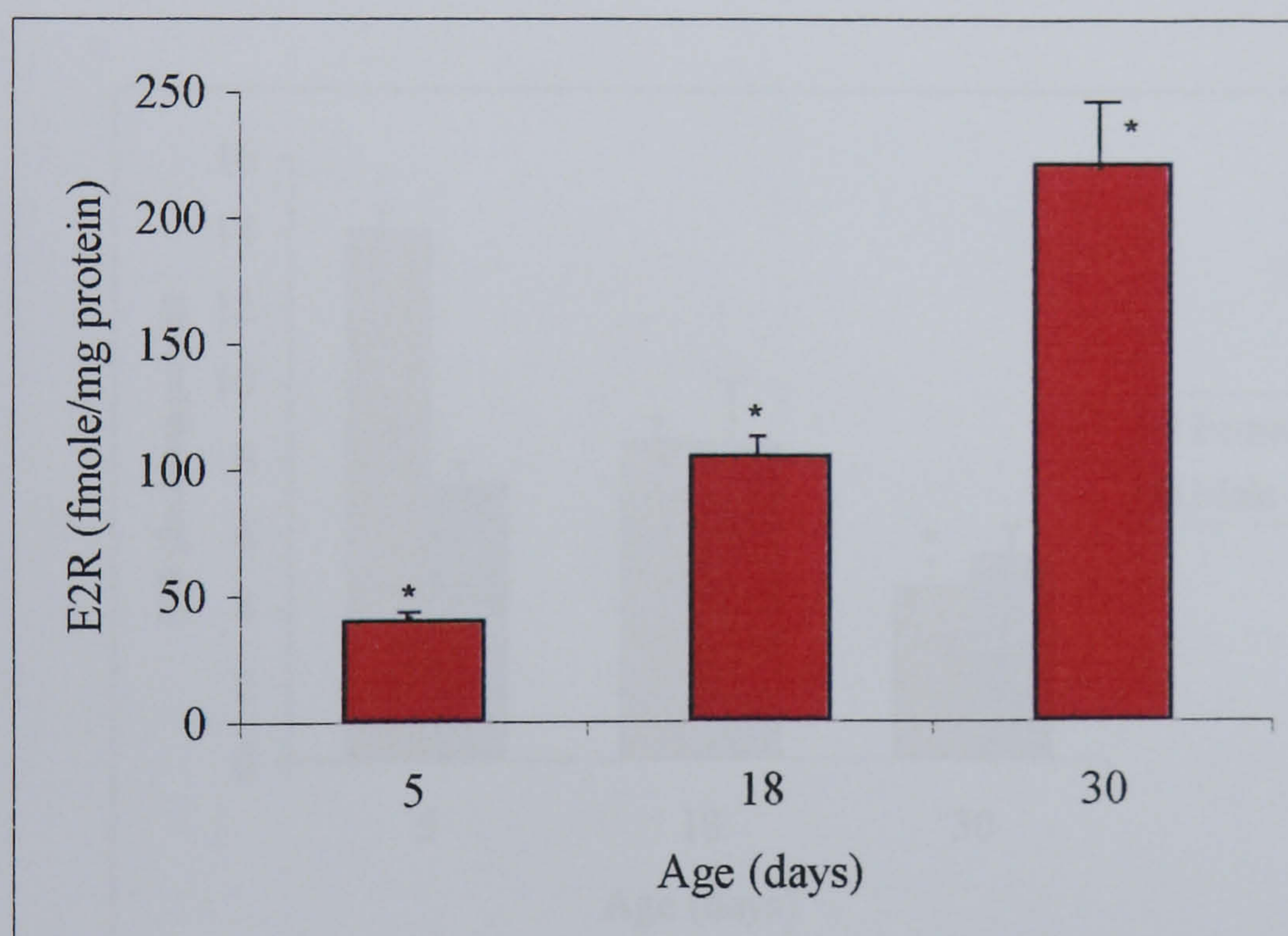


Figure 4.4: Concentration of cytosolic oestrogen receptors (E2R), (fmole/mg protein) in the uterus of Wistar rats at 5, 18 and 30 days. Bars represent the mean \pm SEM of 5-10 observations/group. (*Differs between females in the three age groups, $p < 0.05$; Kruskal-Wallis One-Way ANOVA on Ranks, Dunn's Multiple All Pairwise Comparison).

4.3.4.2: Hypothalamus

The concentration of oestrogen receptors in the hypothalamus of female and male Wistar rats is shown in Figure 4.5. In females, there was a progressive decrease with age in the concentration of oestrogen receptors in the hypothalamus ($p < 0.05$). There were higher concentrations (approximately double) of binding sites in the hypothalamus of 5-day-old females than in males of the same age group ($p < 0.05$). However, at 18 and 30 days, there were no significant differences in the concentration of oestrogen receptors in hypothalamus between the sexes ($p > 0.05$).

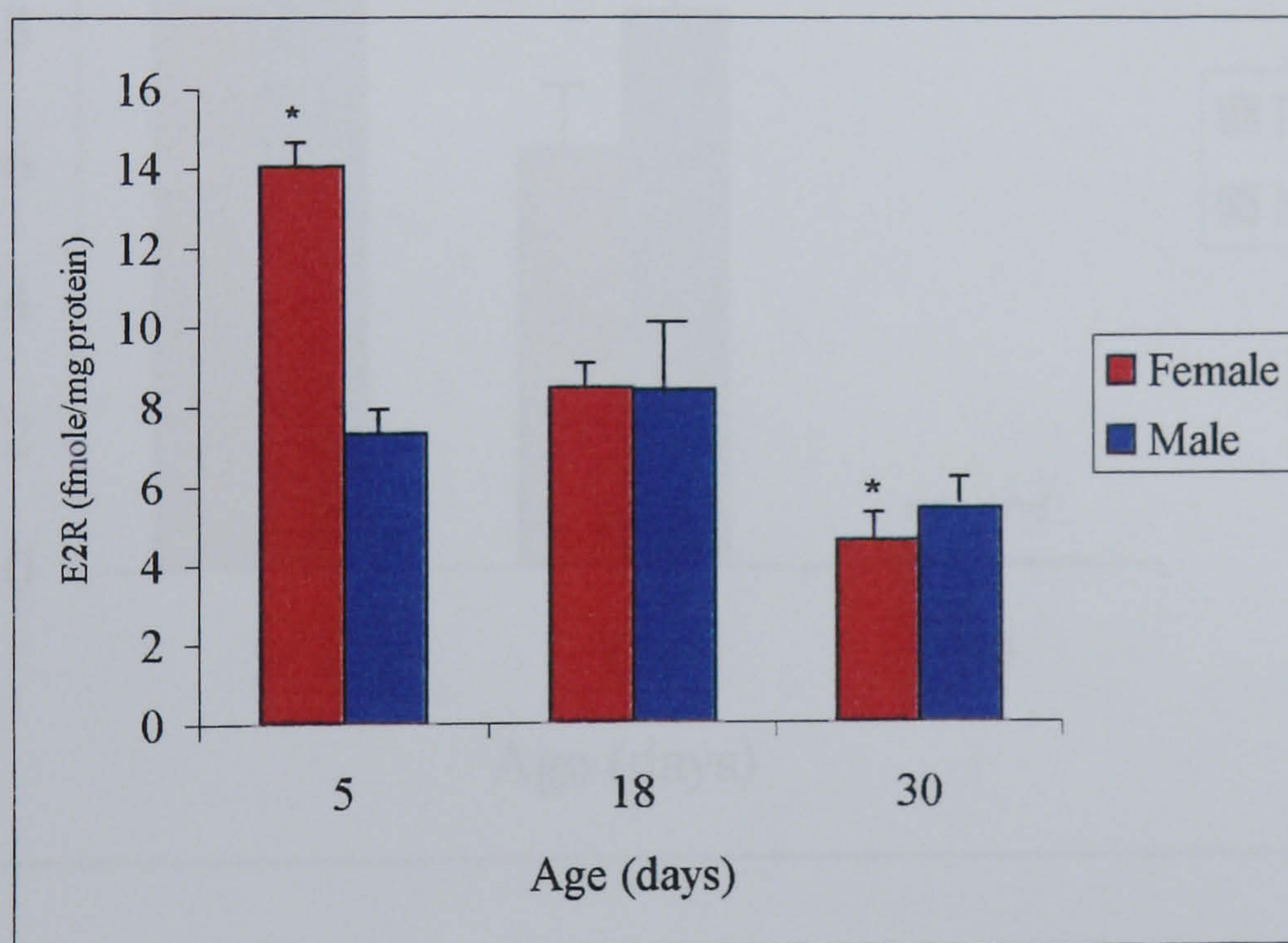


Figure 4.5: Concentration of cytosolic oestrogen receptors (E2R), (fmole/mg protein) in the hypothalamus of female and male Wistar rats at 5, 18 and 30 days. Bars represent the mean \pm SEM of 5-10 observations/group. (*Differs between males and females at 5 days, and between females in all age groups, $p < 0.05$; Kruskal-Wallis One-Way ANOVA on Ranks, Dunn's Multiple All Pairwise Comparison).

4.3.4.3: Cortex

The concentration of oestrogen receptors in the cortex of female and male Wistar rats is shown in Figure 4.6. In cortex, there were no differences in concentration of oestrogen receptors in 5-day-old and in 18-day-old rats ($p > 0.05$). By 30 days, however, no binding sites were detectable in either sex.

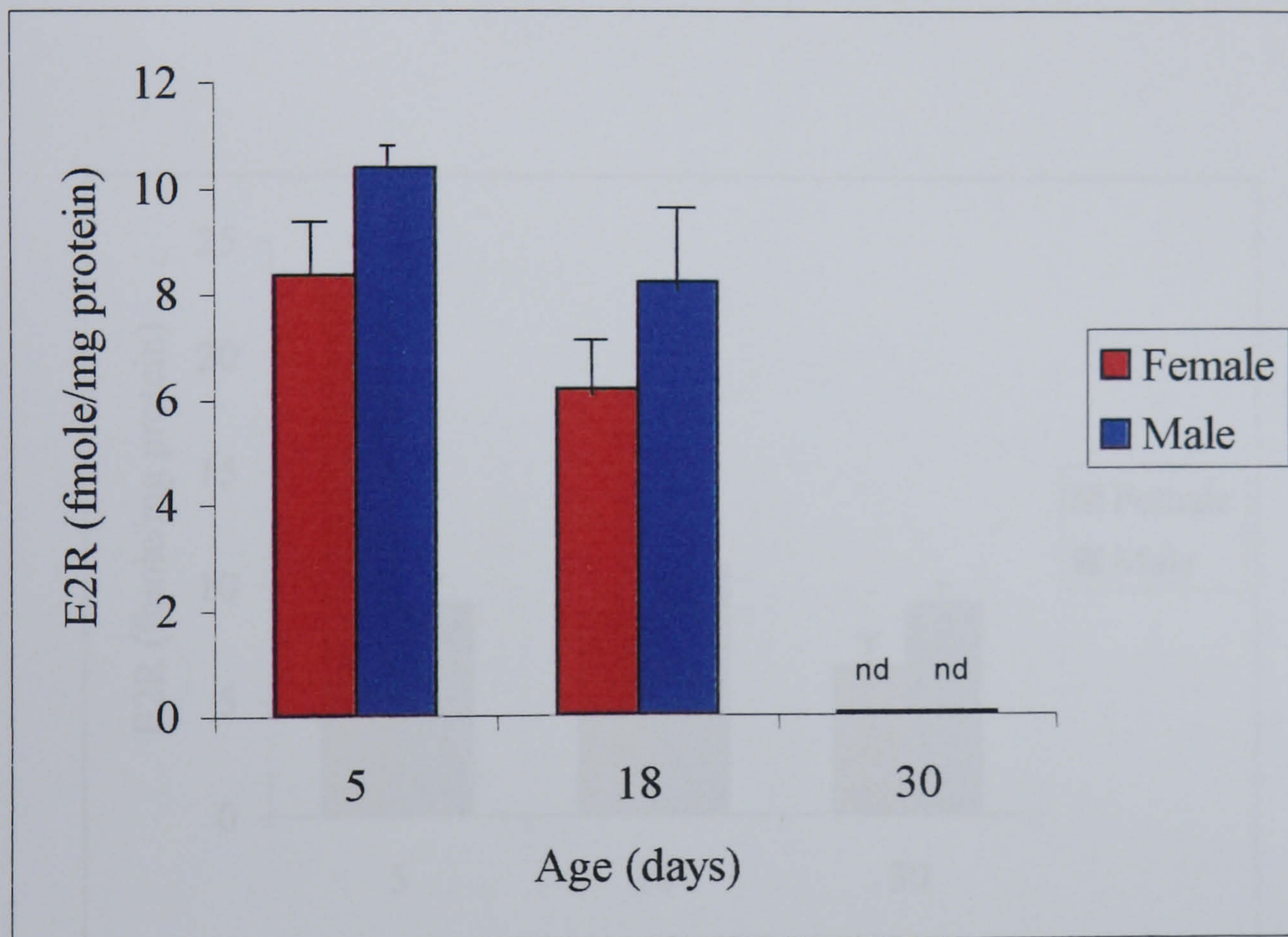


Figure 4.6: Concentration of cytosolic oestrogen receptors (E2R), (fmole/mg protein) in the cortex of female and male Wistar rats at 5, 18 and 30 days. Bars represent the mean \pm SEM of 5-10 observations/group. nd = not detectable.

4.3.4.4: Thymus

The concentration of oestrogen receptors in the thymus of female and male Wistar rats is shown in Figure 4.7. In thymus, the concentration of binding sites was similar in both sexes and in all three age groups ($p > 0.05$) with the exception of 5-day-old females. In this group, the concentration of binding sites was not only greater than in males of the same age group, but also higher than in females in any of the other groups ($p < 0.05$).

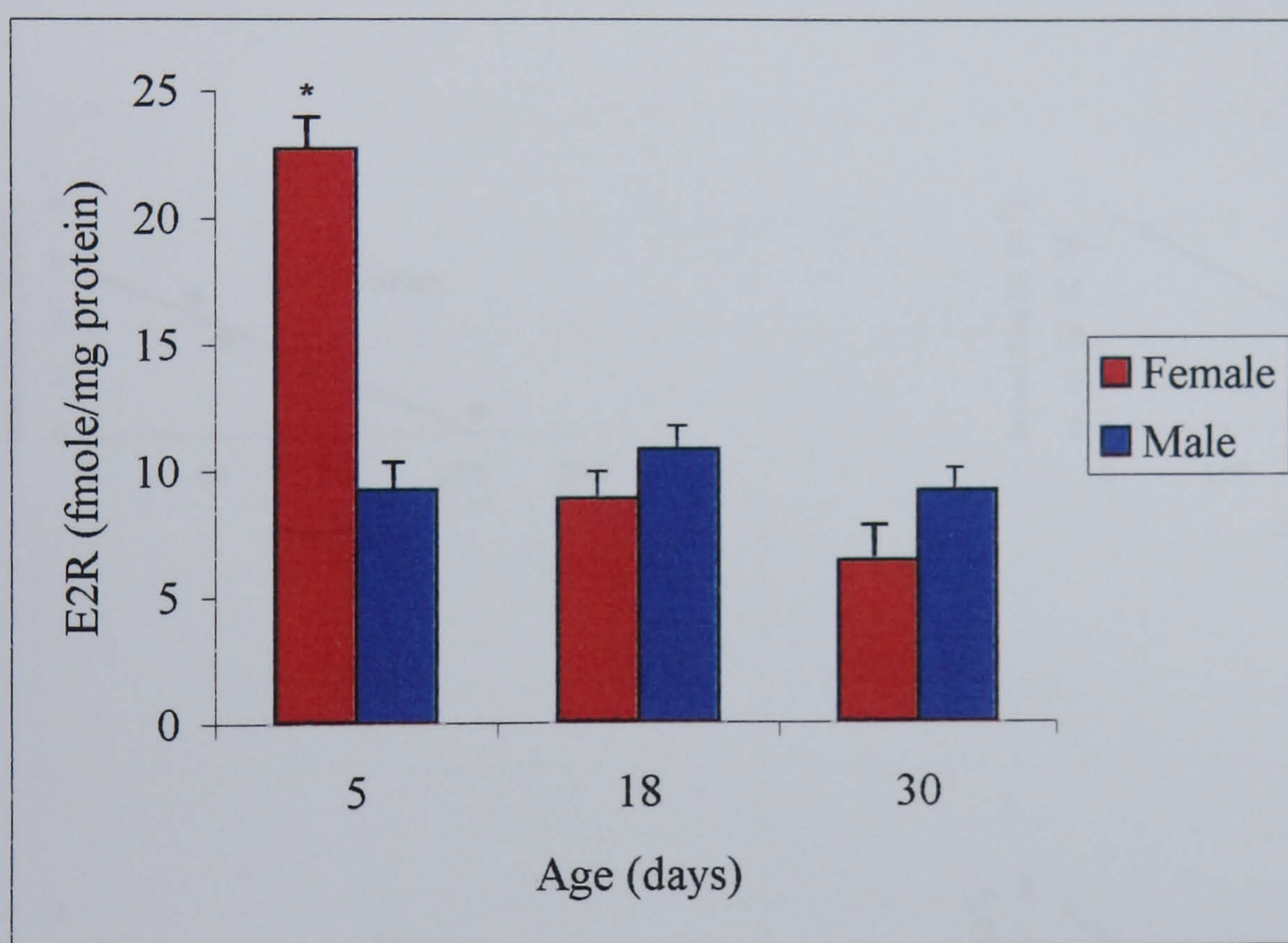


Figure 4.7: Concentration of cytosolic oestrogen receptors (E2R), (fmole/mg protein) in the thymus of female and male Wistar rats at 5, 18 and 30 days. Bars represent the mean \pm SEM of 5-10 observations/group. (*Differs between males and females of the same age group, $p < 0.05$; Kruskal-Wallis One-way ANOVA on Ranks, Dunn's Multiple All Pairwise Comparison).

4.3.5: Measurement of the molar dissociation constants of the oestrogen receptor (K_d) in uterus, hypothalamus, cortex and thymus.

Molar dissociation constants (K_d) were calculated by Scatchard plots as described in Section 2.2.5.4. In all tissues, and under the conditions of the experiments, binding isotherms yielded linear Scatchard plots, and the results (Table 4.2) were consistent with the presence of a high-affinity binding receptor for the ligand. (Figure 4.8).

