ANDROGEN AROMATIZATION AND CYTOSOL ESTRADIOL-RECEPTORS IN THE MEDIATION OF MASCULINE SEXUAL BEHAVIOR IN JAPANESE QUAIL

by

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INTRODUCTION

Many of the complex biochemical interactions that exist between the endocrine and nervous systems are involved in the mediation of sexual behavior. In males, the influence of exogenous gonadal steroids on behavior has been extensively investigated. In all but a few species tested, implantation or injection of aromatizable androgens or estrogens has resulted in the expression of mating behavior, whereas non-aromatizable androgens have been incapable of exerting a similar behavioral influence.

In vitro and *in vivo* studies have localized androgen aromatization within specific areas of the central nervous system, predominately the hypothalamic and limbic regions. Implantation and lesioning studies have implicated these regions in the mediation of sexual behavior. In addition, proteins that selectively retain androgens and estrogens have been localized within these same areas of the brain by autoradiography. Although these steroid receptors have been isolated from several species and characterized by a variety of biochemical techniques, the extent of their role in the expression of sexual behavior is unknown. In all probability, the mechanism by which gonadal steroids regulate masculine mating behavior is based on steroid-receptor interactions as well as on metabolic factors. It was the purpose of the present study to examine the relative importance of androgen metabolism pattern and estradiol-receptor interactions to the mediation of masculine mating behavior in lines of Japanese quail selected bi-directionally for adult mating frequency.

REVIEW OF LITERATURE

An immense amount of data has been collected pertaining to the role of the aromatization of androgens to estrogens in the mediation of masculine sexual behavior. For this reason, the review of literature presented herein includes information on a number of pertinent aspects. Those included are: (1) Behavioral studies, (2) *in vitro* and *in vivo* androgen metabolism, (3) steroid-receptor definition and (4) androgen and estrogen receptors.

Behavioral Studies

Aromatizable and nonaromatizable androgens have been administered to castrated males of both mammalian and nonmammalian species. By comparison within some species, injections or implants of aromatizable androgens were shown to elicit male mating behavior while non-aromatizable androgens failed to elicit this behavior. In these studies, mating behavior was induced in castrated rats treated with the aromatizable androgens, testosterone and androstenedione, or their propionated forms (Luttge and Whalen, 1970; Whalen and Luttge, 1971; Feder, 1971; Beyer *et al.*, 1973; Larsson *et al.*, 1973; Parrott, 1974; Parrott, 1976). Only aromatizable androgens prevented the increase in refractory period duration that is typical of the newly castrated rat (Parrott, 1975). On the other hand, treatment with the nonaromatizable 5α and 5β reduced androgens, dihydrotestosterone, androstanedione,

androsterone, or androstanediol, did not restore mating behavior (McDonald et al., 1970; Whalen and Luttge, 1971; Feder, 1971; Beyer et al., 1973; Larsson et al., 1973; Parrott, 1976). The latter compounds did act peripherally, however, to restore the accessory sex glands after castration (Luttge and Whalen, 1970; McDonald et al., 1970; Whalen and Luttge, 1971; Feder, 1971; Beyer et al., 1973; Parrott, 1975).

The response of castrated male mice to androgen injections was somewhat different from that of the rat. Both testosterone and androstenedione restored mating behavior to the castrated rat, whereas in the mouse, testosterone but not androstenedione was effective (Luttge and Hall, 1973). In both species dihydrotestosterone exerted its effect on the secondary sex characteristics.

Testosterone and testosterone propionate injections as well as implants were capable of restoring mating behavior to physically or functionally castrated male Japanese quail (Beach and Inman, 1965; Adkins, 1977; Adkins and Pniewski, 1978; Adkins, 1978; Balander, 1978; Adkins et al, 1980). Testosterone and/or nonaromatizable 5α and 5β reduced androgens stimulated development of the proctodeal gland and testes along with vocalization and strutting (Adkins, 1977; Adkins and Pniewski, 1978, Adkins, 1978; Balander, 1978; Adkins et al, 1980).

Mating behavior in castrated male hamsters, rabbits, monkeys and guinea pigs is not considered to be dependent on the aromatization of androgens. This has been shown to be true for the hamster by Christensen et al. (1973), Whalen and DeBold (1974), and Payne and

Bennett (1976); for the rabbit by Beyer and Rivaud (1973), and Agmo and Sodersten 1975; for the monkey by Phoenix (1974); and for the guinea pig by Alsum and Goy (1974).

Conclusions regarding the aromatization of testosterone to estrogen have been derived not only from comparisons of the effects of aromatizable and nonaromatizable androgen treatments, but also from research involving aromatase inhibitors and anti-estrogens. Aromatase inhibitors interfere with hydroxylation of the C_{19} atom of the androgen molecule. Androst-1,4,6-triene-3,17-dione (ATD), aminoglutethimide and 4-hydroxy-androstenedione are aromatase inhibitors which depress the typical behavioral responses of castrated male rats given exogenous testosterone (Christensen and Clemens, 1975; Morali et al., 1977; Booth, 1978). The aromatase inhibitor, ATD, has also been found to be effective in male quail (Adkins et al., 1980) and to inhibit the normal sexual differentiation of neonatal rats (McEwen et al., 1977).

Anti-estrogens affect mating behavior by competing with estrogens for cytosol receptor sites, and thereby depress the sexual behavior of castrated males treated with testosterone. Two anti-estrogens that are effective in rats are CI-628 and ICIO-46474 (Luttge, 1975; Beyer et al., 1976; McEwen et al., 1977; Sodersten, 1978). Adkins and Nock (1976) noted that CI-628 was also effective in male quail.

Androgen Metabolism

Pathways of androgen metabolism and estrogen biosynthesis are shown

in Figures A.1 and A.2 of Appendix A, respectively. Androgens are metabolized via a series of A-ring reductions followed by reduction of the 17-keto group. The reactions are catalyzed by oxidoreductase enzymes in the presence of NADPH and oxygen. Studies involving homogenates of rat brain (Rommerts and van der Molen, 1971) and ventral prostate (Nozu and Tamaoki, 1973) have shown that reductase activity is localized in the nuclear and microsomal fractions and that dehydrogenase activity is in the cytosol fractions. The biosynthesis of estrogens from androgens proceeds through successive hydroxylations of the C_{19} atom and then through a spontaneous conversion to the aromatic 18-carbon estrogen molecule. The mechanism of action for the conversion of androgen to estrogen is known and researchers agree that both oxygen and NADPH are required although some controversy exists in defining the rate limiting step (Heftman, 1970; Thompson and Siiteri, 1973; Goto and Fishman, 1977). Specifically, the mechanism involves enzymatic hydroxylation of the C_{19} methyl group of androstenedione, oxidation of the C_{19} hydroxyl group, and hydroxylation of the A-ring (Appendix Figure A.3). The hydroxylase and oxidoreductase enzymes that are involved are collectively referred to as aromatase enzymes. Conversion of the final aliphatic compound, 2-hydroxy-19-androstenedione, to the aromatic compound, estrone is thought to be non-enzymatic.

Androgen metabolism has been studied by <u>in vivo</u> and <u>in vitro</u> techniques in a variety of species. Both techniques have demonstrated that androgens are metabolized in central and peripheral tissues. The

advantages and disadvantages inherent to the *in vivo* and *in vitro* methods prescribe a need for obtaining data by both techniques. *In vitro* experiments may be faulted because organ isolation disrupts the integrity of tissues. Also, the addition of necessary cofactors to isolated tissue systems may introduce artifacts which would not be present in the intact animal. On the other hand, experiments with isolated tissue systems provide valuable information on target organ function.

In Vitro Experiments

The conversion of testosterone to dihydrotestosterone in target organs is of particular interest in view of the fact that exogeneous dihydrotestosterone as well as testosterone acts peripherally to restore the integrity of accessory sex characteristics following castration. When added to prostate organ cultures, dihydrotestosterone induced epithelial hyperplasia to a greater extent than did testosterone (Robel *et al.*, 1971). In the rat, the prostate, epididymis, seminal vesicles, penis, preputial gland, and scrotum all supported high rates of conversion to dihydrotestosterone (Bruchovsky and Wilson, 1968; Gloyna and Wilson, 1969; Wilson and Gloyna, 1970; Stern and Eisenfeld, 1971; Hansson *et al.*, 1974). Massa *et al.* (1972) compared conversion rates of testosterone to dihydrotestosterone in rat prostate to conversion rates in areas of the brain and found the prostate to be most active in its production of dihydrotestosterone. In addition to dihydrotestosterone,

the prostate has been shown to actively produce androstanediol and androstenediol (Robel et al., 1971; Genot et al., 1975). Significant quantities of dihydrotestosterone were also produced by the bullfrog testis (Callard et al., 1978a); the epididymis (Nakamura and Tanabe, 1972, 1973), comb, wattle and uropygial glands of the chicken and duck (Wilson and Gloyna, 1970); and by the rat liver (Ghraf et al., 1973). Studies of this nature have been extended to other species, including man, baboon, dog, lion, mouse, guinea pig, cat, bobcat, bull, and rabbit. Metabolism of testosterone to dihydrotestosterone was observed in prostatic tissue of these species, but was limited to the growth period in several of them.

A series of studies conducted by Callard and co-workers (1977, 1978a, b, c) revealed that the aromatization of androgen to estrogen is a "primitive" characteristic of the vertebrate central nervous system. That is, all classes of vertebrates, with the exception of the jawless Agnatha, actively synthesized estrogens from androgen precursors.

Behavioral studies wherein castrated male animals were given exogeneous hormone treatments have provided indirect yet strong evidence that androgen metabolites and their subsequent aromatization to estrogens are responsible for the expression of male mating behavior. Definitive evidence lies in the direct assessment of the aromatization process. Therefore, many experiments have been conducted to follow the metabolism of radiolabeled testosterone and androstenedione in a variety

of central nervous system (CNS) tissue preparations from numerous species. By far the rat is the most extensively studied species.