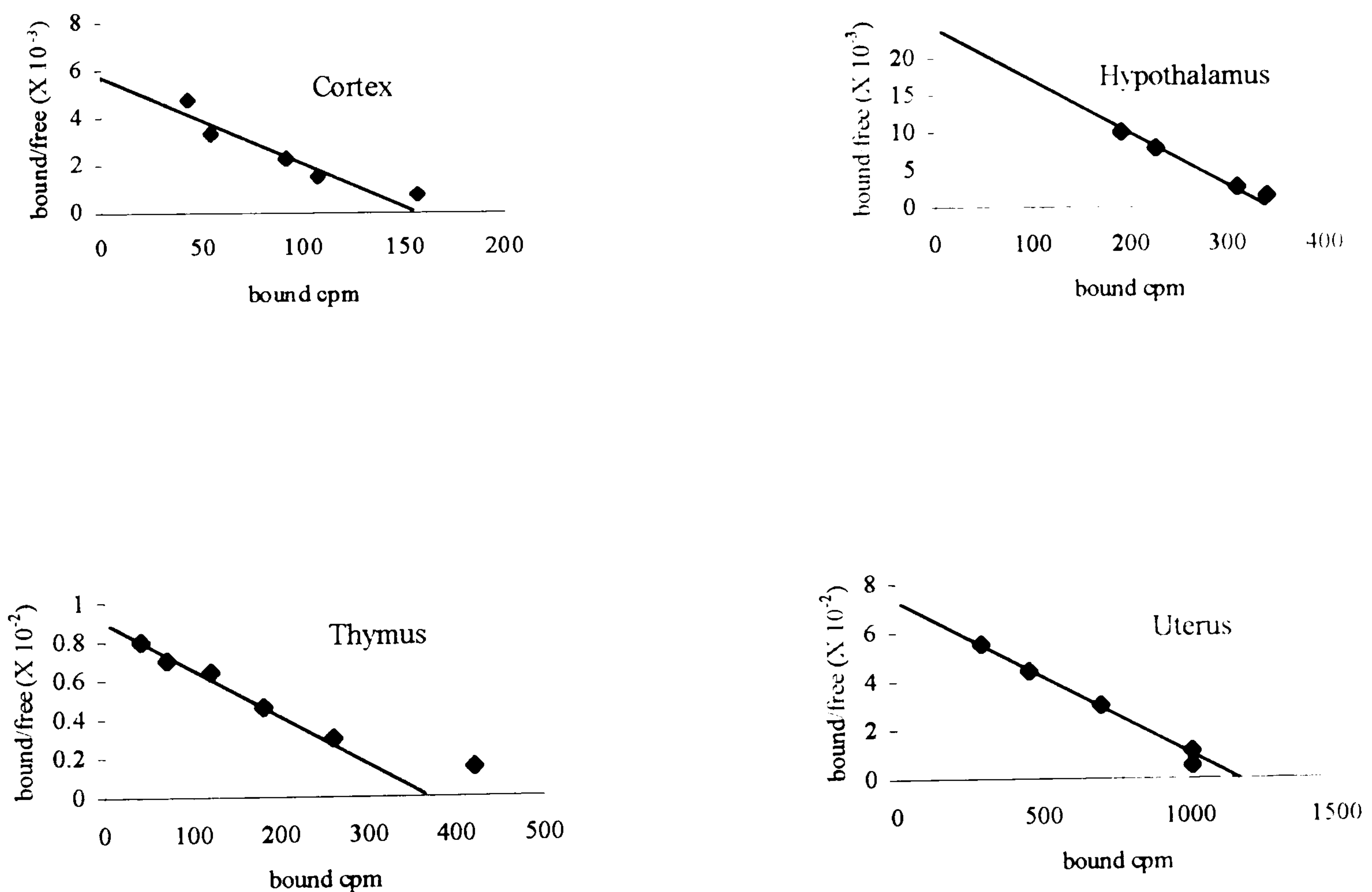


Figure 4.8: Examples of Scatchard analysis of moxestrol binding to immature male and female rat cortex, hypothalamus, thymus and uterus cytosols.

No significant differences were found in the value of apparent affinity of the oestrogen receptor for ^3H -moxestrol in any of the tissues examined ($p>0.05$). The value of the dissociation constant in all the tissues was of the order of 10^{-9} M (Table 4.2). In cortex, the binding constant was the same as in other tissues in rats of 5 and 18 days ($p>0.05$). By 30 days, however, no binding was detectable in this tissue in either male or female rats.

Table 4.2: Molar dissociation constant (Kd) of cytosolic oestrogen receptor in uterus, hypothalamus cortex and thymus of male and female Wistar rats at 5, 18 and 30 days old mean \pm SEM; number of observations 6-10; (Kruskal-Wallis One-way Analysis of Variance on Ranks; Dunn's All Pairwise Multiple Comparison Method) n.d. = not detectable by this method.

| Sex | Tissue | 5 days Kd x 10^{-9} M | 18 days Kd x 10^{-9} M | 30 days Kd x 10^{-9} M |
|--------|--------------|----------------------------|-----------------------------|-----------------------------|
| Female | Uterus | 2.53 \pm 0.75 | 1.31 \pm 0.18 | 2.23 \pm 0.32 |
| Female | Hypothalamus | 4.52 \pm 1.17 | 2.16 \pm 0.19 | 2.75 \pm 0.68 |
| Male | Hypothalamus | 3.33 \pm 0.44 | 2.46 \pm 1.17 | 3.82 \pm 1.06 |
| Female | Cortex | 2.55 \pm 0.62 | 2.22 \pm 0.27 | n.d. |
| Male | Cortex | 1.44 \pm 0.36 | 3.06 \pm 1.84 | n.d. |
| Female | Thymus | 2.20 \pm 0.51 | 1.89 \pm 0.83 | 3.24 \pm 0.57 |
| Male | Thymus | 1.71 \pm 0.39 | 1.79 \pm 0.58 | 2.62 \pm 0.41 |

4.3.6: The free fraction of oestradiol in the serum of female and male Wistar rats at 5, 18 and 30 days

The results of the measurement of the free fraction of oestradiol in the serum of Wistar rats at 5, 18 and 30 days are shown in Figure 4.9. The free fraction of oestradiol in the serum was low at 5 and 18 days (less than 1.8% of alphafetoprotein-bound oestradiol) but had risen by 30 days to similar levels in both sexes of 6.25%, which is the level found in adult rats. At 30 days, alphafetoprotein levels are low as the carrier protein is synthesised by the foetus during the prenatal period and is gradually metabolised in the neonate.

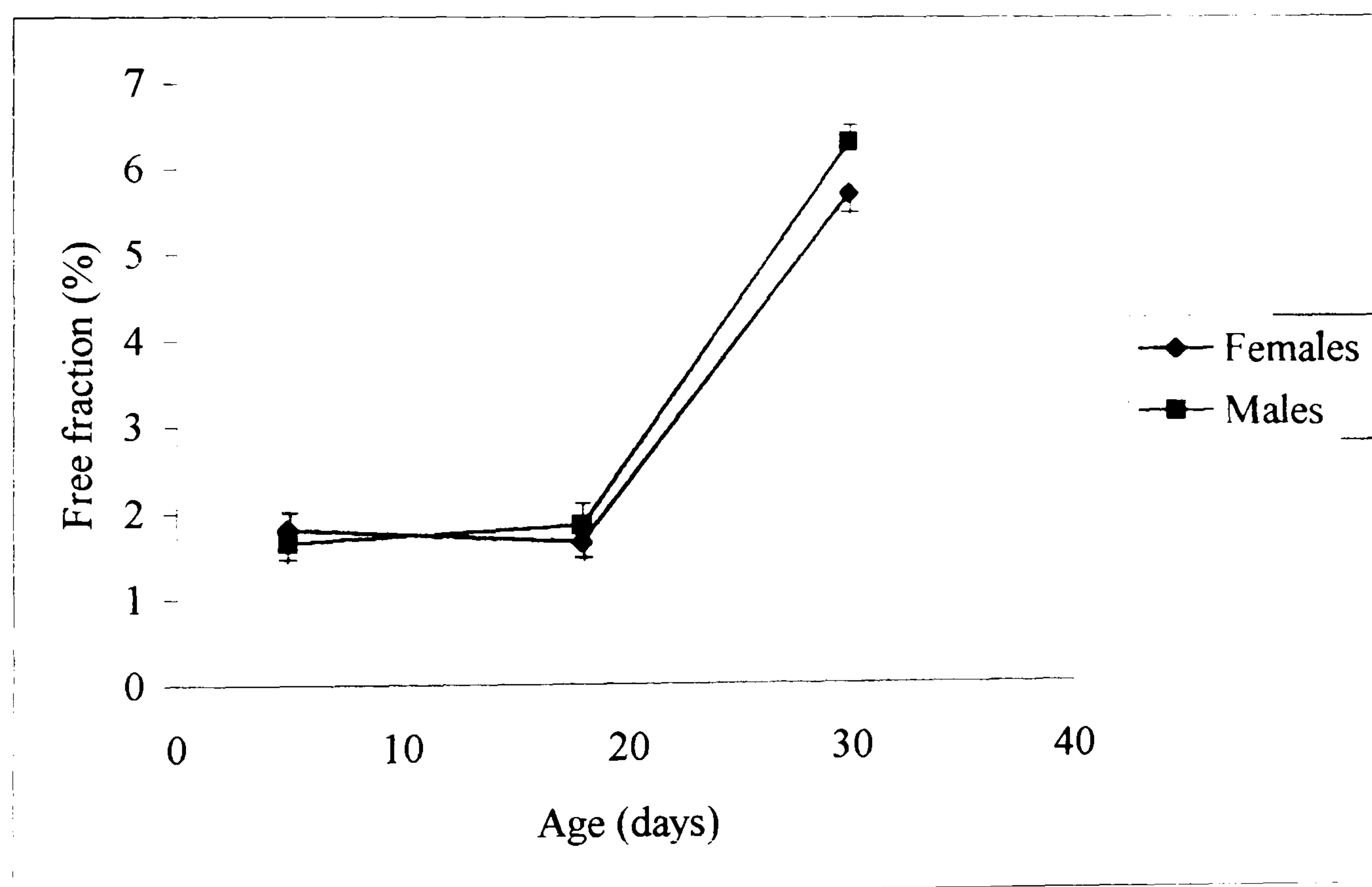


Figure 4.9: The free fraction of oestradiol in serum of female and male Wistar rats at 5, 18 and 30 days. Bars represent the mean \pm SEM of 5-10 observations per age and sex group.

4.4: Discussion

The aims of this study were to examine the binding characteristics of the cytosolic oestrogen receptor in the uterus, two brain regions, (the hypothalamus and the cortex), and the thymus of immature female and male rats and to establish whether there were similarities or differences in its concentration and dissociation constant in these tissues. Such findings could have physiological significance.

The three age groups chosen for this developmental study were identified as being appropriate for a number of reasons. Among the many changes taking place during this stage of development are several that appear to be dependent upon oestrogen. The uterus reaches its potential for reproduction during this period (Greene and Press, 1984). Sexual differentiation of the central nervous system in rats, as in most mammalian species, results, in part, from gender differences in gonadal hormone environment in the brain during the perinatal period (Keefer and Holderregger, 1985; O'Keefe and Handa, 1990; reviewed by Pilgrim and Hutchison, 1994). The free fraction of circulating oestradiol is low at 5 days and begins to rise at about 20 days as puberty approaches, reaching adult levels at 30 days (Greenstein, 1992). There is much evidence that involution of the thymus is related to increasing concentrations of sex hormones with approaching puberty (Chiodi, 1940; Grossman, 1984; Greenstein, 1986). Moxestrol, a synthetic oestrogen, was used for determining the binding characteristics of the oestrogen receptor in the three age groups. The dissociation constant of the oestrogen receptor for oestradiol is lower ($0.16 \times 10^{-9}\text{M}$) than for moxestrol ($0.73 \times 10^{-9}\text{M}$) in some rat tissues. However, in Scatchard analyses

that compared the binding characteristics of these two ligands, the same concentration of oestrogen receptors was found although the K_d value differed somewhat (Dahlberg, 1982; Kuhn-Velten *et al*, 1984). Moxestrol, unlike oestradiol, does not bind to alphafetoprotein. In immature rats, the high level of alphafetoprotein could interfere with the binding assays (Gerlach *et al*, 1983; Puig-Duran *et al*, 1979). Since the objective of this work was to compare the K_d and concentration of the cytosolic oestrogen receptor in immature rats, moxestrol was the more suitable agonist than oestradiol for this purpose.

4.4.1: Body and tissue weights

Between the age of 5 and 30 days, Wistar rats increased in weight. At 5 days, males and females had similar body weights and the increase in weight between this age and 18 days was similar in both sexes. However, 30-day-old males were heavier than females of the same age by which time, free oestrogen levels were rising in both sexes (see Figure 4.9). Oestrogen is necessary for bone maturation and in the pre-pubertal growth spurt in males and females (reviewed by Lee and Witchel, 1997). In rats, there is synergism between growth hormone and testosterone and this finding may account, in part, for the weight difference found between males and females (Zachmann, 1992).

Uterus weight increased progressively in each of the age groups (Figure 4.2) although the tissue-to-body weight ratio of this tissue did not change between the ages of 5 and 18 days (Table 4.1). By 30 days, however, the tissue-to-body weight ratio of the uterus was significantly greater ($p < 0.05$) than at the earlier ages in response to the increasing availability of oestrogen in circulation. The increased uterus weights were similar to the changes found by Brown *et al*

(1994) in their study of pubertal development of oestrogen receptors. During this period, oestrogen receptor mRNA is present in the uterine stroma and increases from day 1 to day 14 of postnatal life. It is not present at birth in the luminal epithelium and does not become abundant in this tissue until postnatal day 7. However, in the developing uterus, the oestrogen receptor is abundant in the glandular epithelium of the uterus by postnatal day 21 (Fishman *et al*, 1996).

The weight of the thymus also increased progressively with age and the increase was similar in both sexes. In both sexes the ratio of tissue-to-body weight increased from 5 to 18 days. In females, the value of the tissue-to-body weight ratio at 30 days was similar to its value at 18 days. In males at 30 days, however, the ratio of thymus to body weight was significantly less than in females of the same age. Wise and Klindt (1995) made a similar finding when considering the thymus of developing male and female cattle. In rats, oestrogen levels rise between 24 and 30 days in both sexes (Figure 4.9) and this response could slow the rate of thymus growth compared to the overall body weight gain in the males (Table 4.1) which is mainly directed by growth hormone. It is well-known that oestradiol administration causes thymic atrophy in rodents (Chiodi, 1940; Dougherty, 1952) and the thymus at this age may be responding to the increasing levels of free oestradiol in circulation. The effects of increasing testosterone may also be among several causes of thymic regression at this period of development and could explain the difference observed between males and females by 30 days. Greenstein *et al* (1988a) showed that orchidectomy increased the tissue-to-body weight ratio of thymus in 10-week-old rats, while testosterone administration blocks the regenerative effect. It is also possible that

in the thymus, aromatase activity in males may convert circulating testosterone to oestradiol.

4.4.2: Receptor studies: oestrogen receptor concentration

Uterus

The determination of the concentration of cytosolic oestrogen receptors in Wistar rats showed that in uterus there was a progressive increase with age in the number of receptors per mg of protein ($p < 0.05$) (Figure 4.4). At 30 days, the concentration of cytosolic oestrogen receptors in the uterus of Wistar rats was half the value found by Grossman *et al* (1979a) in Sprague-Dawley rats of the same age but was higher than the value found in 55-60-day-old Sprague-Dawley rats by Hagenfeldt and Eriksson (1988). Daher showed that cytosolic oestrogen receptor concentration differed between autoimmune-prone and non-autoimmune-prone mice. The difference between the results of Grossman's study and the results found in this study may be due to strain differences or to the different methods used in the two studies. Results of other research groups whose results are shown in Table 4.3.

During this period, the concentrations of free oestradiol are rising as alphafetoprotein, the molecule that sequesters maternal oestrogen in the blood of new-born mammals, disappears from the circulation (Greenstein, 1992; Keel and Abney, 1984). Between 5 and 30 days, the ovaries, in rats, begin to synthesise and release oestradiol and this increase is associated with increased growth of the uterus and of cytosolic oestrogen receptor concentration in this tissue (Gorski *et al*, 1968).

Table 4.3: Concentration of oestrogen receptors in uterus from different species of rodent.

| Authors | Tissue | Animals | Oestrogen receptor concentration (mean± SEM) (fmoles/mg protein) |
|-------------------------------|---------------|-----------------------------------|---|
| Dhafer, 1992 | Uterus | 21-day-old-Balb/c mice | 112±25 |
| Dhafer, 1992 | Uterus | 21-day-old-MRL-lpr/lpr mice | 556±78 |
| Grossman <i>et al</i> , 1979a | Uterus | 30-day-old Sprague-Dawley rats | 531±25 |
| Hagenfeldt and Eriksson, 1988 | Uterus | 55-60-day-old Sprague-Dawley rats | 123±8 |

Hypothalamus

The concentrations of cytosolic oestrogen receptors in the hypothalamus of males were similar in all the age groups (Figure 4.5). In females, there was a significant decrease in the concentration of oestrogen receptors between 5, 18 and 30 days although at 18 and 30 days there were no differences between the sexes. At 5 days, the concentration of receptors in females was approximately double and significantly higher than the value found in males of the same age. A comparison of the results obtained here and those of other groups is shown in Table 4.4.

Table 4.4: Concentration of oestrogen receptors in hypothalamus of different strains of rats reported by other groups.

| Authors | Tissue | Animal | Oestrogen receptor concentration (fmol/mg protein) (mean± SEM) |
|-------------------------------|------------------------------------|--|--|
| This study | hypothalamus | Wistar rats | |
| | | males 5 days | 7.31±1.10 |
| | | female 5 days | 14.08±1.96 |
| | | males 18 days | 8.43±2.52 |
| | | females 18 days | 8.47±0.93 |
| | | males 30 days | 5.41±0.65 |
| | | females 30 days | 4.63±0.99 |
| Brown <i>et al</i> , 1994 | preoptic area of the hypothalamus | 30-day-old female Sprague Dawley rats | 20 |
| Brown <i>et al</i> , 1994 | hypothalamus | 30-day-old female Sprague Dawley rats | 25 |
| O'Keefe J and Handa R, 1990 | hypothalamus | 5-day-old Sprague-Dawley rats (male and female pooled) | 30 |
| O'Keefe and Handa, 1990 | hypothalamus | 18-day-old Sprague-Dawley rats (male and female pooled) | 20 |
| O'Keefe and Handa , 1990 | hypothalamus | 30-day-old Sprague-Dawley rats (male and female pooled) | 20 |
| Kuhnemann <i>et al</i> , 1994 | hypothalamic ventro-medial nucleus | Wistar rats | |
| | | 5 days males | 18 |
| | | 5 days females | 28 |
| | | 10 days males | 8 |
| | | 10 days females | 20 |

The present study differed in some respects from the results of Brown *et al* (1994) and O'Keefe and Handa (1990). The higher concentration of hypothalamic oestrogen receptors in Sprague-Dawley rats that Brown *et al* (1994) reported may be due to strain differences with respect to the Wistar rats used in the present study. The different techniques used in Brown's study (autoradiography) could also account, in part, for the different results obtained for 30-day-old rats. O'Keefe and Handa (1990), using binding techniques, found a slightly higher concentration of cytosolic receptors in the hypothalamus of 5-day-old rats compared with older age groups and no sex differences in any of the age groups studied. Results of their study showed higher concentrations of oestrogen receptor in the hypothalamus than the results of the present study and the difference could be due to the different strain (Sprague-Dawley) rat used. On the other hand, results of the present study were in agreement with those of Kuhnemann *et al* (1994), who detected higher concentrations of oestrogen receptors in 5-day-old female Wistar rats than in males of the same age group, with the sex difference still present at 10 days. By 18 days, in the Wistar rats used in the present study, no sex differences in hypothalamic oestrogen receptor concentrations were detected.

Changes over time of oestrogen receptor concentration in hypothalamus could be due to a number of factors. The decrease in oestrogen receptor concentration in females between 5 and 30 days could be due to the increasing availability of oestradiol as alpha-fetoprotein concentrations decrease and as the ovaries begin to synthesise and release oestradiol. A consequence of increased oestradiol would be binding of the receptor and subsequent translocation to the

nucleus and hence a reduction in cytosolic receptor concentration would be observed. Alternatively, the binding of receptor could down-regulate receptor synthesis and lead to reduced cytosolic receptor concentrations as replenishment of receptors declined. In males, on the other hand, locally synthesised oestradiol, by conversion from testosterone, is available at 5 days. Binding of oestradiol in males to the oestrogen receptor and to the subsequent translocation of the bound receptor to the nucleus would result in a decrease in cytosolic oestrogen receptors in males at 5 days. Measurement of the nuclear receptors as well as of cytosolic receptors by autoradiography or by immunohistochemistry could identify whether the hormone-receptor complex was bound to the DNA in the nucleus. There are higher concentrations of oestrogen receptor mRNA and of the nuclear oestrogen receptor itself in the hypothalamus of 5-day-old male rats than in females of the same age (Adcock and Greenstein, 1986). Moreover, in female rats, treated at 5 days with oestradiol, the concentration of oestrogen receptor mRNA is the same as in males, the nuclear oestrogen receptor concentration also rises to the same level as in males at 5 days and treatment of neonate females with testosterone produces the same result (Adcock and Greenstein, 1986). Higher concentrations of nuclear receptors could be due to the presence of high concentrations of oestradiol, aromatized from testosterone in the hypothalamus in rats of this age. Translocation of receptor-bound oestradiol from cytosol to nucleus in hypothalamus of males in the present study could explain the higher concentrations of (untranslocated) cytosolic receptors found in the hypothalamus of 5-day-old female rats compared with the males. As circulating oestradiol concentrations rise in females during the period up to 30

days, the cytosolic receptor levels would fall, if translocation to the nucleus occurred, to levels similar to those found in males.