In CNS tissue isolated from the rat, the bulk of *in vitro* conversions of testosterone to androstenedione, and testosterone and androstenedione to estrogens occurred in hypothalamic and limbic tissues while the cortex and pituitary were much less active (Naftolin *et al.*, 1972; Ryan *et al.*, 1972; Denef *et al.*, 1973; Weisz and Gibbs, 1974a; Genot *et al.*, 1975; Naftolin and Ryan, 1975; Canick *et al.*, 1977; Selmanoff *et al.*, 1977). Specifically, the anterior portion of the hypothalamus was most active (Naftolin *et al.*, 1972; Ryan *et al.*, 1972; Naftolin and Ryan, 1975; Selmanoff *et al.*, 1977). These data are consistent with those of Johnston and Davison (1972) who found that implantation of testosterone propionate into the anterior hypothalamic-preoptic area was a greater stimulus for mating in castrated rats than was implantation in the posterior hypothalamus. In spite of the numerous reports cited above, both Jaffe (1969) and Perez *et al.* (1975) found higher levels of aromatization in pituitary sections versus hypothalamus, hippocampus and cerebral cortex. The explanation for this discrepancy may lie in the *in vitro* technique employed, that is, in the tissue preparation and experimental conditions.

In addition to the rat, aromatization of androgens in CNS tissues has been demonstrated in the adult rabbit (Reddy *et al.*, 1972), fetal rabbit (George *et al.*, 1978), rhesus monkey (Flores *et al.*, 1973a), cow (Sholiton and Werk, 1969), dog (Perez-Palacios *et al.*, 1970), human

fetus (Naftolin, et al., 1971a, b), chicken (Nakamura and Tanabe, 1974), European starling (Massa et al., 1977), dove (Steimer and Hutchison, 1980), bullfrog (Callard et al., 1978a), turtle (Callard et al., 1977, 1978b; Lisboa et al., 1978), snake and several amphibians (Callard et al., 1978b).

Androgen metabolism in the CNS of rats and rabbits has been influenced by the hormonal status of the animal prior to sacrifice. Both aromatization and reduction of androgens increased following castration (Kniewald et al., 1971; Massa et al., 1972; Reddy et al., 1972; Denef et al., 1973; Kniewald and Milkovic, 1973; Massa et al., 1975; Naftolin et al., 1975). Kniewald et al. (1971) attributed the increase in reductase activity to the elevated level of follicle stimulating hormone (FSH) which was caused by castration. In support of their hypothesis, the formation of dihydrotestosterone was stimulated to a much higher level when FSH was included in the incubation medium surrounding pituitaries from intact males (Kniewald et al., 1971; Massa et al., 1972). In addition, Reddy et al., (1972) and Naftolin et al. (1975) showed that exogenous testosterone and estradiol treatments decreased or prevented the reduction of testosterone to dihydrotestosterone (Massa et al., 1972); Denef et al., 1973). Progesterone treatment decreased both aromatizing and reducing activities. Some of the more recent studies conducted in this area have shown that, in birds, castration affects both 5α and 5β reductase activities (Balthazart et al., 1979; Massa et al., 1979; Hutchison and

Steimer, 1981).

The reduction of testosterone to dihydrotestosterone is not limited to the peripheral organs. Tissues isolated from the CNS of a variety of species including the rat (Jaffe, 1969; Sholiton and Werk, 1969; Kniewald et al., 1971; Massa et al., 1972; Denef et al., 1973; Perez et al., 1975; Selmanoff et al., 1977), rabbit (Reddy et al., 1974), guinea pig (Sholl et al., 1975), dog (Perez-Palacios et al., 1970), chicken (Nakamura and Tanabe, 1974), starling (Massa et al., 1977), quail (Massa et al., 1979), and turtle (Lisboa et al., 1978) also actively reduced testosterone to dihydrotestosterone in vitro. In fact, testosterone reduction predominated over aromatization in the central nervous system (Massa et al., 1977). Therefore, it is conceivable that in addition to its peripheral activity, dihydrotestosterone acts at the level of the CNS in synergism with estrogen. This has been suggested by Baum and Vreeburg (1973), Beyer et al. (1975), Perez-Palacios et al. (1975), Davidson (1977), Balthazart and Hirschberg (1979), and Landau (1980).

In Vivo Experiments

Studies conducted in vivo have demonstrated a considerable uptake of radiolabeled androgens by both brain tissue and accessory sex organs. The uptake of ^{3}H-testosterone occurred within one minute of an intravenous administration to normal or functionally hepatectomized rats, and at least 90% was metabolized by the prostate with the principal metabolite being dihydrotestosterone (Bruchovsky and Wilson,

1968; Bruchovsky, 1971). In mature castrated rats, Stern and Eisenfeld (1971) found the seminal vesicles and the hypothalamus to be very receptive and the cerebrum to be slightly receptive to ^{3}H-testosterone within one hour of its injection. At that time, 70% of the label had been removed from the plasma.

Whalen and Rezek (1972) traced the metabolism of ^{3}H-testosterone and ^{3}H-dihydrotestosterone in CNS tissues of mature castrated rats and found testosterone, dihydrotestosterone, androstenedione, androstanediol, and androstanedione to predominate. Compared to muscle tissue, the accessory sex organs of adult castrates were significantly more receptive to ^{3}H-testosterone and its metabolites (Buric et al., 1972); Becker et al., 1973). Autoradiograpy of brain tissues from rats injected with radiolabeled androgens has localized androgen binding within specific neurons of the forebrain, pituitary and limbic regions (Pfaff, 1968; Sar and Stumpf, 1972, 1973, 1977). Luttge et al. (1976) found the uptake of ^{3}H-dihydrotestosterone to be higher in the pituitary than in any other brain region of castrated mice. Regarding the metabolites of testosterone, Schmidt et al. (1972, 1973) noted varied effects on cell proliferation and metabolism among target tissues of immature castrated rats. As a result, they proposed the existence of multiple intracellular sites of androgen action; that is, different androgens may act independently of one another in peripheral tissues due to their unique patterns of accumulation and metabolism.

Additional studies with rats have utilized radiotracer techniques

to follow the synthesis of estrogens from ^{3}H-testosterone in hypothalamic and limbic tissues of adult gonadectomized-adrenalectomized males (Lieberburg and McEwen, 1977), neonatal females (Weisz and Gibbs, 1974b), and neonatal males (Lieberburg and McEwen, 1975). Very little estrogen was synthesized in cortical or pituitary tissues although the pituitary did synthesize dihydrotestosterone. Flores et al. (1973b) observed the same compartmentalization of estrogen synthesized from ^{3}H-androstenedione in rhesus monkeys. Their novel experiment involved the perfusion of an isolated brain with ^{3}H-androstenedione via an artificial apparatus rigged to preserve normal blood flow and tissue permeability patterns.

In vivo uptake and metabolism of radiolabeled androgens have also been studied in nonmammalian vertebrates. In Aves, androgen retention has been reviewed by Zigmond (1975). In castrated male doves, ^{3}H-testosterone was localized within the forebrain and midbrain, primarily in the hypothalamic and preoptic nuclei and the nucleus intercollicularis (Stern, 1972; Zigmond et al., 1972; Martinez-Vargas et al., 1974). The nucleus intercollicularis was also implicated as a major site of androgen retention in songbirds, namely the chaffinch (Zigmond et al., 1973) and zebra finch (Arnold et al., 1976). In the male chicken, Meyer (1973) observed high levels of ^{3}H-testosterone uptake and retention in the area of the third ventricle, extending from the preoptic area to the medial hypthalamus. In the laying hen, Wood-Gush et al. (1977) localized androgen retention among forebrain

hemispheres including the ventral hyperstriatum, dorso-lateral neostriatum, and to a lesser extent the dorsal hyperstriatum and the nucleus intercalatus hyperstriatum. In the rooster, ^{3}H-testosterone was localized primarily in the hypthalamic-preoptic area, as well as in the nucleus taeniae and nucleus intercollicularis (Barfield et al., 1978). Concerning non-CNS tissues, Dube et al. (1975) observed that in castrated cocks, the uptake and metabolism of ^{3}H-testosterone was higher in the lung and wattle than in breast muscle, earlobe or comb. In the frog (Kelly et al., 1975, 1978), paradise fish, lizard and mink (Morrell and Pfaff, 1978), androgens were concentrated primarily in the hypothalamic, preoptic and limbic regions.

Considerable overlap has been found to exist among those brain regions which concentrate estrogens and those which concentrate androgens. The primary sites of ^{3}H-estradiol localization in both intact and ovariectomized females, and in intact male rats were nuclei of the hypothalamic-preoptic and limbic areas (Stumpf, 1968; Zigmond and McEwen, 1970; Pfaff and Keiner, 1973). Pfaff et al. (1976) noted a similar pattern of localization of ^{3}H-estradiol in ovariectomized rhesus monkeys. In chicken embryos, ^{3}H-estradiol has been detected within specific hypothalamic and preoptic nuclei as early as 10 days of incubation (Martinez-Vargas et al., 1975). Later in embryonic development, estrogens began to accumulate in other regions of the brain. In addition to hypothalamic and preoptic nuclei, estrogens have been localized within the amygdala and the midbrain (Martinez-Vargas et

al., 1976) and the thalamus (Kim et al., 1978) of the dove, and within the ventral and dorsal hyperstriatum, and the nucleus intercalatus hyperstriaticus of the chicken (Wood-Gush et al., 1977). The localization of ^{3}H-estradiol in amphibians (Morrell et al., 1975; Kim et al., 1978; Morrell and Pfaff, 1978) and insectivores (Stumpf and Sar, 1978) was similar to that of birds and mammals.

Steroid-Receptor Definition

The dogma of steroid hormone action is based upon the concept of receptors. It assumes that the formation of a receptor-steroid complex is a prerequisite for a particular steroid-mediated response. According to the model of O'Malley and Means (1974), hormone enters the target cell and binds to a specific receptor. Once bound to hormone, the cytoplasmic receptor becomes "activated", thereby enabling its transport (in conjunction with the bound hormone) into the nucleus of the cell. Within the nucleus, the "activated" receptor-steroid complex is bound to certain sites on the genome, thereby inducing the formation of new RNA. Some of the newly formed RNA strands are "specific" mRNA species. These strands are transported to the cytoplasm of the cell where they are bound to ribosomes and translated into proteins. It is these newly synthesized proteins which ultimately bring about the functional changes associated with the steroid-mediated response.

Clark and Peck (1979) have expanded the model of O'Malley and Means (1974) to include a number of receptor characteristics. They indicated

that a receptor should have both a high affinity and a limited capacity for a hormone. This is because physiological levels of hormone are very low (10^{-10} to 10^{-8} M) but, nevertheless, capable of eliciting a saturable response. Furthermore, a receptor should express steroid specificity to minimize interference from other signals. Tissue specificity should be expressed, as well, so that only the appropriate target organs are stimulated. Most importantly, they indicated that the formation of a hormone-receptor complex must be correlated with some biological response.

The word "receptor" has also been used as a general term to label any putative macromolecule which intracellularly binds steroid. As a means of clarification, receptors are commonly regarded as either "specific" or "nonspecific"; this classification refers to receptor stereo-specificity for the steroid ligand. While both types of receptors are proteins, their individual interactions with a given steroid are quite different. Steroid binding by specific receptors is stereo--selective and occurs with a particularly high affinity (K_d of 10^{-10} to 10^{-9} M; Salhanick and Callard, 1979) but a relatively low capacity, whereas the nonspecific species of receptors is not stereo-selective and binds with a much lower affinity and a higher capacity for steroid.[1]

[1]The nonspecific receptor capacity for steroid is so much greater that in the presence of excess steroid in vitro, the specific receptor sites become saturated well before the nonspecific sites. For this reason, estimates of nonspecific receptor-steroid affinity constants usually cannot be obtained using the concentrations appropriate to the study of high affinity sites.

Specific receptors are often referred to as the "true receptors" because, when bound to steroid, they are capable of moving from the cytoplasm into the nucleus of a cell where they may change the pattern of gene expression (Salhanick and Callard, 1979). By definition, nonspecific receptors cannot enter the nucleus and so are restricted to the cytoplasm. This distinction between specific and nonspecific receptors may serve to indirectly regulate the amount of a given hormone which is available for binding to specific receptors, and ultimately to regulate the degree of responsiveness of a cell to the hormone (Clark and Peck, 1979).

Androgen Receptors

The androgen-resistant mutant mouse strain known as testicular feminization (Tfm) has been used to study androgen binding in hypothalamic-preoptic area tissues. These mice display 5-15% of the androgen binding capacity of their wild-type counterparts (Fox, 1975, 1977). The drastically reduced level of androgen binding seen among the Tfm mice is consistent with the phenotypic androgen resistence of the strain, and, therefore, has made them useful as a genetic control for receptor studies (Fox, 1975).

To determine if only reduced numbers of receptors were responsible for the characteristic decrease in androgen binding among Tfm mice, Fox (1975) studied androgen binding in the presence of excess testosterone *in vitro*. Alternatively, it was hypothesized that diminished binding

may also have resulted from an abnormally low steroid affinity rather than a decrease in receptor number alone. However, no increase in androgen binding was detected when these assays were conducted using a combination of column chromatography and sucrose gradient sedimentation. Rather, binding assays conducted in the presence of excess testosterone or estradiol implicated a binding component with a high affinity for estradiol only, and another component with a high affinity for both androgens and estrogens. As a result, receptor action was thought to be under the influence of relative concentrations of androgens and estrogens.

Androgen and estrogen receptors from wild-type and Tfm mouse hypothalamic-preoptic area tissues may be distinguished from one another on the basis of their adherence to DNA-cellulose columns, and by their column elution profiles (Wieland et al., 1978). Furthermore, the column elution patterns of mutant and wild-type receptors bound to DNA are tissue dependent and, therefore, may not be transferred from the cytosol of one genotype to another (Wieland and Fox, 1979). In other words, the phenotypic expression of the mutant genotype was mediated not only by a reduction in numbers of receptors, but also by the inherent properties of the receptor molecule.

In addition to their focus on receptor characteristics, Wieland and Fox (1979) recognized that androgen metabolites may play an active role in eliciting an androgen response. Upon observing differences in elution patterns among wild-type receptors bound either to testosterone

or to the testosterone metabolite, dihydrotestosterone, they proposed two mechanisms of receptor hormone action. One mechanism requires that a particular receptor will bind a restricted type of hormone structure to initiate a unique biological response. The other mechanism permits a class of receptors to bind more than one hormone structure and become activated according to the bound hormone. The latter interpretation considers that the activity of a receptor-steroid complex is implicit within the ligand structure while the former possibility considers activity to be dependent upon the nature of the receptor.

Estrogen Receptors

Using a gel filtration assay (Ginsburg et al., 1974), Ginsburg et al. (1975a) found that castration of rats increased the ratios of available estradiol binding sites in the anterior versus mid to posterior hypothalamus, and in the amygdala versus the pituitary. These researchers explained their findings by pointing out that the primary sites of androgen aromatization are the anterior hypothalamic and limbic regions, and that in intact males, estrogens are more actively synthesized from androgen precursors. A larger proportion of the total number of estradiol binding sites would be occupied in the intact male and would be unavailable for in vitro binding. Although the number of estradiol binding sites varied with the physiological status of the animal (gonadectomized males, or cycling or ovariectomized females), steroid binding affinity remained constant (Ginsburg et al., 1975b;

Barley et al., 1977). In a later report, Ginsburg et al. (1977) discussed the possibility of estradiol receptor heterogeneity. That is, although all estradiol receptors in various brain extracts were estradiol selective, not all displayed the same binding affinities for the various estrogens and anti-estrogens.

Fox and Johnston (1974) and Fox (1977b) identified estradiol receptors in cytosols of mouse brain by DNA-cellulose affinity chromatography. The isolated cytosol receptors had a high affinity but a limited capacity for estradiol, and were capable of converting to a heavier nuclear form in the presence of DNA (Fox, 1977a). Adult-like estradiol receptors were isolated from the embryonic mouse brain as early as four days prior to parturition (Vito and Fox, 1979). The ontogeny of these cytosol proteins has been discussed (Fox, et al., 1978).

Although the literature contains a number of reports describing estrogen binding in uterine tissue, only a few studies involving brain tissues have been reported. This is because, in comparison to uterine tissue, the number of cytosol estrogen receptors in brain tissues is known to be very small. For example, 100 mg of rat hypothalamic tissue has been found to contain approximately 0.5 picomoles of estrogen receptor as compared to 5.92 picomoles in 100 mg of uterus (Clark and Peck, 1980). The relatively small concentration of estrogen receptor combined with the characteristically high lipid content of brain tissue has made it very difficult to accurately study steroid

binding (Kelner et al., 1980). The latter researchers (1980) have, however, developed a method to circumvent these problems when studying brain nuclear estrogen receptors. As a result, they were able to study nuclear estrogen binding in both hypothalamus and pituitary (Kelner et al., 1980; Kelner and Peck, 1981). In particular, they were able to show a correlation between nuclear estrogen receptor numbers and RNA polymerase activation in rat hypothalamus (Kelner et al., 1980).

JUSTIFICATION

Although the work of Adkins and co-workers provides convincing evidence that the aromatization of androgens to estrogens is required for the expression of masculine sexual behavior in Japanese quail (Adkins, 1977; Adkins and Pniewski, 1978; Adkins, 1978), a question still remains regarding in what capacity aromatization is required. That is, does the pattern of androgen metabolism restrict male mating behavior or is the availability and/or efficiency of steroid-receptor action restrictive? The purpose of the present study was to examine the relative importance of androgen aromatization and estradiol-receptor interactions to the mediation of male mating behavior in lines of Japanese quail selected bi-directionally for adult mating frequency. In this study, the line difference in mating frequency was used as evidence that some physiological change had occurred to alter the observed behavior. By comparing the selected lines of quail it was possible to assess whether certain speculated neuroendocrinological changes have indeed resulted from selection for mating behavior.

Experiments with both chickens (McCollum et al., 1971; Jones, 1974) and quail (Cunningham et al., 1977; Balander, 1978) that have been selected bi-directionally for mating behavior have shown that endogenous androgen titers were not responsible for the difference in observed mating behavior. In these experiments, injections of graded levels of testosterone into caponized males failed to raise the mating frequency of the Low Mating (LM) lines up to the level of the High Mating (HM)

lines. Neonatal injections of testosterone also had no effect on subsequent mating behavior in the selected lines of chickens (Benoff, 1979).

Further evidence with chickens suggests that line differences in mating frequency do not stem from a differential ability of the hypothalamus to concentrate testosterone or its metabolites (Benoff et al., 1978). Thus, neither endogenous androgen titers nor the ability of the hypothalamus to concentrate androgens appear to be responsible for the line difference in mating frequency. Instead, Balander (1978) has suggested that selection for mating frequency has affected the capacity for aromatization in the LM line quail. In his experiment, testosterone propionate injections stimulated mating in only the HM line birds, but mating was stimulated in both HM and LM line birds injected with 5 mg/d estradiol benzoate. Nevertheless, these data do not preclude the possibility that selection for mating frequency has altered the physical properties of the steroid receptors themselves. In mice, for instance, steroid receptors of the Tfm mutant are modifications of the wild-type receptors. To resolve this issue, the present study has been conducted to investigate the relative importance of androgen metabolism pattern and estradiol-receptor interactions to the regulation of quail sexual behavior. It was felt that such a study was necessary to further elucidate the mechanism by which gonadal steroids mediate the expression of male mating behavior in the HM and LM line Japanese quail.