Treatment of female rats at 5 days with dihydrotestosterone, which is not convertible to oestradiol, does not produce the same increase in oestrogen receptor mRNA in 5-day-old females as does oestradiol or testosterone treatment (Greenstein *et al*, 1988a) and this finding suggests that oestradiol treatment enhances the synthesis of oestrogen receptor mRNA. Treatment of pre-pubertal female rats with alphafetoprotein delays vaginal opening and reduces the number of nuclear oestrogen receptors in the hypothalamus and the preoptic area as well as in the uterus (Greenstein, 1992). This finding of lower concentrations of nuclear receptor when oestradiol is not available would be consistent with a role for oestradiol in the translocation of hormone-bound receptor from the cytosol to the nucleus of the cell. The higher concentrations of oestrogen receptors in the hypothalamus of 5-day-old females in the present study could suggest that in neonate males and females, oestrogen receptors were present in the cytosols, but that in males, circulating testosterone was converted to oestrogen in some cells through the activity of aromatase. At 5 days females had little free circulating oestradiol and therefore their tissues were not exposed to oestradiol at the same concentrations as were those of the males until a slighter later age (18-30 days) when the ovaries began to synthesise and release oestradiol.

Cortex

The concentration of receptors in cortex cytosols was similar in both sexes at 5 and 18 days (Figure 4.6). There did appear to be a slight reduction of receptor concentrations between 5 and 18 days, but the difference did not reach levels of statistical significance.

Kuhnemann *et al* (1994), using quantitative *in vitro* autoradiography and Wistar rats, reported similar results to the present study of brain cortex cytosolic receptor concentration at 5 and 10 days with no differences between the sexes in cortical oestrogen receptors. O'Keefe and Handa (1990), using binding assays, similar to the techniques used for this present study also reported similar results of an age-dependent trend to decreased oestrogen receptor concentration in cortex, with no sex differences and no receptors detectable by 30 days in either sex. These results could suggest that in cortex, increasing concentrations of available oestradiol were bound to cytosolic receptors which were translocated to the nucleus. Brown *et al* (1994) reported increased concentrations of nuclear oestrogen receptor and concomitant decreased cytosolic receptors in various brain areas of rats at puberty and/or after administration of oestradiol. This finding could explain the observation of the disappearance of detectable oestrogen receptors in the cortex in the present study. High concentrations of oestrogen receptor mRNA have been detected in the cortex of adult rats using *in situ* hybridisation techniques (Pelletier *et al*, 1988). Loss of cytosolic receptor in the cortex could, therefore, have been due to altered post-translational processing of oestrogen receptor mRNA or to the development of receptor-degrading enzymes in this brain area following the early postnatal period. The

sex difference between males and females in the hypothalamus was not shown in the cortex and this result could be indicative of the presence and activity of aromatase in the hypothalamus, but not in the cortex. Beyer *et al* (1994) found significantly higher concentrations of aromatase-immunoreactivity in the hypothalamus of developing male mice than in the females. Moreover they found little aromatase immuno-reactivity in the cortex and no sex differences in this area of the brain. It is possible that no oestrogen receptors are present in the brain cortex after the early postnatal period (Table 4.5).

Table 4.5: Concentration of cytosolic oestrogen receptors in brain cortex and amygdala tissue at different ages (days) in various strains and species of rodent.

(nd = not detectable).

| Author | Tissue Animal | Sex and age | Mean oestrogen receptor concentration (fmoles/mg protein) |
|--------------------------------|------------------------------------|------------------------------|---|
| This study | Cortex Wistar rats | Female 5 | 10.5 |
| | | Male 5 | 8.5 |
| | | Female 18 | 6.0 |
| | | Male 18 | 6.0 |
| | | Female 30 | nd |
| | | Male 30 | nd |
| Kuhnemann <i>et al.</i> , 1994 | cortical amygdala Wistar rat | male 5 | 17 |
| | | female 5 | 17 |
| | | male 10 | 8 |
| | | female 10 | 12 |
| Brown <i>et al.</i> , 1994 | amygdala Sprague- Dawley rat | female 30 | 4 |
| O'Keefe and Handa, 1990 | cortex Sprague- Dawley rats | male and female pooled 5 | 20 |
| O'Keefe and Handa, 1990 | cortex Sprague- Dawley rats | male and female pooled 18 | 2 |
| O'Keefe and Handa, 1990 | cortex Sprague- Dawley rats | male and female pooled 30 | nd |
| Shughrue <i>et al.</i> , 1990 | cortex perinatal mice | age 1-8 age 8-24 | increase decrease |

Thymus

The concentration of oestrogen receptors measured in thymus cytosols by other research groups is shown in Table 4.6. The concentration of oestrogen receptors measured in thymus cytosols from male and female rats at 30 days were similar to those reported by Grossman *et al*, (1979b) in the thymus of adult male Sprague-Dawley rats and in cattle. These values were a little lower than the concentration (22.2fmole/mg protein) found by Brodie *et al* (1980) in the thymus of rats of the same sex, age and strain as those studied by Grossman *et al* (1979b). Using enzyme immunoassay techniques, Kawashima *et al* (1992) reported higher levels for whole thymus in mice. In the present study, significantly higher concentrations of cytosolic oestrogen receptors were found in thymus of 5-day-old females (22.7 fmoles/mg) than in females of the older age groups and in males of all the age groups investigated. Grossman *et al* (1979a) found no sex differences at these ages. The result reported here for thymus showed the same trend as in the hypothalamus for the same age group of females and might reflect similar mechanisms of translocation to the nucleus and/or down-regulation of oestrogen receptor synthesis in this tissue.

The microenvironment of the hypothalamus and thymus differs in many ways. Nevertheless, these tissues may be similar with respect to their ability to convert testosterone to oestradiol and to differ in this respect from the cortex, in which there was no evidence of sex differences in oestrogen receptor activity at any age. Different nerve growth and other growth factors may be produced locally in the hypothalamus and thymus and may interact with oestradiol and the oestrogen receptor in analogous ways to give locally relevant responses. In the

developing basal forebrain, oestrogen receptors co-localise with nerve growth factor receptors (Toran-Allerand *et al*, 1992) and oestrogen-sensitive neurons express mRNAs for neurotrophins and their receptors during development (Miranda *et al*, 1994). Oestradiol, available through aromatization in prenatal and perinatal thymus tissue in male rats, may in the presence of thymic factors (interleukins or thymic hormones) or other neurosteroids direct thymic development. These pathways would be unavailable to females at this age because of the low concentration of available oestradiol at this early, critical period of development.

Different or similar mechanisms in males and females could be producing the same end results i.e., higher concentrations of cytosolic oestrogen receptors in hypothalamic and thymic tissue in females than in males at 5 days (Figures 4.5 and 4.7). The higher levels of oestrogen in males, produced through aromatization of testosterone to oestradiol, could lead to occupancy of the receptors and a lowering of cytosolic receptor concentration available for measurement. Oestrogen-bound receptors would be translocated into the cell nucleus and become tightly associated with DNA. This could account for the lower concentration of oestrogen receptors in hypothalamic and thymic cytosols. Moreover, as oestrogen classically downregulates its own receptor in the adult brain (Shughrue *et al*, 1990), the higher levels in males resulting from aromatization of testosterone could have a similar effect in the hypothalamus and thymus of male rats as young as 5 days when testosterone concentrations are high. In females, increasing availability of oestradiol, synthesised and released by the developing ovaries and circulating in the blood, could interact with the

receptors in hypothalamus and thymus at 18 and 30 days in a similar manner to that found in males of all three age groups studied. The thymus tissue-to-body weight ratio was greater in females than in males at 30 days. This result could support the conclusion that males at this age were exposed to oestrogen, formed in the thymus from aromatization of testosterone, for a longer period and that oestrogens cause thymic involution (Forsberg, 1995). This explanation is unlikely, however, as the absolute weight of the thymus in both sexes increased over the period. Moreover increased bone and body mass in males may be independent of thymic development.

Oestrogen treatment is known to alter T-cell development in the thymus in mice and results in a decrease in the number of CD4⁺/CD8⁺ cells and an increase in the proportion of CD4⁺ helper T-cells (Rijhsinghani *et al*, 1996b). This change in the ratio of immature to mature T-cells could be due to the interaction of oestradiol with reticuloepithelial cells and/or with the T-cells themselves. Thymosin- β 4 is synthesised and released by the thymus and this hormone has long been known to be important in a number of immune functions (Low *et al*, 1981) as well as in a number of interactions with the hypothalamus and its release of luteinizing hormone-releasing hormone (Rebar *et al*, 1981). Moreover, oestrogen therapy depresses thymosin- β 4 concentrations in women (Suh *et al*, 1985). There is some evidence that the oestrogen receptor can bind to the DNA independently of the presence of oestrogen itself (Gorski *et al*, 1993).

Table 4.6: Concentration of oestrogen receptors (mean \pm SEM) in thymus in various strains and species of rodent.

| Authors | Animal | Sex and age | Oestrogen receptor concentration (fmol/mg protein) |
|-------------------------------|---------------------------|------------------|--|
| Present study | Wistar rats | Female, 5 days | 22.76 \pm 2.37 |
| | | Male, 5 days | 9.26 \pm 1.68 |
| | | Female, 18 days | 8.89 \pm 1.28 |
| | | Male, 18 days | 10.81 \pm 0.95 |
| | | Female, 30 days | 6.41 \pm 0.37 |
| | | Male, 30 days | 9.21 \pm 0.86 |
| Grossman <i>et al</i> , 1979b | Sprague-Dawley rats | Male, 1-2 months | 10.15 \pm 0.39 |
| Brodie <i>et al</i> , 1980 | Sprague-Dawley rats | Male, adult | 22.2 \pm 4.0 |
| Grossman <i>et al</i> , 1979a | Cattle (strain not given) | Male, adult | 6.57 \pm 0.048 |
| Grossman <i>et al</i> , 1979a | Rats (strain not given) | Male, adult | 6.06 \pm 0.365 |
| | | Female, adult | 5.60 \pm 0.308 |
| Kawashima <i>et al</i> , 1992 | C57 BL/6NJcl mice | Female, adult | 24.1 \pm 2.9 |
| Athreya <i>et al</i> , 1989 | NZB mice | Male, adult | 19.3 \pm 1.8 |
| | | Female, adult | 17.3 \pm 1.2 |
| | NZW | Male, adult | 16.7 \pm 2.2 |
| | | Female, adult | 17.2 \pm 1.9 |
| | NZB/NZW F1 | Male, adult | 14.7 \pm 1.5 |
| | | Female, adult | 17.3 \pm 1.1 |

4.4.3: Receptor studies: dissociation constant

The Scatchard analysis obtained using cytosols from uterus, thymus and hypothalamus and cortex yielded straight lines (Figure 4.8). The shape of the plots was consistent with the presence of one class of specific, high-affinity binding site in all the tissues investigated under the experimental conditions used (Figure 2.5). The K_d values obtained for oestrogen receptor were of the order of 1×10^{-9} to 5×10^{-9} M for the tissues examined (Table 4.7). No statistical differences were found between the values in males and females of all three age groups in any of the tissues examined. The values of the dissociation constant of the oestrogen receptor in the tissues examined were similar to results reported by other groups in some instances, but differed from those reported in other cases. The values found in the present study were similar to those found by Danel (1983) and Dhaher (1992). In these two studies, as in the present study, radiolabelled and unlabelled moxestrol were the ligands used to assess total and non-specific binding to the oestrogen receptor and gel filtration was used to separate the bound and free hormone. Differences in the results of the present study from those in Table 4.7 could reflect the different ligands employed or the different methods used to separate the bound and free steroid. Species differences could also account, in part, for the differences in K_d but as the similarities to the present study were found in tissues from humans (Danel, 1987) and mice (Dhaher, 1992), this explanation seems less likely than in the difference in the methodologies employed. However, Dhaher's results for the dissociation constant of the oestrogen receptor for moxestrol in the thymus of immature MRL mice was one order of magnitude lower than the result found for

Balb/c mice in the same study. Athreya (1989) also found lower dissociation constants for the oestrogen receptor in the thymus of NZW/NZB F1 mice. The lower affinity of the receptor in the thymus may be significant in these autoimmune prone strains of mice. Wistar rats are not autoimmune-prone and in this strain, the dissociation constant of the oestrogen receptor for moxestrol in thymus cytosols was similar to the value found in non-autoimmune-prone Balb/c mice (Dhafer, 1992). By 30 days, there was an apparent loss of receptor affinity and/or cytosolic receptor in the cortex. One possible explanation for the difference is that, at this age, oestrogen levels were rising in females and males and could be occupying receptors that were then translocated into the cell nucleus where, tightly bound to DNA, they were no longer detectable in the cytosols from the cortex under the conditions used.

Table 4.7: Molar dissociation constants (Kd) of the cytosolic oestrogen receptor determined in various tissues using different experimental methods.

| Author | Tissue | Animals | Mean Kd ($\times 10^{-9}$ M) (see key for method) |
|----------------------------------|--|----------------------------------|---|
| Present study (see Table 4.2) | uterus hypothalamus cortex thymus | immature Wistar rats | 1.31-4.52 (a. 1) |
| Brodie <i>et al</i> , 1980 | thymus | adult Sprague-Dawley rats | 0.43 (c. 5) |
| Dahlberg <i>et al</i> , 1982 | skeletal muscle | adult rat. strain unspecified | 0.73 (c. 1) |
| Grossman <i>et al</i> , 1979b | thymus | adult Sprague-Dawley rats | 0.25 (c. 5) |
| O'Keefe and Handa, 1990 | hippocampus | immature Sprague-Dawley rats | 0.14-0.55 (a. 2) |
| Yuan <i>et al</i> , 1995 | hippocampus | adult Sprague-Dawley rats | 0.643 (b. 3) |
| Athreya <i>et al</i> , 1989 | thymus | NZB, NZW, NZB/NZW FI mice | 0.09-0.15 (c. 4) |
| Dhaher, 1992 | uterus hypothalamus thymus | immature Balb/c mice | 4.56 7.09 10.9 (a. 1) |

Key: a = gel filtration; b = quantitative autoradiography; c = dextran-coated charcoal; d = enzyme-linked immunoassay; 1 = moxestrol and 3 H-moxestrol; 2 = moxestrol and 3 H-oestradiol; 3 = diethylstilbestrol and 11β -methoxy- 16α - 125 I]iodo-estradiol; 4 = diethylstilbestrol and 3 H-diethylstilbestrol; 5 = oestradiol and 3 H-oestradiol; 6 = diethylstilbestrol and 16α - 125 I]oestradiol.

Table 4.7: (continued) Molar dissociation constants (Kd) of the cytosolic oestrogen receptor determined in various tissues using different experimental methods.

| Author | Tissue | Animal | Mean Kd ($\times 10^{-9}\text{M}$) (see key for method) |
|-----------------------------|-----------------------------------|------------------------|--|
| Dhafer, 1992 | uterus hypothalamus thymus | MRL-lpr/lpr mice | 2.33 0.58 0.79 |
| Sholl and Kim, 1989 | brain | fetal rhesus monkeys | 0.46 (a. 1) |
| Danel <i>et al</i> , 1983 | thymus | human | 2.4 (a. 1) |
| Nilsson <i>et al</i> , 1990 | thymus | humans aged 1-42 years | 0.27 (d. 5) |
| Weusten <i>et al</i> , 1986 | blood monocytes and thymocytes | humans aged 1-48 years | 0.1-0.64 (c. 6) |

Key: a = gel filtration; b = quantitative autoradiography; c = dextran-coated charcoal; d = enzyme-linked immunoassay; 1 = moxestrol and ^3H -moxestrol; 2 = moxestrol and ^3H -oestradiol; 3 = diethylstilbestrol and 11β -methoxy- 16α - ^{125}I iodo-oestradiol; 4 = diethylstilbestrol and ^3H -diethylstilbestrol; 5 = oestradiol and ^3H -oestradiol; 6 = diethylstilbestrol and 16α - ^{125}I oestradiol.

The results of the receptor studies presented here showed similarities and differences compared with the results of other workers. Moxestrol was used as the ligand in order to permit measurement of receptor binding in immature rats. This approach was necessary because moxestrol unlike oestradiol does not bind to alphafetoprotein present in the blood of immature rodents. The dissociation

constant of moxestrol for the oestrogen receptor (K_d of the order of $10^{-9}M$) in the present study was largely similar to the value found by other workers. On the other hand this value differed from the value found by other groups for the binding of oestradiol to the oestradiol receptor (K_d of the order of $10^{-10}M$) indicating the higher affinity of the receptor for oestradiol than for moxestrol. The affinity of different steroids for the oestrogen receptor differs (see Chapter 3) and there is no reason for the affinity of the receptor to be the same for moxestrol and oestradiol. Nevertheless, the results for the measurement of the concentration of oestrogen receptors in the various tissues gave a similar pattern, of lowered cytosolic oestrogen receptor concentration in brain and thymus with increasing age, to results found by other research groups (Tables 4.4, 4.5, 4.6) whether the ligand used was moxestrol or oestradiol.

4.4.4: Free fraction of oestradiol in serum

The free fraction of oestradiol in serum rose in males and females between 5 and 18 days from the low level of 1.8 % of the albumin-bound fraction to its adult value of 6.25% (Figure 4.9). This rise could have resulted in the decreases in cytosolic oestrogen receptor concentration in the hypothalamus and thymus in females at 18 and 30 days compared with the concentration at 5 days in these tissues (Figures 4.5 and 4.7). In males, the concentration of cytosolic oestrogen receptor in these two tissues was the same in all three age groups, perhaps because locally synthesised oestradiol was present earlier in males. It is possible that, in females, the concentrations of cytosolic oestrogen receptors were higher in the hypothalamus and thymus because the receptors were not translocated to the nucleus as they may have been in males, in whom

the receptor can bind locally aromatised oestradiol and be translocated to the nucleus of the cell. Once bound to a steroid response element on the DNA, the receptor dimer can couple with another dimer (or other transcription factor) to create a more stable complex and sufficient residence time at the gene to influence gene transcription (O'Malley and Tsai, 1993). Moreover, the exposure of tissues to oestradiol could have lead to the down-regulation of oestrogen receptor synthesis and consequent lack of replenishment of the cytosolic receptors.

There is a clear need to measure, in the future, the nuclear receptor concentration in the tissues examined in this study. An estimation of the location within each tissue and within cells in the tissues could be made by autoradiography or by immuncytochemistry. Moreover, binding studies could also identify the nuclear DNA-bound receptor in the brain and thymus. Additional studies should address the question of aromatase activity in the cortex and in the thymus. Further studies should also be done in order to measure and compare the expression of the alpha and beta subtypes of the oestrogen receptor in the thymus. Differences between the expression of the sub-types might contribute to the selective action of oestradiol agonists or antagonists in this tissue. Exposure to differential oestradiol concentrations in thymus of developing males and females might contribute to establishment of autoimmune susceptibility which is known to be higher for many autoimmune diseases in females than in males

Conclusion

The development of the weight of the uterus and thymus and the oestrogen-binding characteristics of oestrogen receptors in all the tissues examined in this study showed some similarities and some differences.

The thymus of male and female rats increased in weight between 5, 18 and 30 days. However, in females, the thymus tissue-to-body weight ratio at 30 days was greater than in males at this age (Table 4.1).

The dissociation constant of ^3H -moxestrol for the oestrogen receptor was in the 10^{-9}M range and was similar in all tissues examined in all three age groups.

There were considerable variations in the tissues with respect to oestrogen receptor concentrations. Receptor concentrations in the uterus increased dramatically with age. There were no sex or age differences in receptor concentrations in the thymus, with the exception of its value in females at 5 days, in which it was significantly greater than in males of the same age. A similar result was found in the hypothalamus. In the cortex, no differences were found in the concentration of oestrogen receptor in males and females at 5 and 18 days but, by 30 days, no oestrogen receptor was detectable in this tissue in either sex.