EXPERIMENT I

A Comparison of the *In Vivo* Metabolism of ^{3}H-Testosterone in Brain Tissues of High and Low Mating Line Japanese Quail

INTRODUCTION

The significance of testosterone metabolism in the mediation of masculine sexual behavior in birds has been securely established (Barfield, 1979; Adkins-Regan, 1981). However, the specific mechanism(s) by which testosterone metabolism mediates sexual behavior is yet unknown. A system requiring the aromatization of testosterone to estradiol (Adkins, 1977; Adkins and Pniewski, 1978; Adkins, 1978; Balander, 1978; Adkins *et al.*, 1980; Steimer and Hutchison, 1980; Adkins-Regan, 1981), possibly in conjunction with the reduction of testosterone to dihydrotestosterone (Balthazart and Hirschberg, 1979; Massa *et al.*, 1979; Hutchison and Steimer, 1981), has been implicated in numerous behavioral and *in vitro* studies. While these studies have provided strong support for a regulatory mechanism which is dependent upon relative rates of testosterone aromatization, the evidence is nevertheless indirect. Direct evidence must be obtained by relating *in vivo* patterns of testosterone metabolism to the expression of varying degrees of sexual behavior. The availability of a genetic control for masculine mating behavior in Japanese quail provides a unique opportunity to study this relationship. Therefore, Experiment I has been designed to compare the *in vivo* metabolism of exogenous ^{3}H-testosterone in brain tissues of replicate High and Low Mating line Japanese quail.

MATERIALS AND METHODS

Adult male Japanese quail from replicate High (HM) and Low Mating (LM) lines were bi-directionally selected for cumulative numbers of completed matings for 24 generations (Sefton and Siegel, 1975; Cunningham and Siegel, 1978). At approximately five months of age, 12 males from each of the four mating lines (HM_1,LM_1,HM_2,LM_2) were functionally castrated by two weeks of exposure to a 3:21 hour light:dark photoperiod. Functional gonadectomy was confirmed by the absence of any proctodeal foam gland secretion and by a post-mortem assessment of testicular regression. The experimental protocol was adapted from that of Lieberburg and McEwen (1977).

Birds were weighed and injected in the thigh muscle with 75 μCi (900 ng) of 7-^{3}H-testosterone dissolved in 0.5 ml of a 25% ethanol-saline solution. After one hour of *in vivo* incubation (Benoff *et al.*, 1978), the injected quail were decapitated, the brain exposed, and a uniform block of telencephalic and diencephalic tissue (i.e., cerebral cortex, epithalamus, thalamus, and hypothalamus) was isolated. A coronal cut, made at the level of the posterior commissure, established the posterior boundary. The optic lobes were removed establishing the lateral boundaries and the optic chiasma and pituitary were removed establishing the ventral boundary. The tissue was minced with a scalpel and homogenized in 0.05 *M* ice-cold phosphate buffer (0-4 C, pH 7.55) with a Polytron Pt-10 (Brinkman, Westbury, NY). These homogenates were held frozen until the time of analysis.

At the time of analysis, three 1.5 ml aliquots of homogenates (one from each of three birds of the same sire) were combined into a single test tube as an experimental unit. To this, equal volumes of ^{14}C-testosterone, ^{14}C-dihydrotestosterone, and ^{14}C-estradiol were added as internal standards (9860, 6408, and 10,593 dpm, respectively). All subsequent steps are shown in detail in Figures I.1 and I.2.

Figure I.1 reflects two major modifications on the original protocol of Lieberburg and McEwen (1977). They are: (1) The addition of Step II, the partial purification of the total lipid extract by column chromatography using silicic acid (60/100 mesh, Supelco, Bellefonte, PA), and (2) the omission of estrogen methylation prior to thin layer chromatography (TLC). These modifications were required because of the uniqueness of the quail brain and the particular laboratory environment.

The addition of Step II resulted in a 20% increase in recovery of ^{14}C-estradiol when it was added as an internal standard to an unlabeled test homogenate. Addition of this step was found to be necessary due to the high fat content of the quail brain. It was felt that some non-steroidal lipids interferred with the sodium hydroxide extraction phase by obstructing contact between sodium and estrogen, thereby preventing the formation of estrogen salts. Ultimately this would have created a problem because testosterone and estradiol migrate together during thin layer chromatography and, therefore, any estradiol remaining in the toluene phase could not be distinguished from testosterone. The

end result would be an underquantitation of estradiol and an overquantitation of testosterone. Recovery estimates obtained by this method of adding ^{14}C-steroids to unlabeled test homogenates were 46% testosterone, 57% dihydrotestosterone, and 68% estradiol. Contamination by estradiol in the androgen fraction was 10%, and contaminations by testosterone and dihydrotestosterone in the estrogen fraction were 7% and 6%, respectively. These values represent hormone recoveries before thin layer chromatography.

The methylation of estrogens prior to thin layer chromatography was found to be unnecessary and was omitted from the current protocol. In the original procedure, the synthesis of estrogen methyl esters was necessary to achieve sufficient chromatographic separation of estrone (E_1), estradiol (E_2), and estriol (E_3) (personal communication with B. S. McEwen, 1980). Under the present conditions, adequate separation was achieved by thin layer chromatography on silica gel plates (Eastman Kodak, Rochester, NY) using two developments in chloroform:benzene:ethyl acetate:methanol 65:20:13:2 (vol/vol).

Following hormone separation by thin layer chromatography, a 0.5 cm grid was carefuly penciled on the silica gel plate and several lanes containing the unlabeled steroids, estrone, estradiol, and estriol (or testosterone, androstenedione, dihydrotestosterone, androstanedione, androstanediol, and androsterone for the separation of androgens) were cut from the plate and charred with a mixture of sulfuric acid:methanol 50:50 (vol/vol). In this manner, the individual steroids could be

identified according to 0.5 cm zones and the information extrapolated to the remainder of the plate. The silica gel from each lane was then scraped into mini-scintillation vials according to the standards and counted for radioactivity. The samples were counted for five minutes using a toluene based scintillation fluid in a Beckman LS-133 scintillation counter at 38% efficiency for ^{3}H and 66% efficiency for ^{14}C.

The protocol for the extraction of protein and DNA is shown in Figure I.2. It represents a combination of the methods presented by Lieberburg and McEwen (1977) and Schneider (1957). As indicated, protein and DNA were quantitated by the methods of Lowry (1951) and Burton (1956), respectively.

7-^{3}H-Testosterone (25.0 Ci/mmol), 4-^{14}C-testosterone (51.9 mCi/mmol), 4-^{14}C-dihydrotestosterone (51.75 mCi/mmol), and 4-^{14}C-estradiol (57.0 mCi/mmol) were obtained from New England Nuclear (Boston, MA) and stored in benzene:ethanol 9:1 (vol/vol) at 4 C until needed. At that time, they were evaporated under a stream of nitrogen and reconstituted as follows: ^{3}H-testosterone in a 25% ethanol-saline solution; and ^{14}C-testosterone, ^{14}C-dihydrotestosterone, and ^{14}C-estradiol in toluene. Prior to their use, all radioactive steroids were checked for purity by the thin layer chromatography system described above and were found to be at least 96% pure. Unlabeled steroids were obtained from Steraloids (Wilton, NH) and held refrigerated. All solvents and reagents were of analytic or reagent

grade, and were obtained from Fisher (Raleigh, NC) or Mallinckrodt (St. Louis, MO). Those reagents which were used in the preparation of the thin layer chromatography mobile phase were freshly distilled prior to use and discarded after each run.

Data were adjusted to a body weight norm of 100 g/bird and expressed as picomoles of radioactive hormone per milligram of protein or microgram of DNA. Differences between the mating lines were assessed by single degree of freedom contrasts.

RESULTS AND DISCUSSION

All radioactivity recovered from the thin layer chromatography plates migrated together with the ^{14}C-testosterone, ^{14}C-dihydrotestosterone, or ^{14}C-estradiol internal standards. No other testosterone metabolites were detected. Recoveries of the ^{14}C internal standards were 47 $\pm$ 4% testosterone, 36 $\pm$ 3% dihydrotestosterone, and 47 $\pm$ 3% estradiol (mean $\pm$ SEM, n=16). The data are presented in Table I.1 and the corresponding analysis of variance tables are presented in Appendix Tables B.1-B.3.

In regard to the total amount of radioactivity recovered, no significant differences were found among the mating line quail (Table I.1). These results are consistent with those of Benoff *et al.* (1978) who found no differences in ^{3}H-testosterone uptake among mating line cockerels after 1 hr of *in vivo* incubation. The amount of metabolite radioactivity also did not differ among the lines, thus indicating that both LM and HM line birds have a similar capacity to metabolize testosterone (Table I.1).

In addition, the lines did not differ in the amount of estradiol synthesized as a percent of all metabolite radioactivity (Table I.1). This observation is of considerable importance in that it shows that not only do the LM line birds have the ability to aromatize testosterone, but, moreover, the pattern of testosterone metabolism was not different from that of the HM line birds. Therefore, these data indicate that the selection for mating frequency has not affected the capacity for

aromatization in the LM line quail as Balander (1978) had suggested. Furthermore, because all testosterone metabolite radioactivity was equally divided between estradiol and dihydrotestosterone, the data show that the injected label was aromatized to estrogen and reduced to dihydrotestosterone, equally. Hence, after one-hour of in vivo incubation, neither testosterone aromatization nor testosterone reduction takes precedence for either of the mating lines.

The implications of these results are far-reaching in view of the current status of the literature. That is, some controversy over the dependency of masculine sexual behavior on the aromatization of androgens has recently been expressed in the literature so that two basic theories have been espoused. They are: (1) That the aromatization of androgens is essential for the activation of copulation in male quail (Adkins et al., 1980; supported by Steimer and Hutchison, 1980, with studies of the male dove), and (2) that the structure of an androgen, rather than its ability to undergo aromatization, determines its behavioral effectiveness (Yahr and Gerling, 1978, studies on rats). Perhaps the most popular tenet represents a melding of the two basic theories - that both aromatizable and nonaromatizable androgens play a CNS role in the mediation of masculine sexual behavior (Balthazart and Hirschberg, 1979, studies with chickens; Balthazart et al., 1979, and Massa et al., 1979, studies with quail; Landau, 1980, studies with rats; Hutchison and Steimer, 1981, studies with doves). One specific mechanism which has been proposed is that the 5β-reduction of androgens

might serve as an "inactivation shunt" to prevent the conversion of testosterone into more behaviorally active forms including 5α-dihydrotestosterone, 5α-3α-androstanediol, and androstenedione (Massa et al., 1979), and possibly also to compete with the binding of testosterone to receptors (Hutchison and Steimer, 1981).

In the present study, the LM line quail aromatized as much of the injected ^{3}H-testosterone as did the HM line quail (Table I.1). This suggests that the difference in level of mating frequency between the two lines is not due to a corresponding difference in aromatase activity. This observation, together with the large variation which was observed among individual birds, suggests that a mechanism more subtle than aromatization alone, is responsible for the difference in level of mating frequency. One possible mechanism controlling sexual behavior might depend upon relative patterns of in 5α- and 5β-reduction, as previously suggested by Massa et al. (1979), and Hutchison and Steimer (1981). With the thin layer chromatography system which was used to separate ^{3}H-testosterone from its metabolites, it was not possible to distinguish between the 5α- and 5β-isomers of dihydrotestosterone which were synthesized in vivo. Therefore, if the relative patterns of 5α-and 5β-reduction differed between the mating lines, this difference would have been masked.

An alternative mechanism, which may be regarded as an extension of the aromatization theory, depends upon the availability and/or estrogen binding affinity of specific receptors in the central nervous system.

The latter approach is particularly attractive because it is consistent with the findings of Adkins-Regan and co-workers (see Adkins-Regan, 1981 for a review). Their extensive studies provide convincing evidence that the aromatization of testosterone to estradiol is indeed required for the activation of copulatory behavior in male quail.

In summary, Experiment I has shown that the differences in mating frequency between divergent lines of Japanese quail cannot be attributed to their ability to aromatize ^{3}H-testosterone. In view of the current literature, at least two alternative mechanisms merit further consideration. They are: (1) That the relative patterns of 5α- and 5β-reductase activity represent the mechanism by which testosterone metabolism mediates masculine sexual behavior, or (2) that the availability and/or efficiency of estrogen-receptor action serves to regulate the behavior. This latter mechanism was the objective of investigation in Experiment II.

Table I.1 Total and ^{3}H-testosterone metabolite radioactivity corrected for body weight in telencephalic-diencephalic homogenates one hour after ^{3}H-testosterone injection.[1]

(A) Mean ± SEM of four observations per replicate mating line.

Line	Body Wt. Factor	Protein (mg)	Picomoles/mg protein		DNA (μg)	Picomoles/μg DNA		E_2 % of all
			Total[2]	Metabolite[2]		Total[2]	Metabolite[2]	Metabolite[2]
HM_1	1.01 ± .03	9.4 ± .6	61 ± 22	33 ± 9	379 ± 17	1.4 ± .5	.8 ± .2	50 ± 11
HM_2	1.05 ± .02	10.4 ± 1.0	75 ± 22	44 ± 14	526 ± 115	1.7 ± .6	1.0 ± .4	39 ± 7
LM_1	1.06 ± .03	10.2 ± .7	36 ± 9	26 ± 8	422 ± 49	1.0 ± .2	.7 ± .2	56 ± 7
LM_2	.92 ± .01	10.6 ± .6	95 ± 15	55 ± 8	390 ± 58	2.7 ± .5	1.6 ± .2	36 ± 8

(B) Mean ± SEM by combining replicate mating lines.

H	1.03 ± .02	9.9 ± .6	68 ± 15	38 ± 8	453 ± 60	1.6 ± .4	.9 ± .2	45 ± 6
L	.99 ± .03	10.4 ± .4	65 ± 14	41 ± 8	406 ± 36	1.8 ± .4	1.1 ± .2	46 ± 6

[1]Values reflect multiplication by a normalizing body weight correction factor, computed as 100/g body weight.

[2]No significant single degree of freedom comparisons were found for HM_1 vs LM_1, HM_2 vs LM_2, or HM vs LM.

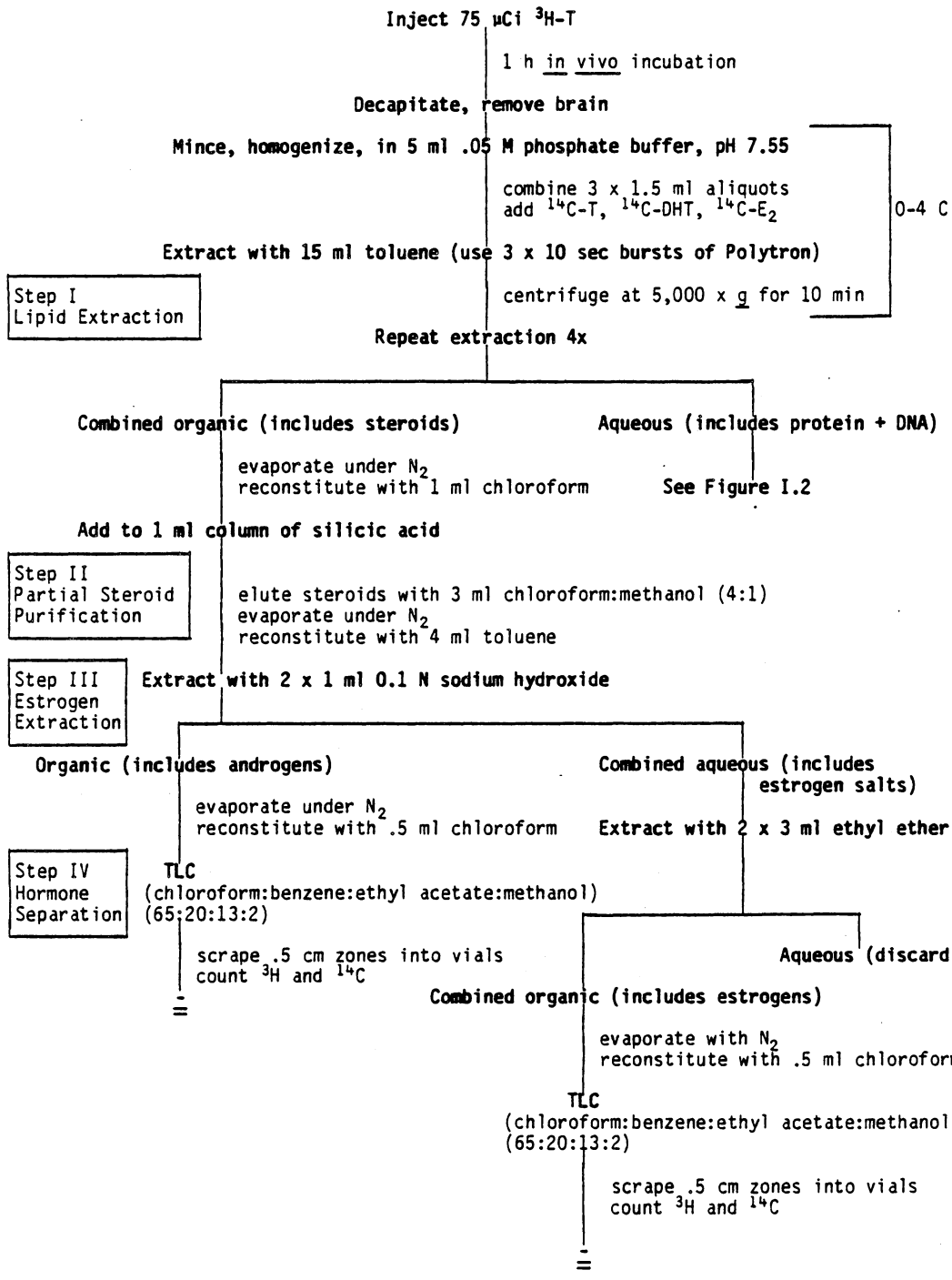


Figure I.1. Protocol for hormone extraction and separation (modified from Lieberberg and McEwen, 1977).