Increasing levels of endogenous free oestrogens in both sexes could have contributed to these developmental changes and the differences observed. Moreover, the aromatization of testosterone to oestradiol in males could have resulted in some of the differences that were found in weight and in oestrogen

receptor concentrations between the tissues in males and females in the three age groups. In order to test the hypothesis that oestradiol-binding to its receptor might be playing a part in sex-related differences in weight-gain patterns and cytosolic oestrogen receptor concentrations during the development of the uterus, brain and thymus, further experiments were carried out on immature male and female Wistar rats using an aromatase inhibitor, 4-hydroxyandrostenedione (4-OHA), clomiphene (CC) an oestrogen antagonist and oestradiol. The results of these studies are presented in Chapter 5.

Chapter five

**Effects of oestrogen receptor antagonists and aromatase
inhibitors on oestrogen receptors in uterus, brain, and thymus of
immature rats**

5.1 Introduction

Greater knowledge and understanding of oestrogen interaction with its receptor has important implications for early development as well as for human reproduction and disease processes. The results of the study described in Chapter 4 revealed some differences in cytosolic oestrogen receptor binding characteristics in the uterus of the three age groups and between the sexes and different age groups in the brain and thymus. In order to investigate these findings further, two compounds were used in this present study: - clomiphene citrate and 4-hydroxyandrostenedione. Clomiphene citrate is a partial oestrogen agonist/antagonist and was used in order to investigate the effects it might have on oestrogen receptor binding characteristics (concentration and dissociation constant) in both sexes. 4-hydroxyandrostenedione is an aromatase inhibitor and was used to study the effect that the inhibitor would have in males when the conversion of testosterone to oestradiol was inhibited.

5.1.1: Clomiphene citrate

In 1940 the William S. Merle Company began to synthesise a group of chloroethylene compounds. Clomiphene citrate (CC), a compound in this group (Figure 5.1), was itself first synthesised in 1956 and tested in 1960 for oestrogenicity and gonadotrophin inhibition as well as for anti-fertility properties. Clinical trials for its use for ovulation induction in animals were conducted from 1961 and the compound was approved for human use as an anti-infertility drug by prescription in 1967 (reviewed by Dickey and Holtkamp, 1996).

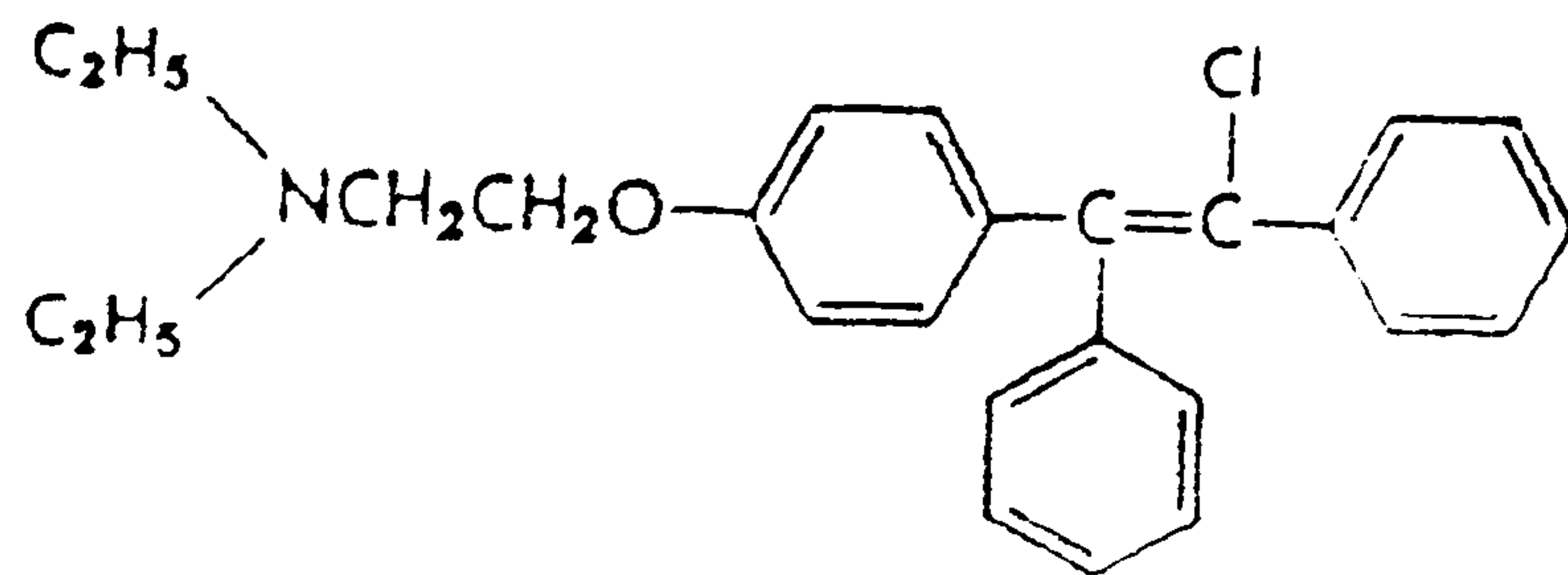


Figure 5.1: Structure of clomiphene citrate.

CC is a non-steroidal racemic mixture of *cis* (38%) and *trans* (62%) isomers. The *cis*-isomer (Zuclomiphene) is mildly oestrogenic as well as anti-oestrogenic, while the *trans*-isomer (enclomiphene) is purely anti-oestrogenic. CC is a competitive antagonist of oestradiol for the cytoplasmic oestrogen receptor in women where it acts at the level of the complex formed in the nucleus between the oestradiol-bound receptor and the DNA (Clarke and Markaverich, 1988). As a result of the antagonism of the oestrogen receptor in the hypothalamus, gonadotrophin-releasing hormone secretion is increased and there is an additional effect of increased sensitivity of the pituitary to this releasing factor (Hsueh *et al*, 1978). As a result, secretion of follicle stimulating hormone and luteinizing hormone is increased and, therefore, an increase in follicular oestrogen synthesis occurs. Additionally, CC may have a direct effect on the ovary, making it more sensitive to pituitary gonadotrophin (Dickey *et al*, 1965). It is the drug of first choice for women suffering from anovulation and for

ovulating women with inadequate follicular or luteal function (Fritz *et al*, 1991). The mechanism of action of CC in stimulating ovulation in humans appears to be interference with oestrogen receptor replenishment in the hypothalamus. The resulting lower concentration of the oestrogen receptor in the hypothalamus alters the pattern of gonadotrophin release and results in enhanced follicular development (Dickey *et al*, 1996). In small mammals, with short oestrus cycles, CC is contraceptive because the effects of both isomers are present throughout the cycle, while in humans, with long cycles, the effects of the anti-oestrogenic isomer are short-lived and the effects of the isomer with oestrogen agonist properties are dominant. In studies undertaken with rats, however, CC protects ovariectomised rats against osteoporosis (Beale *et al*, 1984) and against the loss of cancellous bone although it had no effect on cortical bone histomorphometry (Jimenez *et al*, 1997). Thus, in rats, the compound shows oestrogenic properties in bone in contrast to its antagonistic activity in reproduction.

The effects of CC upon the rat thymus during development are not known. In the study reported here CC was administered alone and in combination with oestradiol in order to determine whether in the uterus, hypothalamus, cortex and thymus of male and female Wistar rats between 15 and 30 days CC might compete with oestradiol for the oestrogen receptor.

5.1.2: 4-Hydroxyandrostenedione

Differentiation of the male brain is associated with the activity of an enzyme complex (aromatase) localised in certain brain areas such as the preoptic, the hypothalamus, and the limbic system (Lauber *et al*, 1994; Roselli and Resko, 1993; Shinoda, 1994). The enzyme appears to be confined to neurones and

absent from glial cells (Abe-Donomae *et al*, 1994). The complex consists of P-450 (arom), a specific form of cytochrome P-450 and the flavoprotein NADPH-cytochrome P-450 reductase (Means *et al*, 1989). Activity of the enzyme, leading to the conversion of testosterone to oestradiol, is a feature of both ovarian and peripheral oestrogen biosynthesis. In pre-menarchal and post-menopausal females, the bulk of circulating oestrogen derives from peripheral conversion of testosterone by aromatase, predominantly in adipose tissue; in females of reproductive age, the ovaries are the source of circulating oestrogen by aromatization of precursor testosterone in the granulosa cells. In males, the testes are the source of testosterone (Funder, 1993).

The relationship between aromatase activity and brain development has been well established in sexual differentiation of the brain and in behaviour (Hutchison and Steimer, 1984; Hutchison, 1993). Moreover, studies using inhibitors of the enzyme have demonstrated that, in the male, several brain areas contain receptors that bind oestradiol, which has been locally produced through aromatization of testosterone (Clancy and Michael, 1994). These areas include for example, the lateral septum, medial preoptic area, and sub-divisions of the hypothalamus, amygdala and bed nucleus of the stria terminalis. The role of aromatase in thymus development has been less documented, although several effects resulting from its inhibition have been shown in this tissue (Greenstein *et al*, 1992; Greenstein *et al*, 1993).

Aromatase inhibitors include steroidal compounds, for example, 1,4,6-androstatriene-3,17-dione (ATD) and 4-hydroxy-androstene-3,17-dione (4-OHA), as well as non-steroidal compounds, such as 4-(5,6,7,8-

tetrahydroimidazo-[1,5- α]-pyridin-5-yl)benzotrile monohydrochloride (CGS 16949A, Fadrazole) (Brodie *et al*, 1987). In the present study 4-OHA, (Figure 5.2), was chosen because it had been used previously in this laboratory and had been shown to effect changes in rat thymus structure (Greenstein *et al*, 1992). 4-OHA is a potent and selective inhibitor of aromatase that inactivates the enzyme by irreversible combination (acts as a 'suicide inhibitor') with the enzyme at its active site (Brodie *et al*, 1979; France *et al*, 1987). Aromatase inhibition has been shown to lead to a decrease in oestrogen receptor concentration in the uterus of ovariectomized rats and in mammary tumours of rats, as well as a decrease in oestrogen concentration *in vivo* (Coombes *et al*, 1987). Clancy and Michael (1994) showed that the lateral septum, medial preoptic area, certain subdivisions of the hypothalamus, amygdala, and bed nucleus of the stria terminalis, were all areas of the male rat brain that contained oestrogen receptors. Using an aromatase inhibitor these studies showed that with the exception of the lateral septum, the other areas bind oestrogens that have been synthesized locally by the activity of aromatase on circulating testosterone.

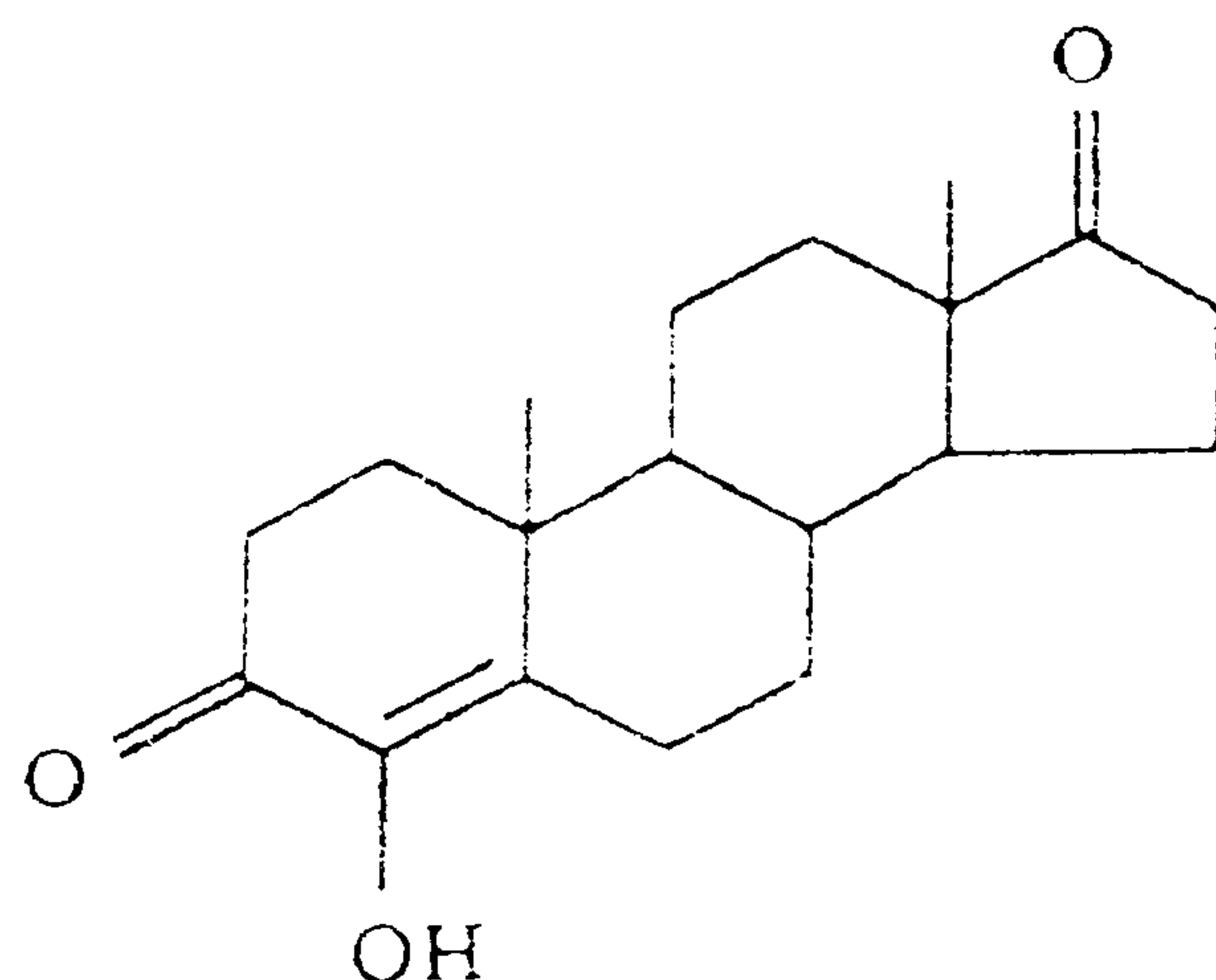


Figure 5.2: Structure of 4-OHA.

The aim of the experiments in which 15 day-old rats were treated with CC, 4-OHA, oestradiol (E2), or CC+E2 was to establish whether:

- a) the oestrogen receptor in the thymus and brain of animals of this age group could be manipulated with CC, a partial agonist/antagonist of oestradiol.
- b) 4-OHA, a compound that inhibits the aromatization of testosterone to oestradiol, affects the K_d of the receptor and its concentration in these tissues.

CC was used as an agonist/antagonist to the oestrogen receptor in order to assess whether it had similar or different effects in the tissues examined. 4-OHA was used in order to assess whether the interaction of oestrogen receptors in the tissues examined might be produced locally from aromatization of circulating testosterone in males. CC, 4-OHA, CC +E2 or E2 alone were implanted into 15-day-old male and female Wistar rats. Body and tissue weights, as well as oestrogen receptor binding and concentration, were estimated in uterus, hypothalamus, cortex and thymus after sacrifice at 30 days. The age chosen for administration of the compounds was 15 days. At this age the free oestradiol concentration in the blood is still low because the hormone is bound to alphafetoprotein. Unbound oestradiol concentrations begin to rise with approaching puberty, and as alphafetoprotein concentrations decrease the free hormone becomes available to the tissues (Greenstein, 1992). Administration of CC, E2, CC+E2 and 4-OHA at 15 days, would allow the time necessary for active plasma levels of the drug to be established before the rise in the oestradiol concentrations. Moreover, the thymus is growing rapidly during this period and drug effects might be more clearly demonstrated than at other ages.

5.2: Materials and Methods

5.2.1: Animals

Male and female Wistar rats (12-days-old) were purchased from Bantin and Kingman as before. The animals were allowed to settle for 48 hours before treatment at 15 days and were housed with their mother in one cage (56 x 38 x 20 cm) in the Biological Services Facility in the Rayne Institute, St. Thomas's Hospital under conditions of controlled lighting and temperature (lights on 08:00-22:00; lights off 22:00-08:00; 19-20°C) until weaning at 21 days. They were allowed free access to steroid-free BK rodent diet and tap water. At 21 days, the mother was anaesthetised and sacrificed and littermates were housed together until sacrifice at 30 days.

5.2.2: Preparation of implants

5.2.2.1 Materials

Clomiphene citrate, 1-[*p*(β -diethylaminoethoxy)phenyl]-1,2-diphenylchloroethylene (CC); oestradiol-17 β were purchased from Sigma Chemical Co, Poole, Dorset, UK. 4-Hydroxy-4-androstene-3,17-dione (4-OHA) was obtained from Innovative Research of America; Ohio, USA. Silastic medical grade tubing (0.335cm ID x 0.465cm OD); Silastic medical adhesive, Silicone Type, were products of Dow Corning Corp, Michigan, USA.

5.2.2.2: Doses

Implants were prepared one day before use as follows. Silastic tubing was cut into lengths approximately 1 cm and the pieces were weighed and each was filled with the steroids to be used. After re-weighing, the pieces of tubing were sealed with Silastic brand medical adhesive. The implants were stored at 4°C until used. The amount of steroid placed in the implants is given in Table 5.1 below:

Table 5.1 Steroids used for implantation into 15 day old male and female Wistar rats.

| Steroid | Amount used / mg |
|---------|------------------|
| CC | 25 |
| E2 | 5 |
| CC + E2 | 25 + 5 |
| OHA | 25 |

The control group were implanted with empty Silastic tubing.

5.2.3: Implantation procedure

The animals were anaesthetised as described in Section 2.2.1 and were weighed. An incision was made mid-way along the dorsal surface of the rat through the skin. The implant was introduced subcutaneously and the wound was closed with stainless-steel clips which were left in place. The animals were then

returned to their mother. Their weight was recorded again at sacrifice at 30 days.

5.2.4: Preparation of tissues

At 30 days, the animals were anaesthetised weighed and sacrificed, blood was collected, the tissues were dissected and cytosol preparations carried out within two weeks (Sections 2.2.1 - 2.2.3).

5.2.5: Assay of cytosols prepared from animals treated with CC, 4-OHA, E2. or CC+E2.

Assays were performed on all the cytosols in the same way as described previously within two months of their preparation and stored at -70°C (Section 2.2.4). Care was taken to assay cytosols from different tissues and different treatment groups on the same day. The results were analysed as before by Scatchard analysis to estimate the binding constant of the cytosolic oestrogen receptor and its concentration (Section 2.2.4).

5.2.6: The free fraction of oestradiol in serum

Estimation of the free fraction of oestradiol in serum was made as described earlier (Section 2.5).

5.2.7: Statistical Analyses

Statistical analyses of the results of this set of experiments were undertaken as described earlier (Section 2.6).

5.3 Results

5.3.1: Body and tissue weights of male and female Wistar rats at 30 days after treatment with CC, E2, CC+E2 or 4-OHA.

5.3.1.1: Body weight

The body weight of male and female Wistar rats in the control and treatment groups are given in Figures 5.3 and 5.4 respectively. Untreated and CC-treated males were heavier ($p < 0.05$) than their female counterparts. Within the female groups, animals treated with E2 or with CC+E2 were significantly lighter ($p < 0.05$) following the treatment than untreated and CC-treated female. Females treated with 4-OHA were similar in body weight ($p > 0.05$) to all the other female groups although their body weight was intermediate between that of the other female groups. Males treated with E2, or CC+E2 were all lighter ($p < 0.05$) than untreated and CC-treated males. Males treated with 4-OHA, like the females of this group, were intermediate in weight between the other groups of males, but not significantly different from any of the other male groups.

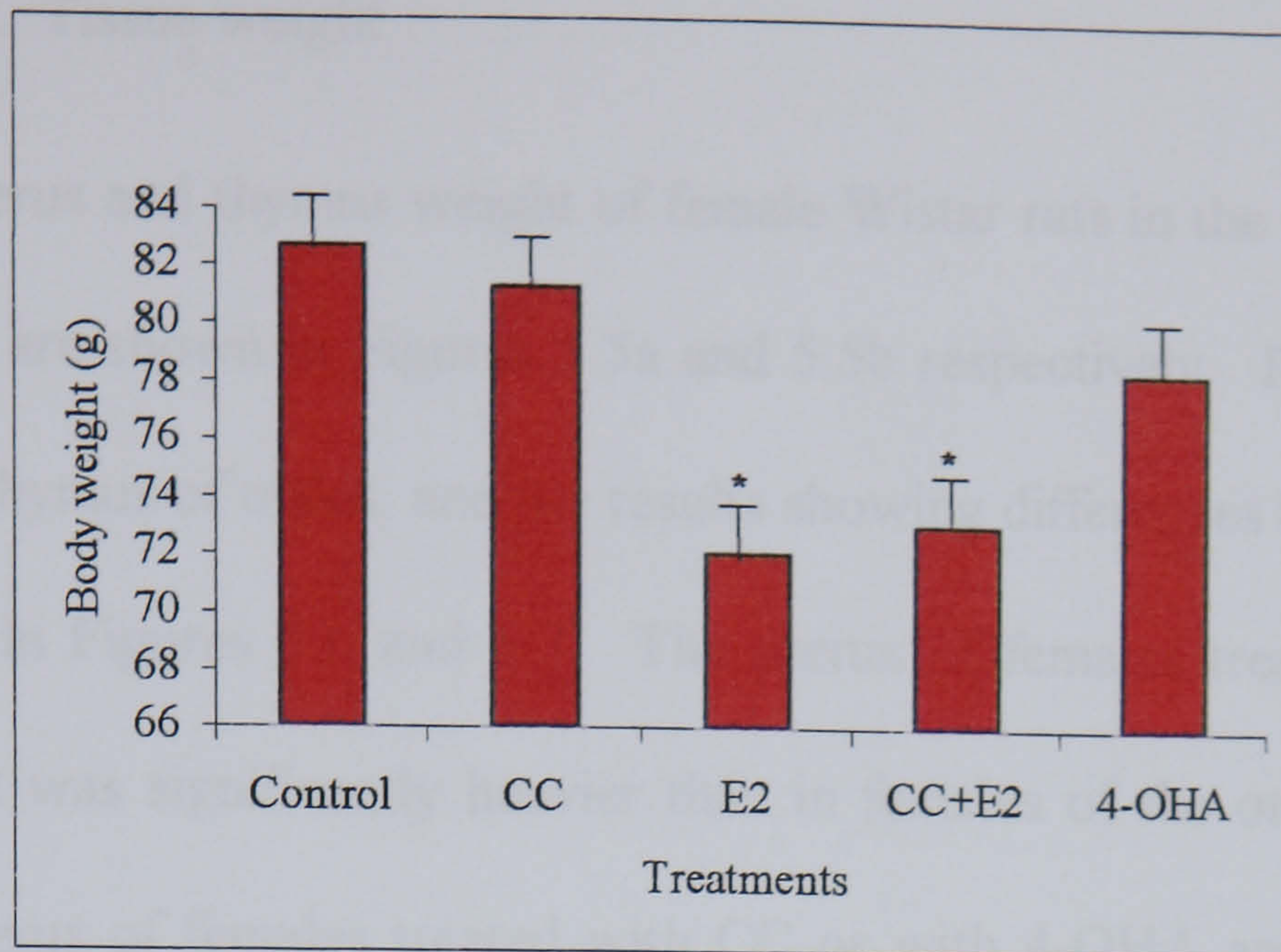


Figure 5.3: Body weights of 30-day-old female Wistar rats in the control group and in the groups treated at 15 days with CC, E2, CC+E2 or 4-OHA. Bars represent the mean \pm SEM of 6-10 observations/group. (*Differs from control CC and 4-OHA groups, $p < 0.05$; Two-Way ANOVA).

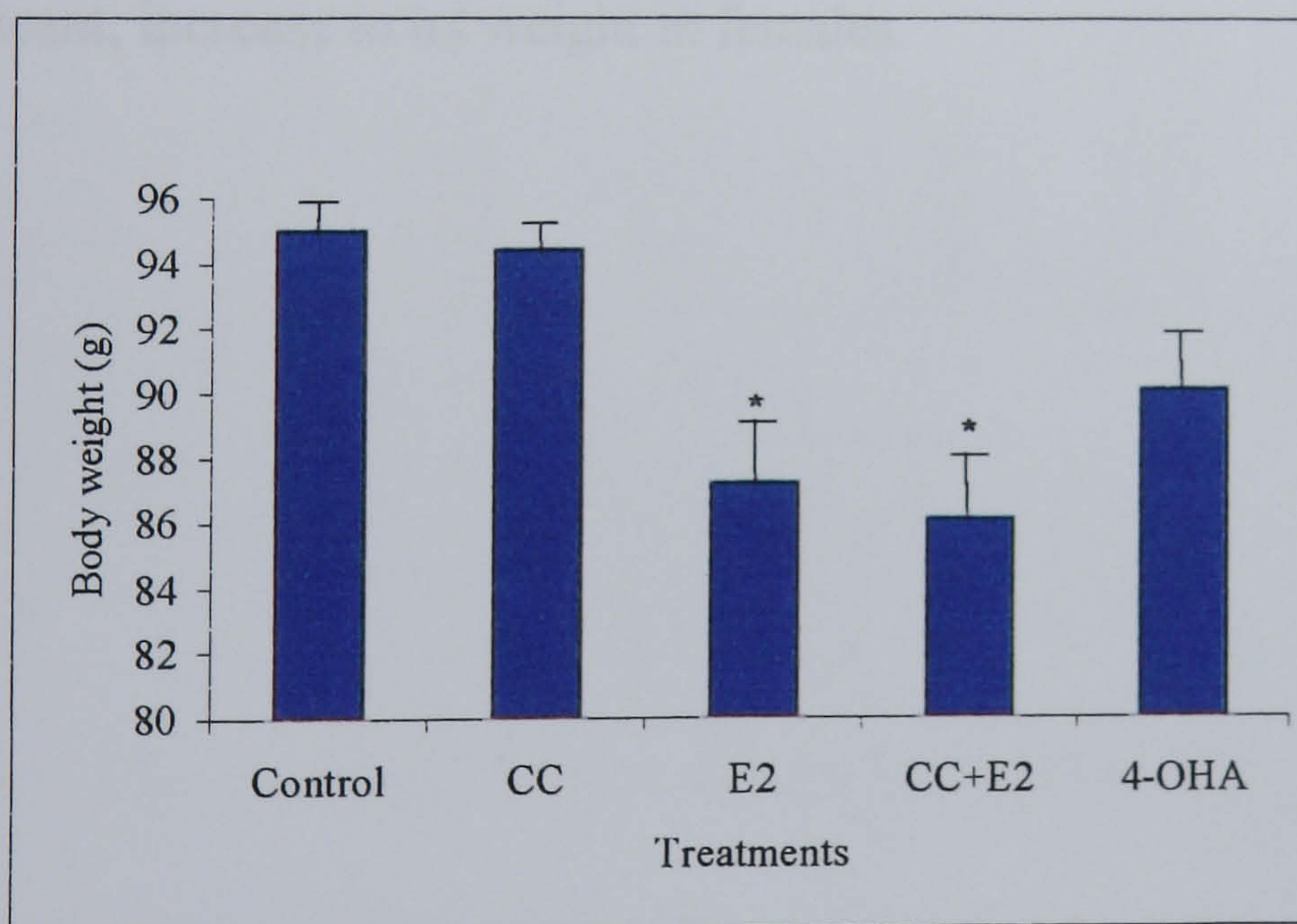
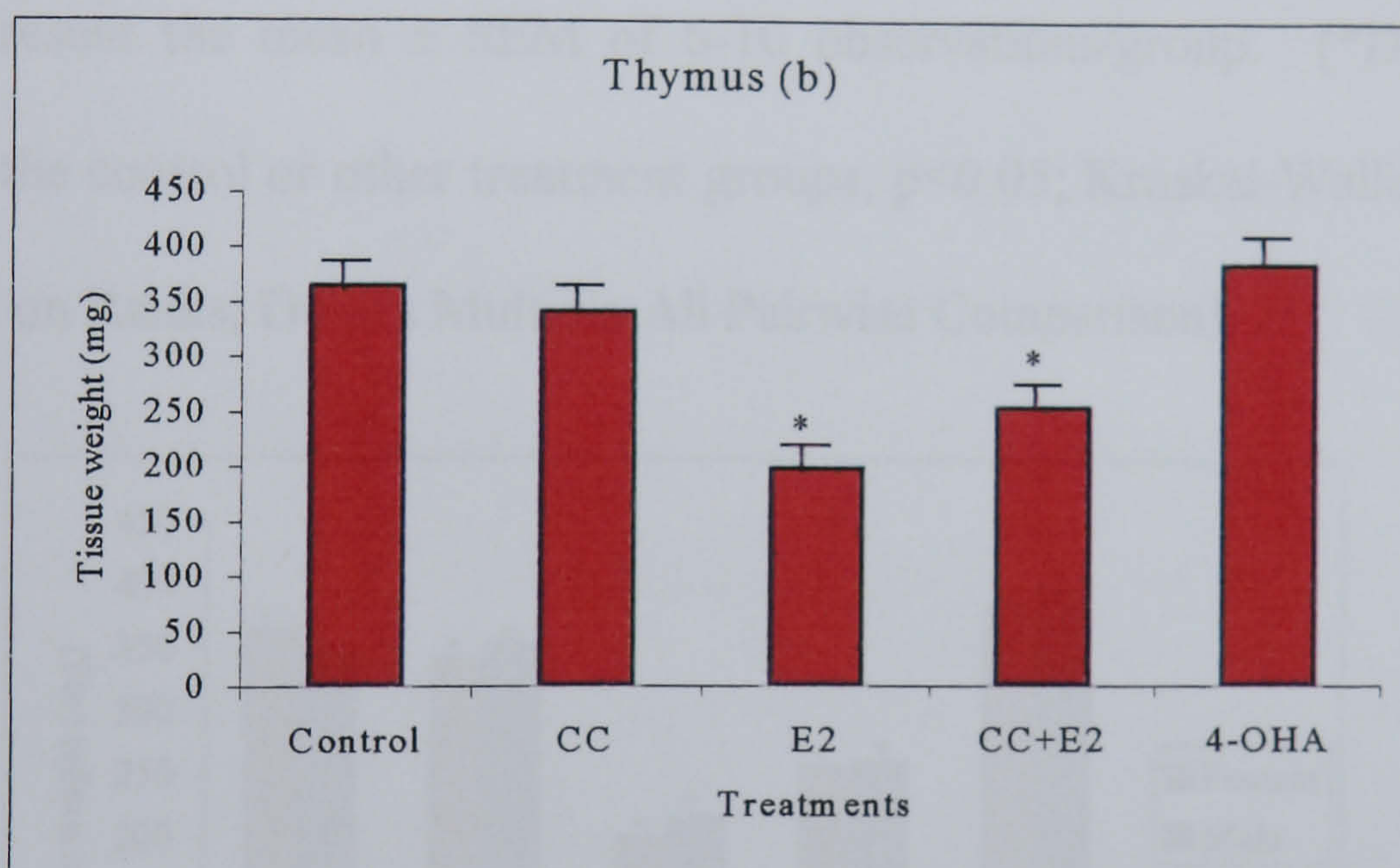
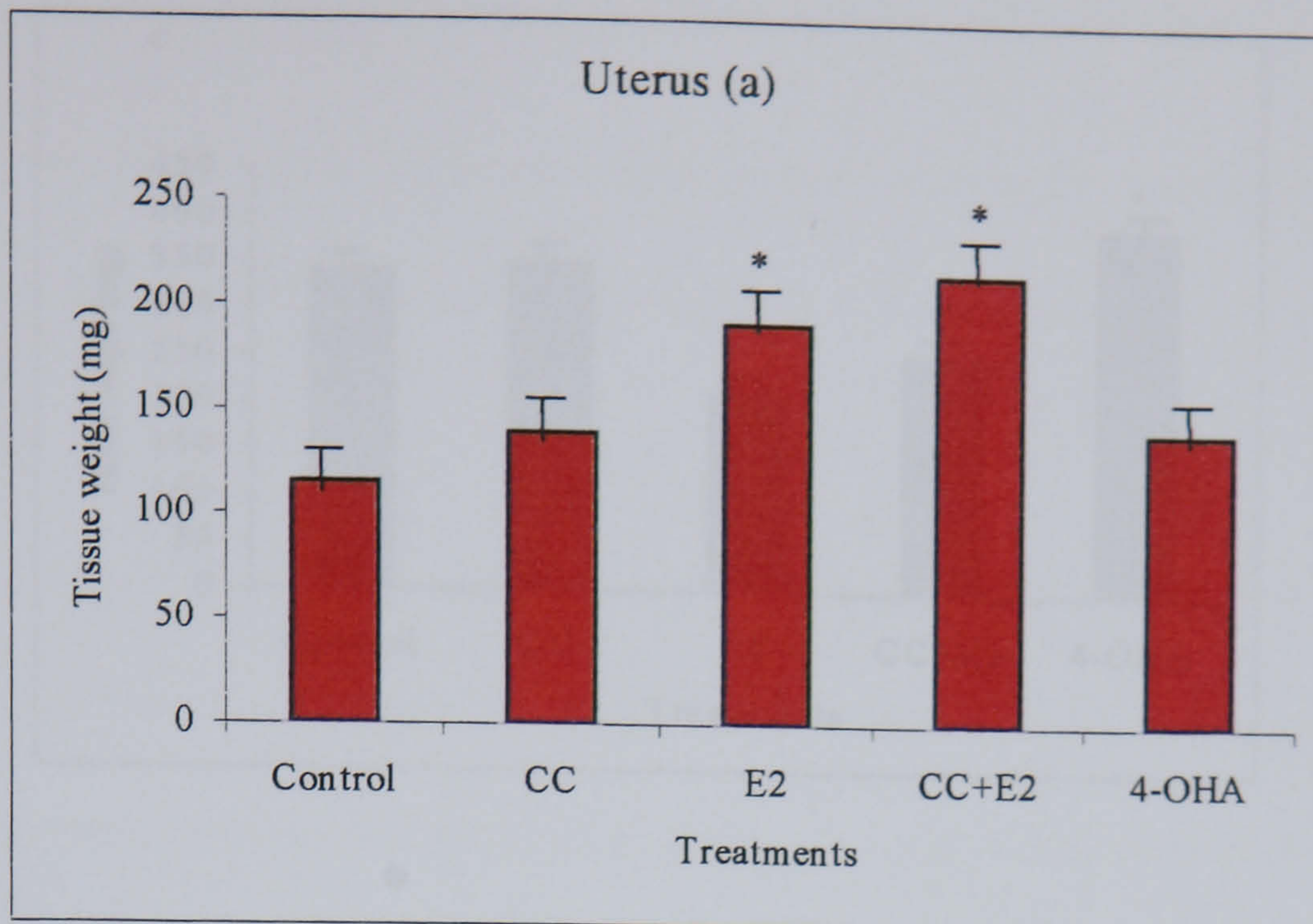


Figure 5.4: Body weights of 30-day-old male Wistar rats at in the control group and in the groups treated at 15 days with CC, E2, CC+E2 or 4-OHA. Bars represent the mean \pm SEM of 6-10 observations/group; (*Differs from control and CC groups, $p < 0.05$; Two-way ANOVA).

5.3.1.2: Tissue weight

The uterus and thymus weight of female Wistar rats in the control and treatment groups are shown in Figures 5.5a and 5.5b respectively. Results for the weight of the thymus of males, and the results showing differences between the sexes are shown in Figures 5.6 and 5.7. The uterus of females treated with E2 or with CC+E2 was significantly heavier than in females of the other groups ($p < 0.05$). The uterus of females treated with CC or with 4-OHA appeared to increase in weight compared with the controls but neither reached levels of statistical significance.

However, the thymus of males and females decreased in weight as a result of treatment with E2 or CC+E2. Treatment with 4-OHA led to an increase in the weight of this tissue in males ($p < 0.05$) and to a slight, but not significant, increase in its weight in females.



Figures 5.5a; b: Uterus (5.5a) and thymus weight (5.5b) of 30-day-old female Wistar rats in the control group and in the groups treated at 15 days with CC, E2, CC+E2 or 4-OHA. Bars represent the mean \pm SEM of 6-10 observations/group. (*Differs within the tissue from females of the control or other treatment groups, $p < 0.05$; Kruskal-Wallis One-Way ANOVA on Ranks; Dunn's Multiple All Pairwise Comparison).

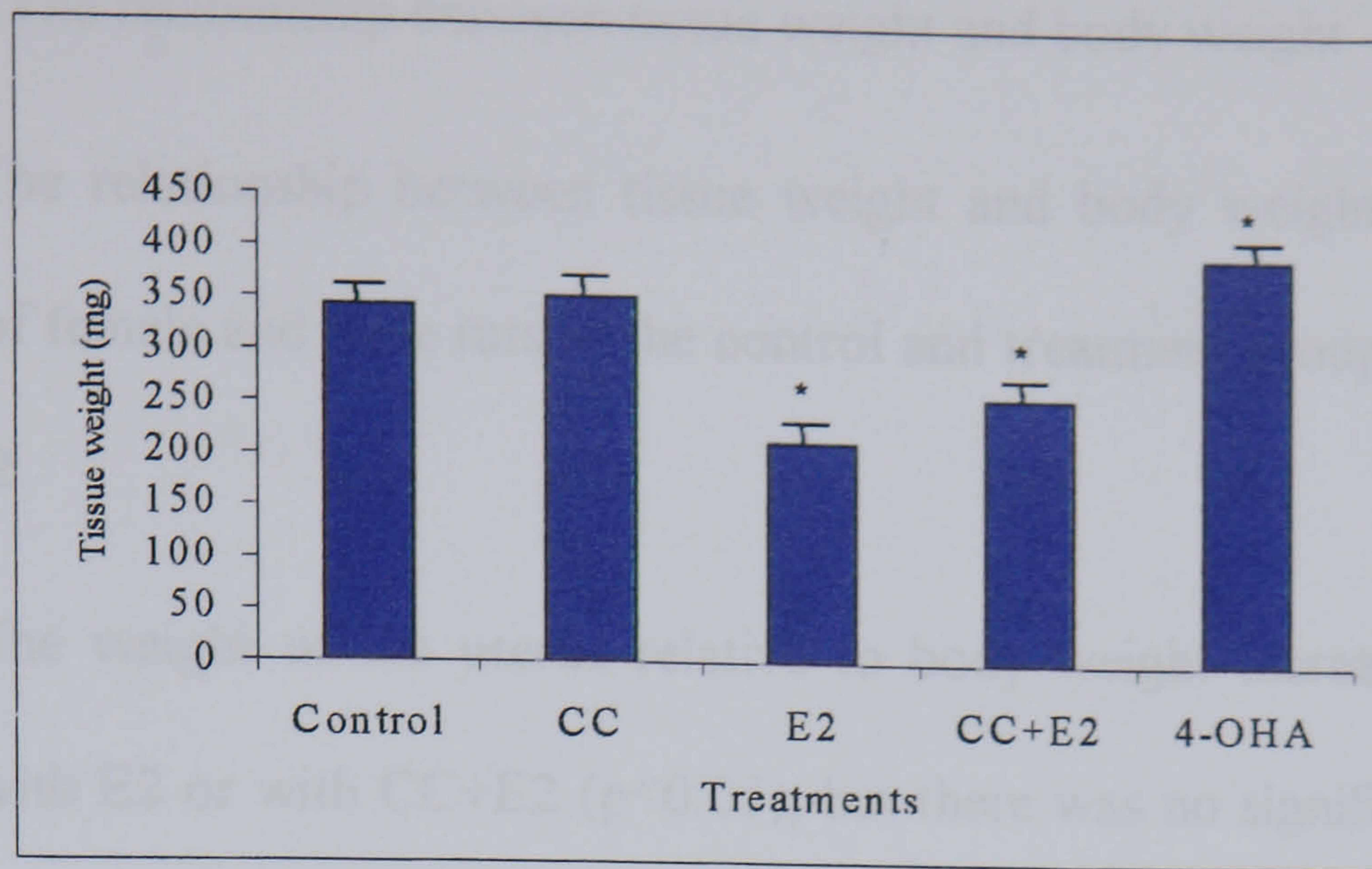


Figure 5.6: Thymus weight of 30-day-old male Wistar rats in the control group and in the groups treated at 15 days with CC, E2, CC+E2 or 4-OHA. Bars represent the mean \pm SEM of 6-10 observations/group. (*Differs from males of the control or other treatment groups, $p < 0.05$; Kruskal-Wallis One-Way ANOVA on Ranks; Dunn's Multiple All Pairwise Comparison).