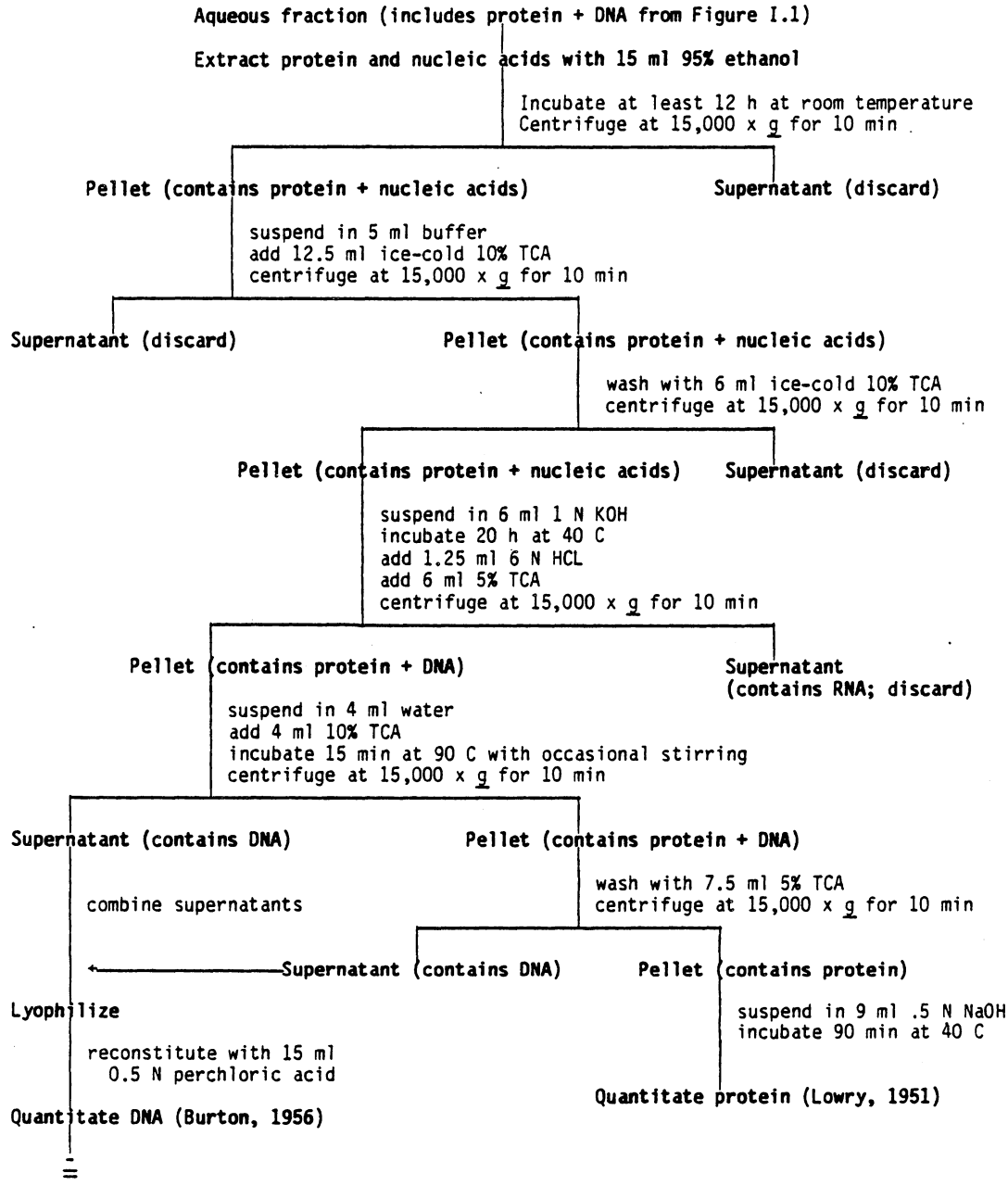


Figure I.2. Protocol for protein and DNA extraction and separation (modified from Lieberberg and McEwen, 1977; and Schneider, 1957).

EXPERIMENT II

A Comparison of *In Vitro* Estradiol-Receptor Binding in Diencephalic Cytosols from High and Low Mating Line Japanese Quail

INTRODUCTION

The results of Experiment I indicated that frequency of sexual behavior was not correlated with aromatase activity. Nevertheless, Experiment I provided the first direct demonstration of aromatization in brain tissues of Japanese quail. Inasmuch as both the High (HM) and Low Mating (LM) lines actively converted testosterone to estradiol, another possible neuroendocrinological mechanism to explain the genetic divergence involves estradiol receptors. Such a mechanism would be consistent with the theory that estrogens rather than androgens are responsible for the expression of masculine sexual behavior in quail (Adkins-Regan, 1981).

A regulatory mechanism involving estrogen receptors is further suggested by the observation that anti-estrogens inhibit copulation (Adkins and Nock, 1976). By virtue of their specificity for cytosolic estrogen binding sites, anti-estrogens compete with estrogens, thereby reducing estrogen binding. To determine if the amount and/or efficiency of cytosolic estradiol binding could explain the difference in mating frequency between the two lines, Experiment II was conducted to compare estradiol binding parameters between the HM and LM lines.

The rationale for this experiment was based upon the presumption that estrogen action in quail brain tissues is dependent upon a system involving cytosol receptors, as has been described for the rodent (Ginsburg et al., 1975b; Fox, 1977a; Clark and Peck, 1979). To date, studies of brain steroid receptors in avian tissues have been limited to

progesterone receptors in laying chickens (Kawashima et al., 1978, 1979a, b, c). However, studies comparing androgen and estrogen receptors from the Testicular Feminization (Tfm) mutant mouse to the wild-type mouse have revealed a correlation between receptor nature and genotype (Wieland and Fox, 1979). This observation suggests that an analogous correlation may also exist between estrogen receptors from HM and LM line brain tissues.

MATERIALS AND METHODS

Diencephalic tissue from functionally castrated adult male Japanese quail of the S_{24} and S_{26} generations of the mating lines (HM and LM described in Experiment I) were used in this study. Birds were decapitated, the brain exposed, and a uniform block of diencephalic tissue (i.e., epithalamus, thalamus and hypothalamus) was isolated. Cornonal cuts made at the level of the anterior and posterior commissure established the anterior and posterior boundaries, respectively. The optic lobes were removed establishing the lateral boundaries and the optic chiasma and pituitary were removed establishing the ventral boundary. The isolated tissue was immediately placed in ice-cold Tris-EDTA buffer (10 mM Tris-HCL, 1.5 mM EDTA; pH 7.4 at 2 C).

Total diencephalic estradiol receptor was assayed by competition studies using the protamine sulfate precipitation method of Clark and Peck (1979). Saturation analyses were carried out over a wide range of steroid concentrations both in the presence and absence of the competitive inhibitor, diethylstilbestrol (DES). In short, aliquots of cytosols from diencephalic homogenates containing receptor were incubated with radiolabeled estradiol alone or in combination with diethylstilbestrol. Following incubation, all proteins including the bound receptors were precipitated with protamine sulfate and any unbound label decanted. Bound label was then eluted from the pellet and counted for radioactivity. The amount of bound label was taken to be an indirect measurement of the amount of estradiol-receptor.

The above method, previously validated for rat studies, was chosen for its ability to detect specific receptor-steriod binding complexes in the presence of large numbers of nospecific binders (E. J. Peck, Jr., personal communication). The quantity of nonspecific binding in neural tissues is, in general, very high relative to the total amount of binding (ibid). Therefore, the application of this method to the study of diencephalic estradiol receptors was considered to be essential.

Although receptor systems in like tissues obtained from different species of animals tend to be similar, the characteristics of each system are unique. For this reason, the protocol already validated for rat hypothalamus could not be assumed to be exactly the same for quail. Furthermore, due to the extreme labile nature of receptor proteins, the optimum conditions for their measurement within any particular system may differ for each laboratory environment. Thus, it was necessary to establish the optimum assay conditions for the present study (Appendix C). The experimental assay procedure which evolved from the preliminary work described in Appendix C follows.

Five saturation analyses were conducted, each with up to 40 diencephalons from mating line quail castrated for an equal number of days. The quail had been functionally castrated for (1) 15 or 16 days, or (2) 24 or 30 days at the time of assay. Diencephalons were pooled in pairs and homogenized in 1 ml ice-cold Tris-EDTA buffer (pH 7.4 at 2 C) with 10 strokes of a Kontes ground glass homogenizer. The homogenates

were combined and centrifuged at 31,800 x g for 30 min at 0-4 centigrade. After centrifugation, the high-speed supernatant (containing cytosol) was diluted to a total volume of 11 ml (5.5 ml for data shown in Figure II.7) with the ice-cold Tris-EDTA buffer.

Aliquots (250 µl) of the diluted cytosol were added to duplicate pairs of 12 x 75 mm plastic culture tubes containing graded amounts of (2,4,6,7,16,17-^{3}H)-estradiol in 200 µl of buffer. For each assay tube, the amount of ^{3}H-estradiol contained per 200 µl of buffer was calculated such that the final (450 µl) concentrations ranged from within 0.2 to 7.0 nM depending upon the individual assay. Appropriate adjustments were made to the ^{3}H-estradiol solutions so that one member of each pair of tubes contained a 100-fold molar excess of diethylstilbestrol (Steraloids, Wilton, NH) for the estimation of nonspecific binding.

After mixing, the tubes were incubated in a 25 C water bath for 60 minutes. Incubation was terminated by chilling the tubes in an ice-water bath for 10 min, after which time 450 µl of ice-cold protamine sulfate (1 mg/ml buffer; Sigma, St. Louis, MO) was added to each tube, followed by mixing. After 15 min of protein precipitation with protamine sulfate, the tubes were diluted to capacity with ice-cold wash buffer (Tris-EDTA + 1% Tween-80) and centrifuged for 15 min at 5,000 x g using a Beckman J-14 swinging bucket rotor at 0-4 centigrade. The supernatant containing unbound ^{3}H-estradiol was decanted and an additional 2 ml of ice-cold wash buffer was added to each tube. The pellets were washed by agitation and recentrifuged as before. The

supernatant was decanted and all residual fluid drained by inverting the tubes on a paper towel.