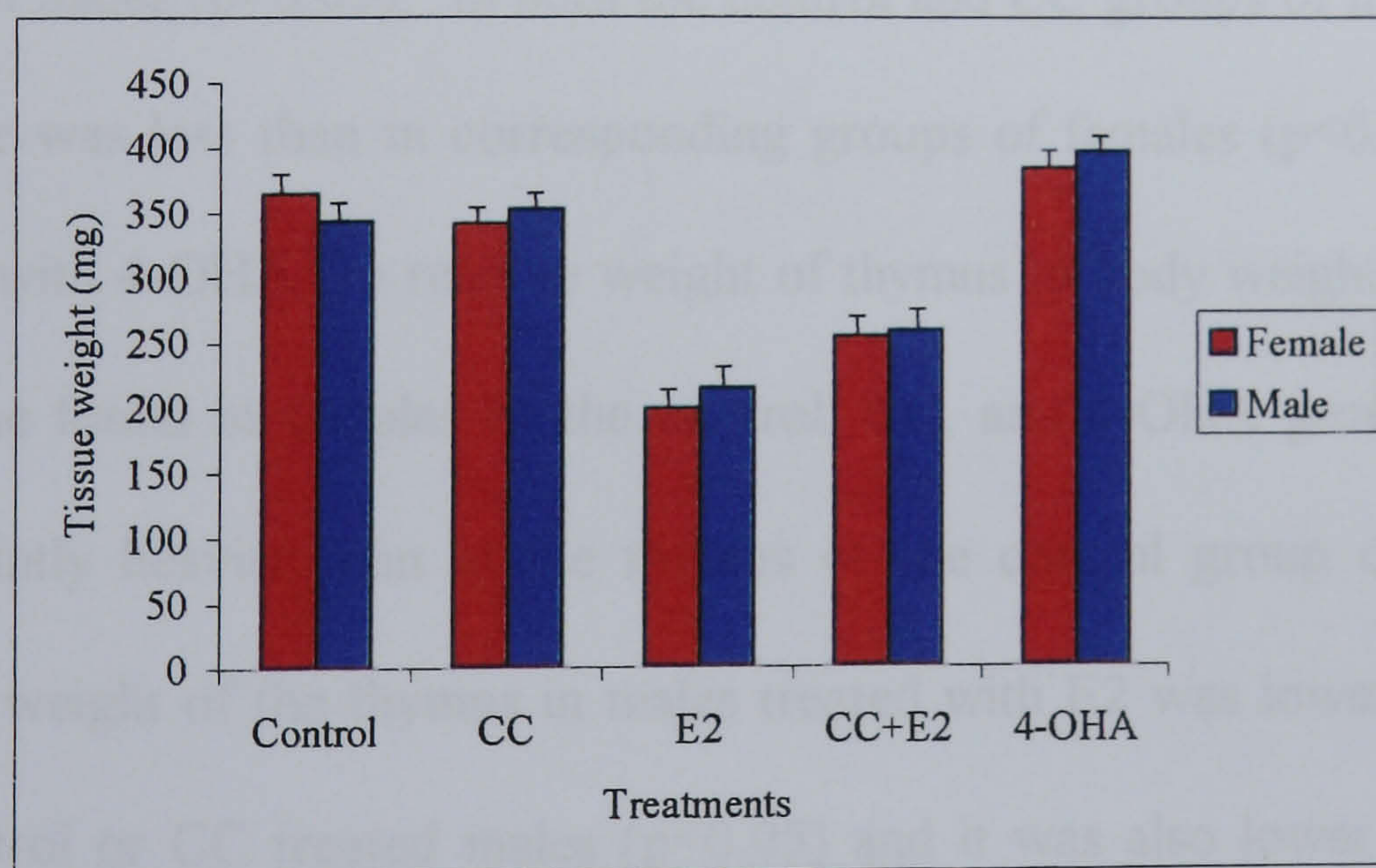


Figure 5.7: Thymus weights of 30-day old female and male Wistar rats in the control group and in the groups treated at 15 days with CC, E2, CC+E2 or 4-OHA. Bars represent the mean \pm SEM of 6-10 observations/group. (Data repeated from figures 5.6, b and 5.7)

5.3.1.3: The relationship between tissue weight and body weight

The relationship between tissue weight and body weight in uterus and thymus of female and male rats in the control and treatment groups are shown in Table 5.2.

The weight of the uterus relative to body weight increased in females treated with E2 or with CC+E2 ($p < 0.05$), but there was no significant difference from the controls in animals treated with CC or with 4-OHA ($p > 0.05$).

The tissue weight relative to body weight of the thymus in females treated with CC or with 4-OHA did not differ from the value in females in the control group ($p > 0.05$), but those treated with E2 or with CC+E2 had decreased thymus weight relative to body weight ($p < 0.05$). As with the females, the tissue to body weight ratio of males treated with CC did not differ from its value in the control group of males ($p > 0.05$). In both the control and CC groups of males, however, its value was less than in corresponding groups of females ($p < 0.05$). In males treated with 4-OHA the relative weight of thymus to body weight was similar to the value found in females of the control, CC, and 4-OHA groups, but it was significantly heavier than in the thymus of the control group of males. The relative weight of the thymus in males treated with E2 was lower than in either the control or CC treated males ($p < 0.05$) and it was also lower than in the 4-OHA treated males ($p < 0.01$).

Table 5.2: The tissue-to-body weight ratio (mean \pm SEM) in female and male Wistar rats, 6-10 per group; (*Differs from control groups within a tissue and sex, $p < 0.05$; Kruskal-Wallis One-Way ANOVA on Ranks; Dunn's Multiple All Pairwise Comparison).

| Sex | Tissue | Control ($\times 10^4$) | CC ($\times 10^4$) | E2 ($\times 10^4$) | CC+E2 ($\times 10^4$) | 4-OHA ($\times 10^4$) |
|--------|--------|------------------------------|-------------------------|-------------------------|----------------------------|----------------------------|
| Female | Uterus | 13.9 \pm 1.1 | 17.2 \pm 2.3 | 26.5 \pm 2.3* | 29.5 \pm 2.3* | 17.9 \pm 1.3 |
| Female | Thymus | 44.1 \pm 2.5 | 42.0 \pm 5.7 | 27.6 \pm 2.4* | 34.8 \pm 2.7* | 48.9 \pm 3.6 |
| Male | Thymus | 32.6 \pm 1.8 | 37.4 \pm 2.6 | 24.6 \pm 1.9* | 30.0 \pm 3.6 | 43.7 \pm 2.0* |

5.3.2: The concentrations of cytosolic oestrogen receptors in uterus, hypothalamus, cortex and thymus of male and female Wistar rats at 30 days, after 15 days treatment with CC, 4-OHA, E2 or CC+E2.

5.3.2.1: Uterus

The concentration of oestrogen receptors in the uterus in control and treated Wistar rats are shown in Figure 5.8. The concentration of oestrogen receptors in the uterus of animals treated at 15 days with CC or 4-OHA did not differ from the value in the control group ($p>0.05$). No receptors were detectable in females treated with E2 or CC+E2.

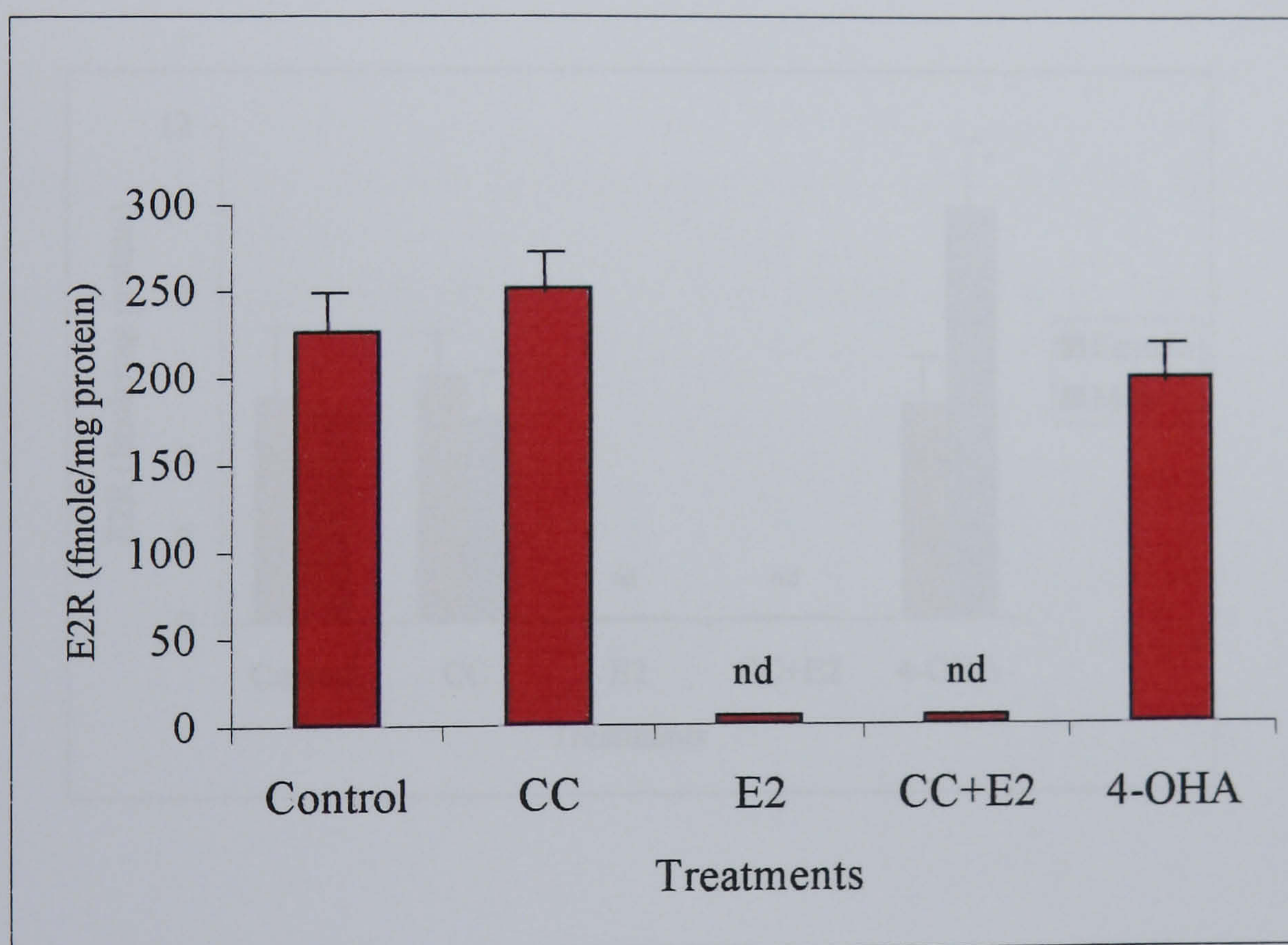


Figure 5.8: Concentration (fmole/mg protein) of cytosolic oestrogen receptors in the uterus of 30-day-old female Wistar rats in the control group and in the groups treated at 15 days with CC, E2, CC+E2 or 4-OHA. Bars represent the mean \pm SEM of 5-9 observations/group; nd, not detectable.

5.3.2.2: Hypothalamus

The concentration of oestrogen receptors in the hypothalamus in control and treated female and male Wistar rats are shown in Figure 5.9. No differences were found in concentration of the oestrogen receptor in the hypothalamus between males and females in the control, or CC-treated groups ($p > 0.05$). Males treated with 4-OHA had significantly higher levels of oestrogen receptors in the hypothalamus than did females of the same treatment group ($p < 0.05$) or than males and females of the control or CC treatment groups ($p < 0.05$). No receptors were detectable in either sex in this tissue after treatment with E2 or with CC+E2.

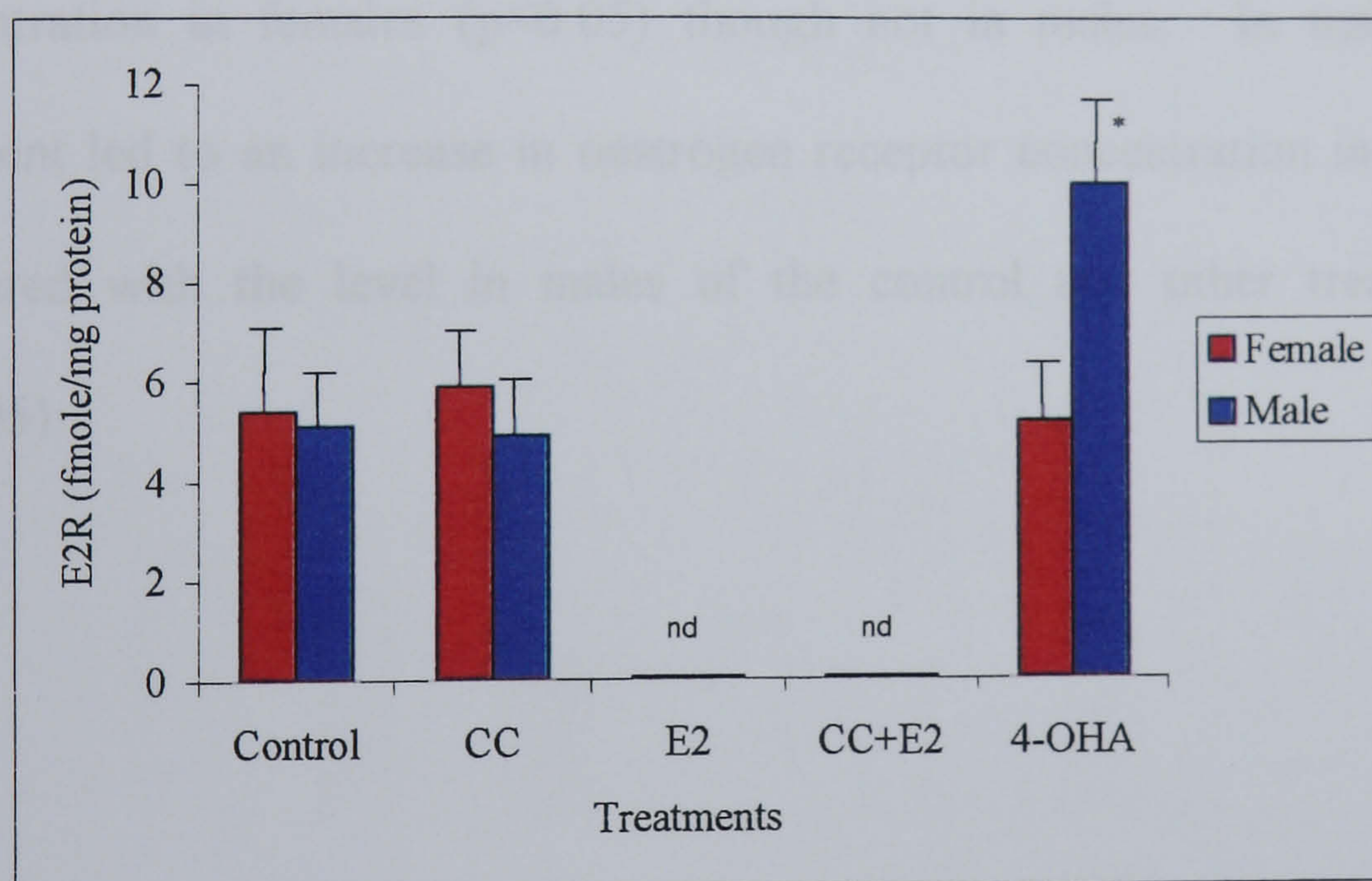


Figure 5.9: Concentration (fmole/mg protein) of cytosolic oestrogen receptors in the hypothalamus of 30-day-old female and male Wistar rats in the control group and in the groups treated at 15 days with CC, E2, CC+E2 or 4-OHA. Bars represent the mean \pm SEM of 5-9 observations/group. nd, not detectable. (*Differs from other groups, $p < 0.05$; Kruskal-Wallis One-Way ANOVA on Ranks; Dunn's Multiple All Pairwise Comparison).

5.3.2.3: Cortex

No cytosolic oestrogen receptors were detectable in the female and male Wistar rats in the control or treated groups. This result was the same as that found in 30-day-old rats in the earlier study.

5.3.2.4: Thymus

The results of the determination of the concentration of oestrogen receptors in the thymus in control and treated female and male Wistar rats are shown in Figure 5.10 and Figure 5.11 respectively. Treatment of females and males with E2 and CC+E2 resulted in a lowering of receptor concentration in the thymus to a level below the detection limit. CC also lowered the receptor concentration in females ($p < 0.05$) though not in males. In males, 4-OHA treatment led to an increase in oestrogen receptor concentration in the thymus compared with the level in males of the control and other treated groups ($p < 0.05$).

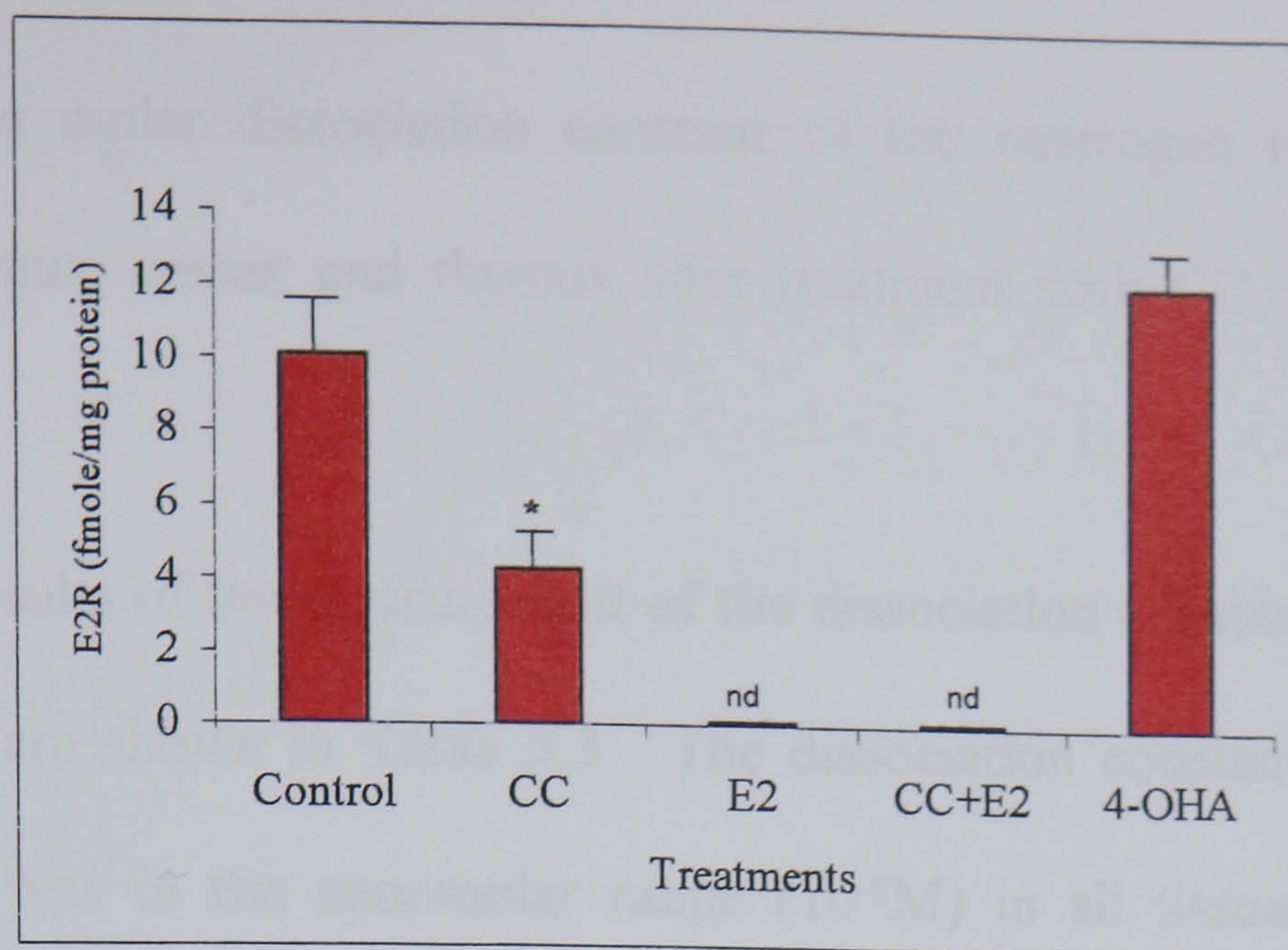


Figure 5.10: Concentration (fmole/mg protein) of cytosolic oestrogen receptors in the thymus of female Wistar rats at 30 days in the control group and in groups treated at 15 days with CC, E2, CC+E2 or 4-OHA. Bars represent the mean \pm SEM of 5-9 observations/group. nd, not detectable. (*Differs from females of the other groups, $p < 0.05$; Kruskal-Wallis One-Way ANOVA on Ranks; Dunn's Multiple All Pairwise Comparison).

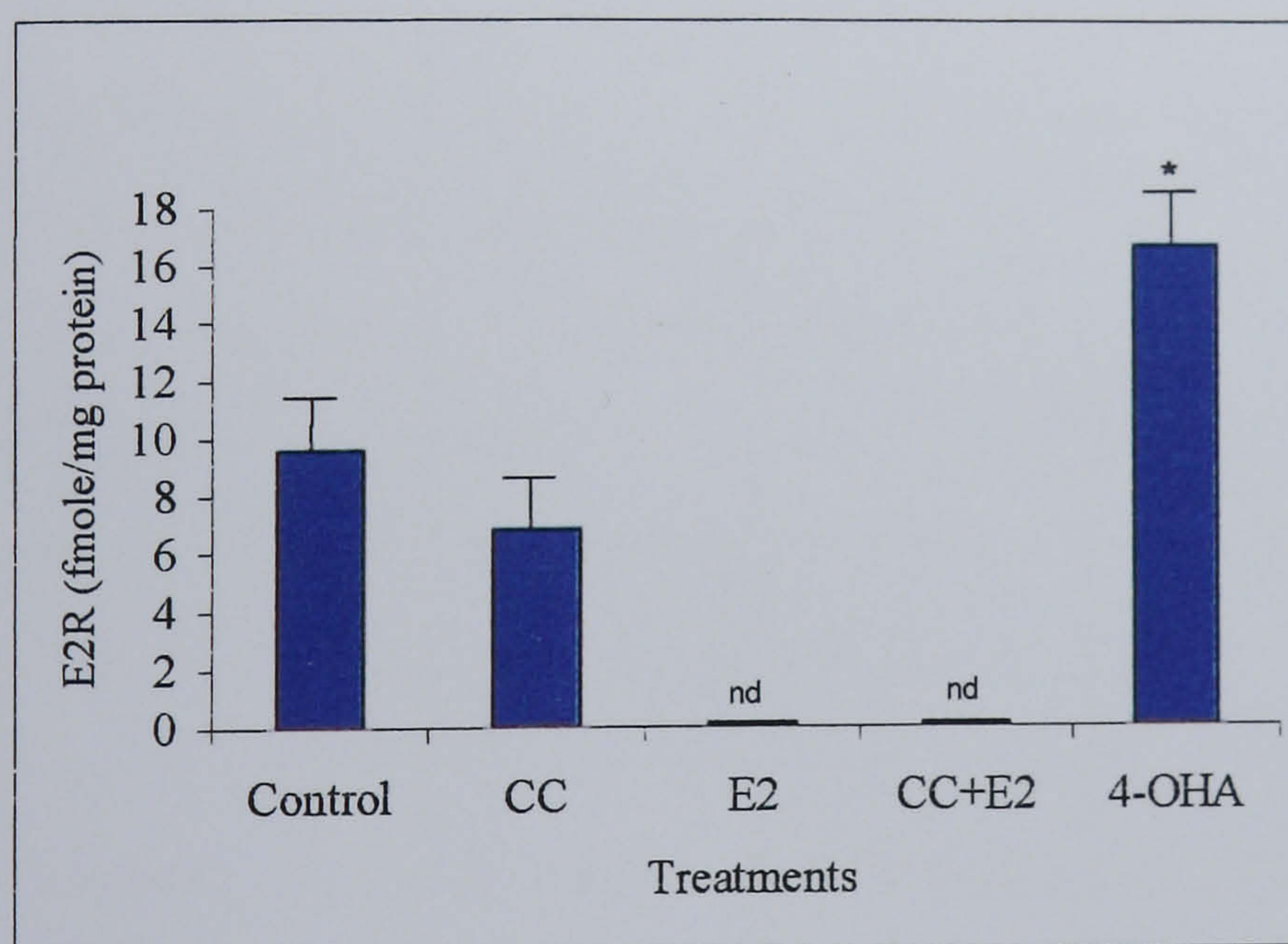


Figure 5.11: Concentration (fmole/mg protein) of cytosolic oestrogen receptors in the thymus of male Wistar rats at 30 days in the control group and in groups treated at 15 days with CC, E2, CC+E2 or 4-OHA. Bars represent the mean \pm SEM of 5-9 observations/group. nd, not detectable. (*Differs from males of the other groups, $p < 0.05$; Kruskal-Wallis One-Way ANOVA on Ranks; Dunn's Multiple All Pairwise Comparison).

5.3.3: The molar dissociation constant of the oestrogen receptor in uterus, hypothalamus, cortex and thymus after treatment with CC, E2, CC+E2 or 4-OHA.

Results of the measurement of the dissociation constant of the oestrogen receptors are shown in Table 5.3. The dissociation constant of the oestrogen receptors was in the nanomolar range (10^{-9}M) in all tissues. There was no significant difference in its value between males and females in any of the treatment or control groups ($p>0.05$). In experiments, when animals had been treated with E2 or CC+E2, no receptors were detectable.

Table 5.3: Values of the molar dissociation constants (K_d , $\times 10^{-9}M$.) of cytosolic oestrogen receptors in the uterus, hypothalamus, cortex and thymus of female and male Wistar rats at 30 days in the control group and in groups treated at 15 days with CC, E2, CC+E2 or 4-OHA. The values represent the mean \pm SEM of 5-9 observations per group. nd, binding not detectable. (No significant differences in the values, $p>0.05$; Kruskal-Wallis One Way ANOVA on Ranks; Dunn's Multiple All-Pairwise Comparison).

| Sex | Tissue | Control $K_d \times 10^{-9}M$ | CC $K_d \times 10^{-9}M$ | E2 $K_d \times 10^{-9}M$ | CC + E2 $K_d \times 10^{-9}M$ | 4-OHA $K_d \times 10^{-9}M$ |
|--------|--------------|----------------------------------|-----------------------------|-----------------------------|----------------------------------|--------------------------------|
| Female | Uterus | 2.72 \pm 0.63 | 1.96 \pm 0.57 | 3.27 \pm 0.63 | 2.48 \pm 0.63 | 1.53 \pm 0.63 |
| Female | Hypothalamus | 3.65 \pm 0.57 | 3.73 \pm 0.57 | nd | nd | 3.29 \pm 0.69 |
| Male | Hypothalamus | 4.03 \pm 1.88 | 3.29 \pm 1.65 | nd | nd | 4.83 \pm 1.56 |
| Female | Cortex | nd | nd | nd | nd | nd |
| Male | Cortex | nd | nd | nd | nd | nd |
| Female | Thymus | 2.37 \pm 0.57 | 2.04 \pm 0.55 | nd | nd | 1.96 \pm 0.55 |
| Male | Thymus | 2.60 \pm 0.89 | 2.02 \pm 1.12 | nd | nd | 3.44 \pm 1.09 |

5.3.4: The free fraction of oestradiol in the serum of female and male Wistar rats at 30 days after treatment with CC, E2 CC+E2 or 4-OHA.

The results of the measurement of E2 in the serum of female and male Wistar rats at 30 days after treatment with CC, E2 CC+E2 or 4-OHA are shown in Table 5.4. There was a small, but significant, increase in the free fraction of oestradiol in the serum of E2 and CC+E2 treated male (9 percent increase) and female (12 percent increase) rats compared with the control groups ($p < 0.05$).

Table 5.4: The free-fraction of oestradiol (mean percentage \pm SEM of 6-10 observations) in the serum of female and male Wistar rats at 30 days after treatment with CC, E2, CC+E2 or 4-OHA. (*Differs within each sex from the control or other treatment groups, $p < 0.05$; Kruskal-Wallis One-Way ANOVA on Ranks; Dunn's Multiple All Pairwise Comparison).

| Sex | Control | CC | E2 | CC+E2 | 4-OHA |
|--------|----------------|----------------|-----------------|-----------------|----------------|
| Female | 5.12 \pm 0.3 | 5.12 \pm 0.3 | 5.72 \pm 0.2* | 5.51 \pm 0.2* | 5.39 \pm 0.4 |
| Male | 5.46 \pm 0.2 | 5.31 \pm 0.4 | 5.92 \pm 0.2* | 5.86 \pm 0.6* | 5.19 \pm 0.2 |

A summary of the results obtained at 30 days following the administration of CC, E2, CC+E2 or 4-OHA to female and male Wistar rats at 15 days is shown in Table 5.5.

Table 5.5: Changes in body and tissue weights, and in oestrogen receptor (E2R) concentration in female (f) and male (m) Wistar rats at 30

days after administration at 15 days with CC, E2, CC+E2 and 4-OHA. ↑ = [E2R] up; ↓ = [E2R] down; - = [E2R] no change; na = not

applicable; nd = not detectable.

| Treatment | Body Weight (BW) | Uterus Weight (UW) | Thymus Weight (TW) | UW / BW | TW / BW | [E2R] Uterus | [E2R] Thymus | [E2R] Hypothalamus |
|-----------|------------------|--------------------|--------------------|---------|---------|--------------|--------------|--------------------|
| CC | f | - | - | - | - | - | ↓ | - |
| | m | na | - | na | - | na | - | - |
| E2 | f | ↑ | ↓ | ↑ | ↓ | ↓ | ↓(nd) | ↓(nd) |
| | m | na | ↓ | na | ↓ | na | ↓(nd) | ↓(nd) |
| CC + E2 | f | ↑ | ↓ | ↑ | ↓ | ↓ | ↓(nd) | ↓(nd) |
| | m | na | ↓ | na | ↓ | na | ↓(nd) | ↓(nd) |
| 4-OHA | f | - | - | - | - | - | - | - |
| | m | na | ↑ | na | ↑ | na | ↑ | ↑ |

5.4: Discussion

5.4.1: Body, tissue and tissue weight related to body weight in female and male Wistar rats treated with CC, E2, CC+E2 or 4-OHA.

Body weight

Treatment of male and female rats at 15 days with E2 and CC+E2 was associated with lower body weight at 30 days, although the males in all groups were heavier than females in the corresponding groups. This result with E2 and with CC+E2 was not unexpected as E2 has been shown previously to inhibit body growth in developing female rats (Jansson *et al*, 1985). The conclusion that reduced body weight was due to the treatment is supported by the finding that the free fraction of oestradiol in serum was higher in the groups treated with E2 and CC+E. Alpha-fetoprotein concentrations fall during the period from 23 to 30 days, so there could have been higher concentrations of free oestradiol available to all the tissues.

E2 treatment could possibly have resulted in decreased body weight in both sexes through an effect on growth hormone. In adult female rats, the growth hormone content of the pituitary is lower than in males and, when male rats are treated with E2, the growth hormone content of the pituitary decreases to the same level as in females (reviewed by Zachman, 1992). There is evidence that growth hormone and testosterone act synergistically in human males to promote growth. The lower weight of the 30-day-old male and female rats treated with E2 or CC+E2 could have been mediated through interactions of E2 with growth hormone. Lack of E2, or insensitivity to its potential effects, leads

to prolonged growth because E2 is a potent stimulator of bone maturation (Lee, 1997) and in the absence of the hormone the bones continue to grow without developing into mature bone.

The effects of treatment of CC+E2 are likely to have been due to E2 because treatment with CC alone had no effect on body weight. These effects might have resulted from the dose of CC given or because CC had no interaction with any other growth factors. As a racemic mixture of the two isomers of CC were given, the anti-oestrogenic properties of the enclomiphene isomer may have predominated over the mildly oestrogenic property of the zuclomiphene isomer of the drug over this period of time (Dickey and Holtkamp, 1996). When E2 and CC were given together, however, E2 effects on body weight seemed to predominate in that the body weight of male and female animals showed a low weight gain pattern similar to that observed in animals treated with E2 alone. This result differs from that of Forsberg (1995) who found that body weight was significantly decreased in neonatal NMRI mice treated with CC. This difference could be due to dose and/or species differences in response to the drug.

Administration of 4-OHA had no significant effect on body weight of either female or male rats. 4-OHA treatment increases the weight of tissues such as the thymus in immature and ageing rats (Greenstein *et al*, 1992). However, there was no evidence in those studies that administration of 4-OHA at the concentration used in the present study, or given as implants, altered overall body weight. It is possible that other hormones, such as growth hormone or its releasing factors, play a more important role in the overall body growth during the first 30 days and that other regulatory pathways not involving testosterone

are more closely associated with body growth during this complex stage of development.

Tissue weight

If oestradiol was associated, at least in part with, decreased overall body weight in male and female rats, what was the effect of oestradiol on tissue weight in rats in the first 30 days of postnatal development?

Uterus

The uterus increased in weight after treatment with E2 and with CC+E2, a result that is in agreement with the result found by Young *et al* (1991) in ovariectomised adult female rats treated with E2 or with CC. Their study showed that E2 restored the weight of the uterus to levels comparable with the intact group and that CC had a smaller effect on this tissue. It is likely that the large increase in uterus weight observed in 30-day-old rats treated with CC+E2 at 15 days was due to the oestrogen effect rather than to CC. There was no significant difference between the weight of the uterus in rats treated with the combination CC+E2 or with E2 alone although the CC+E2 and E2 groups were heavier than other groups. The same result was found in the uterus tissue to body weight ratio, with no significant differences between the control group and the groups treated with CC or with 4-OHA. In the groups treated with E2 or with CC+E2, the tissue-to-body weight ratio was significantly increased from 13.9×10^{-4} (control) to 26.5×10^{-4} (E2) and 29.5×10^{-4} (CC+E2).

Thymus

The thymus, in contrast to the uterus, showed a marked decrease in weight and in tissue-to-body weight ratio in males and females as a result of treatment with E2 or CC+E2. It is well known that both testosterone and oestradiol cause thymic atrophy (Grossman, 1984) and the present result was in agreement with previous studies of the effects of steroid hormones on this tissue. Oestradiol or diethylstilbestrol treatment of gonadectomised Sprague-Dawley rats causes inhibition of thymus growth (Windmill *et al*, 1993), an observation that is in accordance with the results of the present study. Martin *et al* (1994) observed histological and ultrastructural changes in adult rat thymus after oestradiol treatment. Their analysis confirmed lymphocyte depletion in both cortical and medullary regions of the thymus as well as profound modifications in the epithelial component of this tissue. They also found increased vascular permeability in treated animals and this alteration could result in changes in lymphoid traffic into and out of the thymus. These effects may also contribute at an early developmental stage to observed differences in thymus structure and immune function in males and females.

CC had no effect on thymus weight or on the tissue/to body weight ratio. The use of a series of different doses of CC might have clarified whether this drug can cause atrophy or its reversal in the thymus. That steroid hormones can alter thymus architecture is well known, but the potential for CC to act as an agonist or antagonist of E2 needs further elucidation to determine if the drug has any effect on rat thymus at this age.

In males treated with 4-OHA there was an increase in thymus weight and in the thymus-to-body weight ratio. The values reached in male thymus weight and in the thymus-to-body weight ratio were similar to those found in females of the same treatment group. However, the tissue-to-body weight ratio of thymus in males treated with 4-OHA was higher than in the male control group. Between 15 and 30 days postnatally, testosterone levels are high (Myachi *et al.*, 1973) and, although bound to plasma proteins, this hormone is available to the tissues (Manni *et al.*, 1985). It is possible that in the thymus, irreversible binding of 4-OHA to the active site of aromatase (Wei Yue and Brodie, 1997), prevented the conversion of testosterone to oestradiol and thus reduced thymic regression during the period of treatment. In females, little testosterone is present compared with males and, therefore, the effects of an aromatase inhibitor would be minimised.

While the action of oestradiol on the uterus in neonatal and in adult rats is well documented (Sheehan *et al.*, 1981; Woodman, 1997), its specific effects on the epithelial and lymphocyte cell populations of the thymus are less well established and need further investigation. Nevertheless, the results of this study were in agreement with the work of others on the effects of oestradiol treatment on uterus and showed that the thymus was also responsive to E2 at this age. The results of this study also suggest that testosterone might have been aromatized to E2 in the thymus of males in this age group and hence have caused developmental changes in the structure of this tissue.

5.4.2: Effects on the concentration of cytosolic oestrogen receptor

Uterus

The concentration of the oestrogen receptor in uterus cytosols of E2 and CC+E2-treated rats decreased compared with the control group. This result was not surprising as E2 alone, or in combination with CC, could have occupied cytosolic oestrogen receptors and been translocated into the nucleus from where it would not have been recovered. CC treatment alone did not alter the concentration of receptors that were detected in the uterus, and this result suggests that CC had neither agonist nor antagonist activity in the uterus at the concentration used. CC treatment in adult female rats can be oestrogenic or anti-oestrogenic depending upon the dose and isomers used. CC (the racemic mixture) is mildly oestrogenic in the uterus of adult ovariectomised female rats and results in cytoplasmic oestrogen receptor concentrations similar to those found in E2-treated ovariectomised females. Zuclomiphene, the *cis* isomer, is strongly anti-oestrogenic and treatment results in a much lower concentration of cytosolic oestrogen receptors and concomitant decrease in cytosolic progesterone receptor concentration (Young *et al*, 1991).

Treatment with 4-OHA had no effect on cytosolic oestrogen receptor concentration in uterus. Treatment of adult intact Sprague-Dawley rats with 4-OHA results in a reduction in the concentration of free plasma oestrogen *in vivo* and may also inhibit the synthesis of oestrogen receptors in the uterus (Zhou and Brodie, 1995). However, in the immature female rats used in the present study, little testosterone is likely to be available for aromatization to oestradiol. This may explain why the result found in the present study differed from that of Zhou

and Brodie (1995) i.e. the difference might be related to the different age of the rats used in which ovarian synthesis of oestradiol was inhibited by 4-OHA. In the uterus, oestradiol has the effect of increasing the expression of its receptor.

Hypothalamus

E2 and CC+E2 treatment resulted in a decreased oestrogen receptor concentration in the hypothalamus to a level that was undetectable by the methods used. These results suggest that the receptors might have been occupied by E2 and translocated to the nucleus. Oestrogen receptor mRNA synthesis decreases in the hypothalamus of ovariectomised rats following E2 treatment (Lauber *et al*, 1991) and this result would suggest, that in this present study, E2 administration at 15 days down-regulates the synthesis of the oestrogen receptor in the hypothalamus.

The response in the hypothalamus to 4-OHA treatment differed between males and females in that 4-OHA increased the concentration of oestrogen receptor in males, but had no effect in females. 4-OHA treatment of females in this age group, where E2 levels are low, may have had no effect on oestrogen receptor concentration because there was little testosterone present. In male rats, however, the concentration of oestrogen receptor in the hypothalamus increased after treatment with 4-OHA, a result that is in agreement with studies by Clancy *et al* (1995). This result could have been due to inhibition by 4-OHA of local synthesis of oestradiol from testosterone *via* aromatase. 4-OHA treatment could also have lead to decreased rate of translocation of oestradiol-bound receptor from the cytosol to the nucleus. In the control group, however, no differences were observed between male and females. This finding would

argue against the role of aromatization in oestrogen receptor differences between males and females. Using *in vitro* autoradiography techniques, Kuhnemann *et al* (1994) found higher concentrations of cytosolic oestrogen receptor in the hypothalamus of female Wistar rats at 10 days than in males but, in other studies in 30-day-old rats, no sex differences were detected (see Table 4.5). As males have higher levels of aromatase activity in the brain (MacLusky *et al*, 1985) the fact that in this study no sex differences were found in the control group could have been due to the increasing concentrations of free oestradiol in both sexes between 15 and 30 days.