Bound steroid was eluted from the protamine-sulfate-protein pellet by incubation with 2 ml of scintillation fluid composed of toluene: dioxane:methanol:napthalene:PPO:POPOP (36.2:35:21.7:7.3:4.6:0.08 wt/vol) for 15 min at 40 C, followed by mixing. The scintillation fluid containing the eluted steriod was poured into mini-scintillation vials and the tubes rinsed with an additional 3 ml of scintillation fluid, which, in turn, were added to the mini-vial. Each vial was counted for ^{3}H for 20 min (resulting in a 1-5% counting error) in a Beckman LS-133 scintillation counter at 29% efficiency.

Saturation curves of nonspecifically bound steroid versus free steroid were constructed by simple linear regression using the model:

$$y = mx + \xi \text{ where } \xi \text{ follows } N(o,\sigma^2).$$

Saturation curves of total binding were constructed by the nonlinear regression method of Marquardt (1963) using algorithms generated by SAS (Helwig and Council, 1979) from the model:

$$y = mx + \frac{\alpha x}{x+\beta} + \xi \text{ where } \xi \text{ follows } N(o,\sigma^2).$$

and m was substituted from the nonspecific equation above. The resulting parameter estimates were confirmed upon comparison to those obtained with the Gauss-Newton and Steepest Descent Methods (Appendix Table D.11).

Saturation curves of specific binding were obtained from the difference between total and nonspecific curves, that is, $y = \frac{\alpha x}{x+\beta}$.

Estimates of the dissociation constant (K_d) and number of receptors (B_{max}) were obtained from Scatchard Transformation (1949) of the specific binding curve (Figure II.1), and the significance of each of these parameter estimates was determined by Student's t-test. Validation of this method of parameter estimation has been described in Appendix D.

Receptor concentrations were expressed per milligram cytosol protein where cytosol protein content was determined by the method of Lowry (1951) using bovine serum albumin as a standard. All receptor assays were conducted using the same stock solution of ^{3}H-estradiol dissolved in absolute ethanol. A concentrated solution of (2,4,6,7,16,17-^{3}H)-estradiol (147 Ci/mmol) in toluene:ethanol 9:1 (vol/vol) was initially obtained from Amersham (Arlington Heights, IL). Immediately upon its receipt, the contents of the vial were evaporated under a stream of nitrogen and reconstituted in absolute ethanol. This solution was found to be at least 94% pure using the thin layer chromatography system described in Experiment I.

RESULTS AND DISCUSSION

15 and 16-day functional castrates

Results of saturation analyses conducted with HM_2 and LM_2 line tissues are shown in Figures II.2 and II.3, respectively. Corresponding analysis of variance tables have been included in Appendix Tables D.1 and D.2. As functional castrates, the quail had been subjected to a 3:21 light:dark photoperiod for either 16 days (HM_2) or 15 days (LM_2). For the HM_2 line quail, an apparent K_d of 0.40 $\pm$ .06 n$\underline{M}$, and a B_{max} of 10.4 $\pm$ .4 fmoles/mg protein were obtained. Using Student's t-test, both estimates were found to be significantly different from zero for $P \leq .001$ (Appendix Table D.1), indicating that the data were fit to an appropriate statistical model which represented a single-class, specific binding site system. For the LM_2 quail, an apparent K_d of 0.41 $\pm$ .20 n$\underline{M}$, and a B_{max} of 9.5 $\pm$ 1.0 fmoles/mg protein were obtained. As shown in Appendix Table D.2, significance levels for the LM_2 estimates were $P \leq .001$ for the K_d, and $P \leq .06$ for B_{max}. Again, it was concluded that the data were fit to an appropriate statistical model.

Comparisons of the HM_2 and LM_2 estimates for K_d and B_{max} values were not significant ($P > .1$), indicating that diencephalic cytosol receptors of both mating lines bind estradiol with equal affinity and equal capacity. In order to substantiate the experimental rationale for using diethylstilbestrol as a competitive inhibitor of estradiol binding, and thereby rule out the possibility of cooperative interaction

between binding sites, double reciprocal plots (Figure II.4) were constructed from the total and nonspecific estradiol binding data presented in Figures II.2 and II.3. In both the HM_2 and LM_2 line double reciprocal plots, addition of diethylstilbestrol resulted in an increased estimate for K_d without a corresponding alteration in B_{max}. By reflecting the mutually exclusive nature of ligand binding to estradiol receptors, these results implied that diethylstilbestrol acted as a competitive inhibitor of estradiol binding (Clark and Peck, 1979). Thus, the possibility of cooperativity between adjacent sites was ruled out, and the inclusion of diethylstilbestrol was considered to be a valid method for in vitro estimation of nonspecific estradiol binding in quail diencephalon. As a result, the similarity between corresponding binding parameters for the divergent mating lines was interpreted to mean that neither receptor concentration nor estradiol binding affinity were responsible for the line differences in mating frequency.

Though no line differences were detected from the HM_2 and LM_2 line binding studies, these results demonstrate the presence of cytosol estrogen receptors in avian brain. In addition, the data provide the initial report of avian brain cytosol estrogen binding parameters. Previously, Clark and Peck (1979) suggested that avian brain tissues might not contain cytosol estrogen-receptors because avian blood estrogen levels are higher than mammalian levels (nM versus pM) and, if cytosol receptors function as a cellular reservoir for estrogens, the advantage conferred by cytosol receptors may be superflous in avian

brain tissues.[1] However, the interpretation of a species-dependent presence of cytosol estrogen receptors becomes doubtful upon comparison of avian and mammalian protein-normalized receptor concentrations. That is, an average concentration range of 9.5 to 10.4 fmoles receptor/mg cytosol protein was obtained in the present report, as compared to a reported 4.7 to 6.0 fmoles/mg protein for intact rat hypothalamus (Cidlowski and Muldoon, 1976).

On the other hand, Clark and Peck (1979) have reported a cytosol concentration of 25 fmoles of receptor/rat hypothalamus in comparison to an average value of 4 fmoles/quail diencephalon for the present report. Therefore, when presented on a target tissue basis, receptor concentrations appear to be much higher in the rat hypothalamus than in the quail diencephalon, but appear to be lower on a cytosol protein basis. If tissue size alone were responsible for the observed differences in tissue receptor levels, a proportional reflection in cytosol protein level would also be expected. These results could be interpreted to mean that the nonspecific binding proteins function more as a cellular hormone reservoir than do the specific binding proteins. Furthermore, because estimates for the quail estradiol-receptor K_d were of similar magnitude to published figures for both male and female rat

[1]Though the authors were referring to female blood levels, nanomolar blood levels of estradiol have also been found in 17 day male chick embryos (Woods and Brazzill, 1981).

hypothalamic receptors (on the order of 10^{-10} M, Ginsburg et al., 1974; Ginsburg et al., 1975; Barley et al., 1977; Clark and Peck, 1979), the quail receptor species appears to chemically resemble the rat receptor in spite of their tissue concentration differences. This implies an inter-species similarity of steroid-receptor nature, a point in agreement with Baulieu's (1979) suggestion that receptor-steroid interactions are qualitatively similar across species.

24 and 30-day functional castrates

When additional saturation analyses were conducted with mating line quail that had been exposed to a relatively prolonged restricted lighting regime of 24 days, virtually no specific binding was obtained. The data are plotted in Figures II.5 and II.6 and their corresponding analysis of variance tables are in Appendix Tables D.3 and D.4. As shown, the nonlinear parameter estimates for K_d and B_{max} were not significantly different from zero. These results indicate that the data were fit to an inappropriate statistical model, possibly because receptor tissue levels were below sensitivity of the assay procedure. That is, by 24 days of exposure to a restricted lighting regime, the gonads had been functionally inactivated long enough for receptor degradation to surpass steroid-induced receptor synthesis. Because the latter assays were conducted with tissues from only replicate mating line #1, the experiment was repeated with tissues from HM_2 line birds which had been subjected to the restricted lighting regime for 30 days

(Figure II.7). Whereas nonlinear parameters were successfully estimated using a single-class specific binding site model for HM_2 line birds castrated for only 15 days (Figure II.2), such estimates for K_d and B_{max} could not be formulated after 30 days of castration.

The inability to accurately measure receptor after 24 days of functional castration suggests the existence of a "shut-off" mechanism for synthesis of cytoplasmic receptors, i.e., the basal level of receptor which was present at 15 days. If the basal level that was measured at 15 days of castration was maintained by adrenal steroids, as suggested by Clark and Peck (1979), the absence of measurable receptor at 24 days may represent diminished adrenal sex-steroid production.

For the assays described in Figures II.5 and II.7, double reciprocal plots of total and nonspecific binding were constructed as before to rule out the possibility of cooperativity between adjacent binding sites and to establish that diethylstilbestrol did behave as a competitive inhibitor of estradiol binding (Figure II.8). Plots corresponding to Figure II.6 were omitted because total and nonspecific binding were equal for this data set and, therefore, their double reciprocal transformations would be superimposed. Both of the double reciprocal plots presented in Figure II.8 indicated the presence of competitive inhibition by showing that the addition of diethylstilbestrol resulted in a higher estimate for K_d (by the double reciprocal method) without causing a change in B_{max}. Thus, prolonged castration did not appear to alter the ligand-independent nature of

estradiol binding sites though it did result in quantitatively immeasurable levels of receptor. It is more likely that the failure of these assays to estimate receptor binding parameters was the result of reduced receptor synthesis in response to subthreshold steroid levels rather than a change in the receptor mechanism, per se. This interpretation would be consistent with the data on estradiol-receptor systems in rat neural tissues. The rat data indicate that estradiol receptors are inducible gene products and that receptor replenishment is dependent both on reactivation of nuclear receptors and de novo synthesis of cytoplasmic receptors (Cidlowski and Muldoon, 1974; Clark and Peck, 1979). Furthermore, that quail estradiol receptors represent an inducible gene product is consistent with the observation that testosterone insensitivity of long-term castrated doves was reversed by estradiol treatment (Hutchison and Steimer, 1981).[1]

Since only a single-class binding site model was evaluated in the present experiment, one other possibile characteristic of the quail estradiol receptor system merits some consideration. That is, in addition to the more familar "Type I" cytosol receptor discussed so far, the possibility exists that another specific receptor species, the Type II receptor, was also present in quail diencephalon. Like the Type I

[1]It should be noted that in the latter report, testosterone insensitivity was inferred from an increased level of 5β-reduction of testosterone, rather than from a behavioral assessment, per se. Also, Hutchison and Steimer considered long-term castrates to be those doves castrated for a 200-day period.

receptor, the Type II species is stereo-selective for estrogen. In contradistinction to the Type I receptor, the Type II species has a lower affinity, yet higher capacity for estradiol (K_d=33 nM versus 0.8 nM), displays cooperativity with respect to estradiol binding, and does not enter the nucleus even when bound to steroid (Eriksson et al., 1978; Clark and Peck, 1979).