The recent discovery of a second oestrogen receptor (ER β) (Kuiper *et al*, 1996) could account for some observed tissue-specific effects of oestrogens. In the rat hypothalamus, there is differential distribution of ER α , the classical oestrogen receptor (Shughrue *et al*, 1996), and ER β and this finding may lead to further elucidation of the role of oestrogens in the developing hypothalamus. In the present study, no account was taken of the possible differential tissue distribution of the two forms of the oestrogen receptor. In the present study, no distinction was made between the sub-types of the cytosolic oestrogen receptor and the measurements made are likely to have included both sub-types.

Cortex

In the cortex, no oestrogen receptors were detectable in the control or treated animals. This result could be due in both sexes to the increasing levels of free oestradiol with consequent down-regulation in the brain of oestrogen receptors, or to translocation of oestrogen receptors to the nucleus from the cytosol. Brain aromatase activity is confined to those areas such as the

hypothalamus that are associated with reproduction (Hutchison *et al.* 1994). The lack of detectable oestrogen receptors in the cortex is unlikely, therefore, to be due to aromatization of testosterone in males. Other studies of the cortex show similar findings, that is, high levels of oestrogen receptor during the early postnatal period and no detectable oestrogen receptor in rats by 30 days (O'Keefe and Handa 1990; Keefer and Holdregger, 1985, Yokosuka *et al.*, 1995). E2 enhances cortical myelination (Curry and Heim, 1966) and these findings supports a developmental role in both sexes for E2 at an early, if limited, stage of development.

Thymus

The concentration of oestrogen receptors in thymus cytosols of female, though not of male, rats decreased after treatment with CC; no receptors were detectable after treatment with E2 or CC+E2 in either sex; receptor concentrations did not change in females, but rose in males, after treatment with 4-OHA.

The result in females treated with CC is suggestive of oestrogen antagonist activity in this group. E2 levels are low in females at this age and the lowering of the oestrogen receptor concentration suggests that the CC-bound receptors could have been translocated into the nucleus of the cell. No reduction of oestrogen receptor concentrations was found in males, perhaps because locally formed E2 at this age may have been bound to the receptor with greater affinity than that of CC. Thymic weight did not change and the functional effect of CC treatment may differ from the thymic weight reduction effects and reduced receptor concentration effects of E2 and testosterone (Greenstein *et al.* 1992). It

has been well-established that CC is a useful drug for the treatment of infertility in women (Dickey and Holtkamp, 1996), can prevent bone loss in ovariectomized rats and can reduce procarbazine-induced infertility in male rats (Weisseberg *et al*, 1995). Little is known, however of the effects of CC on the thymus or on immune function. In two reports, CC used for induction of ovulation in women has been associated with the development of systemic lupus erythymatosus (Ben-Chetrit and Ben-Chetrit, 1994; Canvin and Capell, 1995). Further studies of the effects of CC in adult rats, and in an animal model of autoimmune disease, could perhaps reveal whether the binding of CC to the oestrogen receptor in the thymus is associated with the development of autoimmune disease. Postmenopausal oestrogen therapy has been associated with higher risks of development of systemic lupus erythymatosus (Sanchez-Guerrero *et al*, 1995) and the CC effect could be mediated through mechanisms similar to those of oestradiol in the development of this condition.

Treatment with E2 and with CC+E2 caused the concentrations of oestrogen receptors to decrease in both sexes to a level at which they were undetectable in the assay system being used. This result differed from the results of Kawashima *et al* (1992) who found oestrogen receptor concentration raised in thymus cytosols from adult ovariectomised E2-treated female mice compared with the controls. There is no obvious reason why these results should be different in rats and mice. The effect may have been due to a different dose of E2 or to treatment at a different developmental stage in the thymus. Oestrogen receptors are present in the thymulin-producing cells in the adult female rat thymus (Kawashima *et al*, 1991) and many other thymic factors are produced by

the reticulo-epithelial cells of the thymus (Low *et al*, 1981; Dardenne *et al*, 1974; Grossman *et al*, 1982). Moreover, oestrogens regulate thymic lymphocyte function *via* thymic factors, and oestrogen receptors are localised in the lymphoid fraction of the thymus (Danel *et al*, 1983; Kawashima *et al*, 1992). The effect of E2 and CC+E2 treatment in the present study could have been due to translocation of receptor to the nucleus and/or to down-regulation of receptor synthesis.

4-OHA treatment led to an increase in thymus cytosolic oestrogen receptors in males, but not in females. This finding is in agreement with the results of Greenstein *et al* (1993) who found that aromatase inhibition with 4-OHA may have contributed to the observed increase in thymic cytosolic oestrogen receptors. In males, high concentrations of testosterone are present between 5 and 30 days, and are converted to oestradiol *via* the aromatase pathway. When 4-OHA is administered to males, it combines irreversibly with aromatase and the conversion of testosterone to oestradiol is inhibited. Similar work using a different aromatase inhibitor, 1,4,6-androstatriene-3,17-dione (ATD), gave the same result, an increase in oestrogen receptors in thymus cytosols of male rats (Greenstein *et al*, 1993).

5.4.3: Effects on the molar dissociation constant (Kd)

The molar dissociation constant of the oestrogen receptor for E2 did not change as a result of any of the treatments. This result suggests that none of the treatments applied (with the exception of E2 or CC+E2) acted as oestrogen agonists in that there was no lowering of the apparent affinity of the receptor for oestradiol. The effects of CC treatments vary considerably from oestrogenic to

anti-oestrogenic depending upon the dose used and the end-organ (Young *et al.*, 1991). The concentration of CC in the present study may have been at insufficient doses and use of a range of doses of the pure cis and trans isomers might have led to a more conclusive result about the effects of this drug on the tissues examined. In the immature females in the study described here, with little endogenous oestrogens present, CC may have acted as a weak oestrogen agonist in the thymus, but had no effect at the concentration used in other tissues or in males in which testosterone which is aromatizable to oestradiol, was present.

The results of the present study did not take account of the recent discovery of the second (beta) form of the oestrogen receptor. If there are differences between the distribution of the alpha and beta forms in the brain and thymus or between males and females in these tissues, they could be significant during the development of these tissues. The development of thymic epithelial cells (and other oestrogen-sensitive thymic cells) could be altered between males and females as a result of differences between expression of the two sub-types of the receptor and/or differences between the sexes in the exposure to oestradiol in the thymus during the early perinatal period.

In summary:

The effects of treatment of female and male Wistar rats at 15 days with E2, CC, CC+E2, or 4-OHA varied depending upon the tissue (summarized in Table 5.5). The effect of E2 on body weight and tissue weight as well as on oestrogen receptor concentration in the uterus, hypothalamus, brain cortex and thymus cytosols showed some similarities between the sexes in response to this treatment as well as some differences. 4-OHA also had differential effects in the

two sexes. These findings may have some relevance to different developmental pathways in the tissues examined. They might suggest possible ways of manipulating the microenvironment of the thymus or of ameliorating the iatrogenic effects on immune function of infertility treatments which alter oestrogen or oestrogen receptor concentrations. Autoimmune diseases such as systemic lupus erythematosus may develop against a particular genetic and/or environmental background. It is possible that against such a background (present possibly in autoimmune-prone experimental animals) low oestradiol or CC levels could cause a temporary manifestation of disease while high levels could induce the full-blown symptoms to develop. If thymus structure and function can be manipulated by administration of oestradiol or its inhibitors, this alteration could have important implications for the treatment of diseases that result from the breakdown of immunological self-tolerance.

Chapter six

Final discussion and conclusion

The studies undertaken in this project addressed the following questions

1. Is the cytosolic oestrogen receptor in the thymus of immature female Wistar rats similar to or different from the receptor in the uterus where its ligand binding specificities are well-established?
2. Do the binding characteristics (K_d and concentration) of cytosolic oestrogen receptors in the thymus differ from those found in the uterus and brain and do they differ between the sexes and at 5, 18, and 30 days in Wistar rats?
3. If differences exist, could they be due to differential exposure to oestradiol in the two sexes at different ages and could manipulation of oestradiol concentrations in this age group modify oestrogen receptor binding in the tissues studied?

Gel filtration was chosen as the most appropriate method for determining the specificity of the cytosolic oestrogen receptor in the uterus and thymus and for measuring its dissociation constant in these tissues and in the brain although it is less sensitive than some molecular biological methods for determination of cytosolic oestrogen receptor concentrations. Although time-consuming and needing relatively large sample volumes, the relatively high sensitivity of this method nevertheless, made it most appropriate to estimate oestrogen receptor concentration and to determine the dissociation constant of the receptor.

The cytosolic oestrogen receptor had similar ligand specificities in the thymus of females and in the uterus with the exception of the higher affinity of the receptor in thymus for corticosterone compared with its affinity for this compound in the uterus. This difference could be significant because of the well-

known atrophic effects on the thymus of stress, mediated *via* the hypothalamic/pituitary axis and raised glucocorticoids in many mammals (reviewed by Spagnolo and Gorospe, 1997). The observed differences could have been due to other factors such as growth factors or thymic hormones present in the thymus. Additional factors could have been genetically determined molecular variation of the oestrogen receptor structure and/or differential post-translational modification of the oestrogen receptor protein in this tissue. Moreover, there could be differences in the ratio of expressed ER α and ER β sub-types in the thymus. In the rat there are differences between the relative expression of the two sub-types in various tissues (reviewed by Kuiper *et al*, 1997). Studies of the relative distribution, affinities and specificities of the two sub-types have not been studied in the thymus thus far. The results are consistent with the hypothesis that the rat thymus in this age group does possess high-affinity receptors for oestradiol.

Oestrogen receptors have been well-characterised in the uterus and brain of developing rats and there are clear differences in these tissues in its distribution and concentrations. In the present studies, sex and age differences were found in cytosolic oestrogen receptor concentrations in the thymus as well as in the uterus and brain. Females, at 5 days, had higher concentrations of receptors in thymus than did males of the same age, a result which was similar to the pattern found in the hypothalamus, though not in cortex. Moreover, thymus weight relative to body weight was lower in males than in females by 30 days. These results suggest that in males, during this period when circulating testosterone concentrations are high, oestradiol, locally-formed from testosterone

in the thymus and hypothalamus, may be contributing to the two-fold lower concentration of cytosolic oestrogen receptors found in males compared with females at 5 days. Thus in males, the difference from females in testosterone secretion and aromatase activity during the perinatal period and conversion of this hormone to oestradiol in hypothalamus and in thymus could have developmental significance in the organisation of these two tissues and in adult responsiveness to oestradiol. Moreover, in the thymus, the difference could, when combined with other factors, genetic or environmental, lead to differential development of autoimmune diseases in males and females.

Because free oestradiol concentrations in the serum did not begin to rise until after 18 days in both sexes, it was decided to treat animals at 15 days in order to give the compounds, CC, E2, CC+E2 and 4-OHA, time to be absorbed. Oestradiol treatment caused thymic atrophy in both sexes, a result that was consistent with previous studies and differed from the effect of increase in the weight of the uterus. On the other hand, administration of an aromatase inhibitor was associated with an increase in thymus weight in males, though not in females. This result suggests that in males, aromatization of testosterone to oestradiol inhibited thymus growth by 30 days. By this age in the control group, the weight of the thymus relative to body weight was lower in males than in females. As cytosolic oestrogen receptors were undetectable as a result of treatment with oestradiol, with or without clomiphene, in both sexes, it is possible that cytosolic oestrogen receptors were translocated into the nucleus and became undetectable by the methods used. Aromatization of testosterone to oestradiol in the thymus of males might have been occurring and could have

resulted in the higher concentrations of oestrogen receptors in males, though not in females, after treatment with the aromatase inhibitor. In the control males, receptors could have been translocated to the nucleus; after aromatase inhibition the unbound receptors might have remained in the cytosol. It is also possible that, in the thymus, oestradiol formed either locally through aromatization or after treatment with oestradiol down-regulates the synthesis of oestradiol receptors and hence their replenishment in the cytosol.

The different effects of the drugs in cytosols from uterus, hypothalamus, cerebral cortex and thymus could have been due to structural differences in the receptor in these tissues. This question could be addressed in the future by sequencing the oestrogen receptor gene in the different tissues in order to look for differences in the nucleotide sequences that would alter the amino acid sequence of the receptor. The finding that the receptor had a higher affinity in the thymus than in the uterus for corticosterone might be explained by such a difference, despite the similar affinity shown in both tissues for oestradiol. In the rat, the tissue distribution and level of ER α and ER β mRNA expression differs in that the concentration of the ER α is moderate to high in uterus, testis, pituitary, ovary, kidney, epididymis and adrenals. The concentration of ER β of the receptor is moderate to high in prostate, ovary, lung, bladder, brain and uterus (Kuiper *et al*, 1996). The two sub-types could also exert tissue specific effects if they are able to transactivate different oestrogen response elements on DNA. Moreover, the ER α and ER β can also mediate gene transcription *via* an AP1 enhancer site with different effects being observed dependent on ligand and receptor sub-type. The discovery of the ER β form of the oestrogen receptor

may have some significance for this study if the two forms of this hormone receptor perform different physiological roles in the different tissues or in different cell types within the same tissue. The two forms of the receptor show only 55 percent homology in the ligand binding domain and this difference could be associated with the higher affinity for corticosterone of the cytosolic receptor in the thymus compared with the uterus. Alternatively, given the biochemical richness of the thymic microenvironment, there could also be soluble products such as growth hormone or thymic hormones that might enhance the binding of corticosterone to the oestrogen receptor. These effects could be mediated by post-translational modification ligand-binding site of the receptor by thymic factors.

The question of whether oestrogen receptors were being translocated to the nucleus in response to the various treatments could be addressed by using gel filtration techniques to measure nuclear receptors. Studies previously undertaken in this laboratory (Greenstein, 1992) showed that cytosolic oestrogen receptor concentrations decrease in the hypothalamus and uterus with a concomitant rise in nuclear receptor concentrations after oestradiol administration. If this pattern is due to translocation of the receptor from cytosol to nucleus and to the tight binding of the receptor to the DNA, the same effect may be present in the thymus.

Histology, using immunocytochemistry or autoradiography techniques, could be used to localise nuclear receptors to specific areas within the tissues examined. Reverse transcriptase-polymerase chain-reaction techniques could identify cells synthesising mRNA for the receptor. Studies could be done at

closer age intervals than 15 and 30 days and might reveal more clearly the stages at which differences arose. Treatment with a series of different doses of clomiphene and/or separate use of the two different isomers might also establish whether treatment with this drug could modulate the response of thymic cells to oestradiol. Clomiphene treatment in the present studies did lower the concentration of oestrogen receptors in the thymus of female rats, presumably as a weakly competitive antagonist, as no change was observed in the binding constant in this treatment group. Castration of males at 5 days and/or treatment with oestradiol together with 4-OHA might also clarify the role of aromatase in the development of the hypothalamus and thymus.

Functional differences that relate to the immune responsiveness (for example, mixed lymphocyte reaction or responsiveness to mitogens) of treated rats need to be examined and compared with these parameters in untreated animals. Moreover, an examination of the interaction of sex hormones with other hormones such as growth hormone and glucocorticoids might reveal effects on T-cell differentiation in the thymus, for example, by altering the ratios of T-cell subsets emerging. Several studies have shown that the ER α and ER β differ in their specificity and tissue distribution in many areas of the brain as well as in tissues such as the ovaries, uterus, prostate, kidney, adrenal, bladder and lung (see Chapter 4, Section 1). It would seem timely to study the distribution and specificity of the two oestrogen receptor sub-types in the thymus. Differences between histocompatible Fischer 344 and Lewis rats have been shown in their diurnal and stress corticosterone levels and in the binding of the Type II glucocorticoid receptors in the thymus. The difference in the affinity of the

oestrogen receptor for corticosterone found in the present study might be associated with the ratio of ER α and ER β in the thymus and, moreover, there could be differences between different strains of immune-prone and control strains of animals. The present studies suggest that the specificity of the cytosolic oestrogen receptor in the thymus closely resembles the receptor in the uterus but there was a notable difference in the affinity of the oestrogen receptor in the thymus for corticosterone compared with the uterus.

Differences in receptor concentrations, but not in affinity for oestradiol, were found between different age groups and sexes. Moreover, tissue weights and cytosolic oestrogen receptor concentrations were, in some tissues, differentially altered in treated males and females. The mechanisms for these differences may vary in detail between the tissues examined. Nevertheless, sex differences in the thymus, as in the brain, could be related to differential exposure of males and females to oestradiol at an early stage of postnatal development.

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APPENDIX A

Program for the Hewlett Packard HP48SX programmable calculator (reproduced by kind permission of Dr B. D. Greenstein). The calculator can be used in conjunction with a small Hewlett-Packard infra-red printer, the HP 82240B

The program relies on the fact that the non-specific binding of the ligand is purely a function of the amount of added radioactivity, and directly proportional to it. Therefore only one incubation is needed where the radioactive steroid (usually in the highest concentration used is diluted by a 100-fold excess of unlabelled steroid.. The program asks for the cpm relating to the total radioactivity used in the incubation in which non-specific binding (NSB) is assessed, and for the cpm bound. It calculates the NSB and stores the value. It then asks for the various cpm of the total bound radioactivity in each of the other incubations in which there is no unlabelled steroid, and proceeds to calculate and plots the Scatchards obtained. The program asks for the correction factor which converts all cpm to molar concentrations, and then proceeds to print the Kd, the binding capacity and correlation coefficient.

```
SCAT
<<LBN SPIN TEST CALC
ARR PRO REG
>>

LBL
<< "ENTER DATE, SPACE TISSUE"
440 .5 BEEP {
":DATE
:TISSUE:" {10
}V} INPUT OBJ→PRST
CLEAR
>>

NSPIN
<<CLΣ 'PPAR' PURGE
'ΣPAR' PURGE 450 .5 BEEP
"ENTER nonspecific
BOUND, TOTAL"
":nonspBOUND:
:nonspTOTAL:"
{ 1 0 } V} INPUT OBJ→
PRST DUP 'NST' STO DROP
DUP 'NSB' STO CLEAR
>>

TEST
<<440 .5 BEEP
"ENTER BOUND, TOTAL"
{":BOUND:
:TOTAL:" {1 0
}V} INPUT OBJ→PRST
DUP 'T' STO DROP DUP
'BT' STO CLEAR
>>

CALC
<< 'BT'-NSB*T/NST'→NUM
'SPBOUND'→TAG DUP 'BSP'
STO 'T-BSP'*NUM 'FRE'
```

```
→TAG DUP 'F' STO 'BSP/F'  
→NUM 'BoverF' → TAG DUP  
'BF' STO PRST 3 DROPN  
>>
```

```
ARR  
<<BSP BF 2 →ARRY Σ+  
>>
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PRO  
<<440 .5 BEEP  
"MORE?"  
PRESS 1 ENTER ELSE  
PRESS 0 ENTER"  
{ "ENTER CHOICE:"V}  
INPUT OBJ→→ x  
<<  
IF 'x=1'  
THEN CLEAR TEST CALC  
ARR PRO  
ELSE CLEAR  
"PARAMETERS" PRI CLEAR  
END  
>>  
>>
```

```
REG  
<< "ENTER CORR. FACTOR"  
440 .5 BEEP {"CORF:}V  
} INPUT OBJ* VFYCF CORF  
PRI CLEAR "PLOTING" PRI  
CLEAR CR 1 XCOL 2  
YCOL LINFIT SCATRPLOT  
PRLCD LR CORR 0 PREDX  
'N'→TAG DUP DTAG 'N'  
STO ROT DUP 'SLOPE' STO  
'1/SLOPE*CORF'→NUM 'KD'  
→TAG 'N*CORF'→NUM  
'NMOLAR'→TAG PRST CLEAR  
>>
```