To rationalize their inability to enter the nucleus, it has been suggested that, in addition to the nonspecific receptors, the Type II sites may serve to accumulate estrogens, intracellularly, to compensate for fluctuating blood estrogen levels (Clark and Peck, 1979). Such a function for Type II and nonspecific receptors could explain why Cunningham et al. (1977) and Balander (1978) could not raise the level of mating among LM line birds up to the level of their HM counterparts, following exogenous hormone treatment. The same reasoning would also explain the observation that brain tissues of HM and LM line chickens were able to concentrate equal amounts of ^{3}H-testosterone or its metabolites (Benoff et al., 1978).

In the present study, saturation analyses were designed to measure just Type I receptors because the Type II receptor has been associated only with non-CNS tissues wherein estrogens stimulate cellular hypertrophy and hyperplasia (Eriksson et al., 1978; Kelner and Peck, 1981). To measure Type I receptor, the present study involved ^{3}H-estradiol concentrations ranging from an average of 0.3 to 6.0 nM, whereas in rat brain, Eriksson et al., (1978) measured Type II estradiol

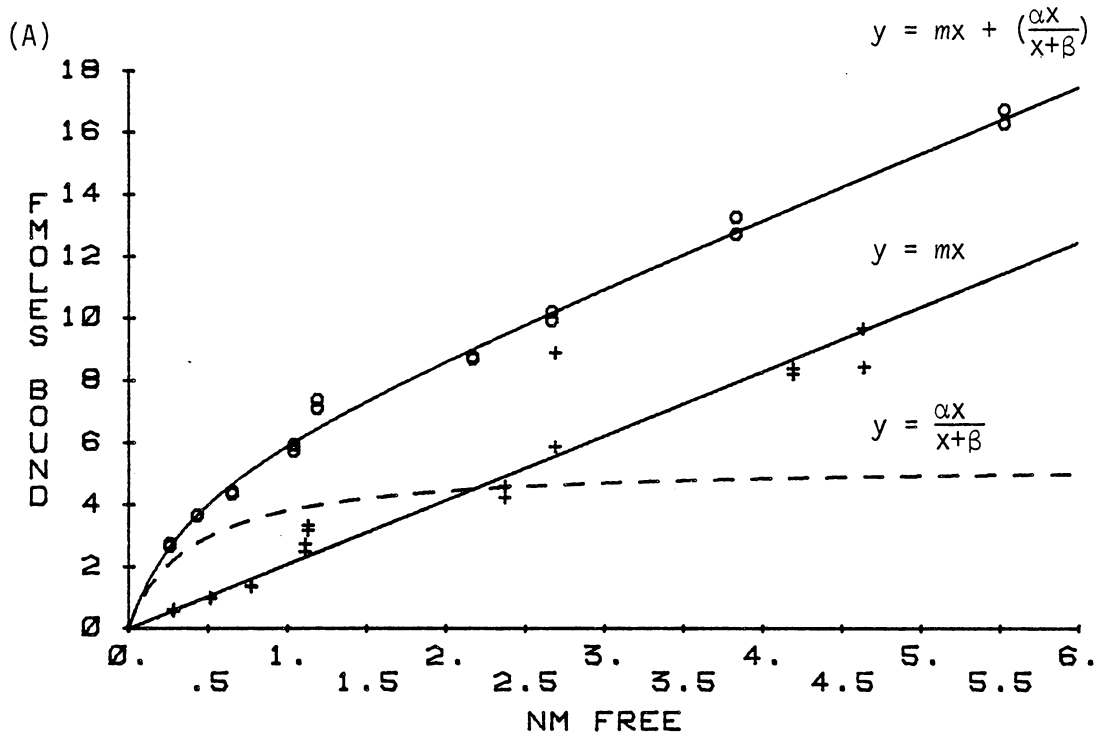
receptors using concentrations ranging from approximately 0.05 to 40.0 nanomolar. Owing to the relative natures of the two types of receptors, neither the concentration range nor the incubation conditions used in the present study, would accurately measure the Type II species. To test for the presence of Type II estradiol-receptors in cytosols from quail diencephalons, it would be necessary to repeat the saturation analyses using conditions appropriate for equilibration of the Type II species.

In summary, saturation analyses with diencephalic cytosols from HM and LM line quail indicate that the number and/or estradiol-binding affinity of a single-class of specific cytosol receptors was not responsible for the line difference in mating frequency. The observation that estradiol-receptors were present in neural tissues of both HM and LM quail suggests an intra-species similarity of steroid hormone action, regardless of genotype. The observation that the K_d for the quail neural estradiol-receptor resembles that of the rat neural receptor suggests an inter-species universality of steroid-receptor nature. That more subtle differences, such as column elution profile, have been identified for the Tfm mutant mouse in comparison to the wild-type mouse (Wieland et al., 1978; Wieland and Fox, 1979), suggests that more extensive biochemical comparisons between HM and LM line quail receptors are required. In addition, consideration must be given to the dynamic relationships between all possible types of specific estradiol receptors, both in the cytoplasm and nucleus of quail hypothalamic-preoptic area neurons.

Figure II.1. Method of parameter estimation for saturation analyses conducted in Experiment II.

A. Saturation curves of total (o—o) and nonspecific (+—+) ^{3}H-estradiol binding were plotted as shown by nonlinear least-squares regression of empirical data. The specific binding curve (-----) was obtained from the difference between total and nonspecific curves, $y = mx + \frac{\alpha x}{x+\beta} - mx = \frac{\alpha x}{x+\beta}$.

B. Estimates for the specific binding parameters, K_d and B_{max} were obtained directly from least-squares regression in Part A. As shown with reference to the traditional Scatchard Analysis, the parameter β represents the apparent K_d and the parameter α represents B_{max}. A mathematical proof for the transformation has been included in Appendix D.


(A)
y = mx + (αx/(x+β))
y = mx
y = αx/(x+β)
BOUND FMOL
NM FREE
0
2
4
6
8
10
12
14
16
18
0.
.5
1.
1.5
2.
2.5
3.
3.5
4.
4.5
5.
5.5
6.

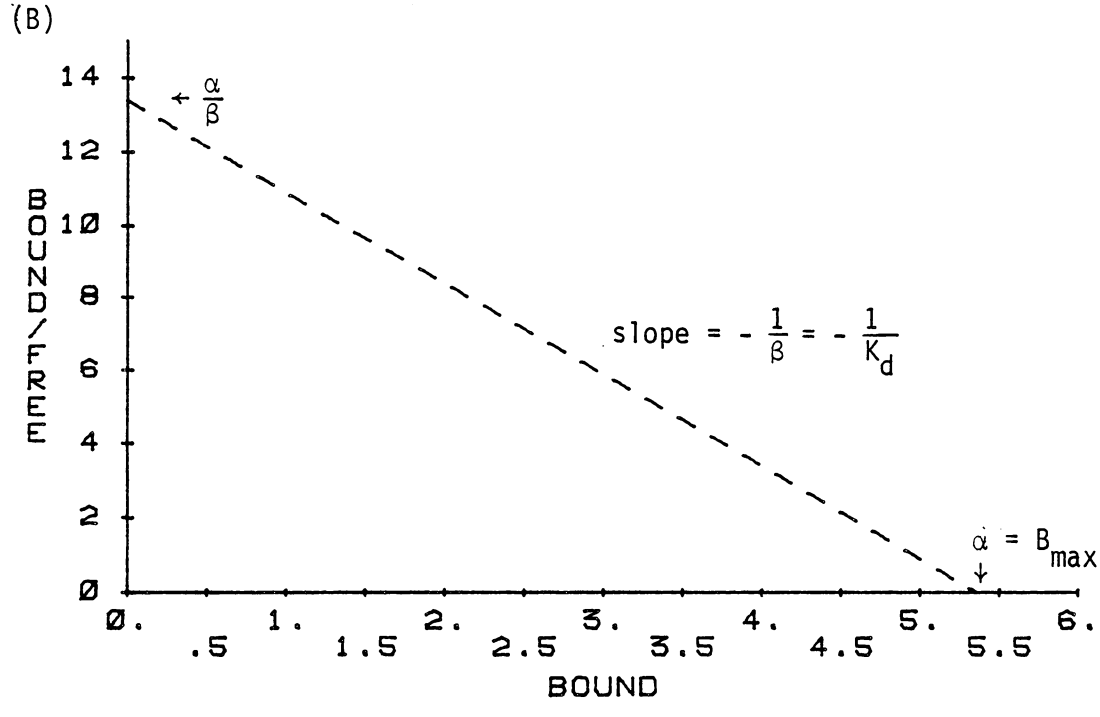

(B)
← α/β
slope = - 1/β = - 1/Kd
α = Bmax
↓
BOUND/FREE
BOUND
0
2
4
6
8
10
12
14
0.
.5
1.
1.5
2.
2.5
3.
3.5
4.
4.5
5.
5.5
6.

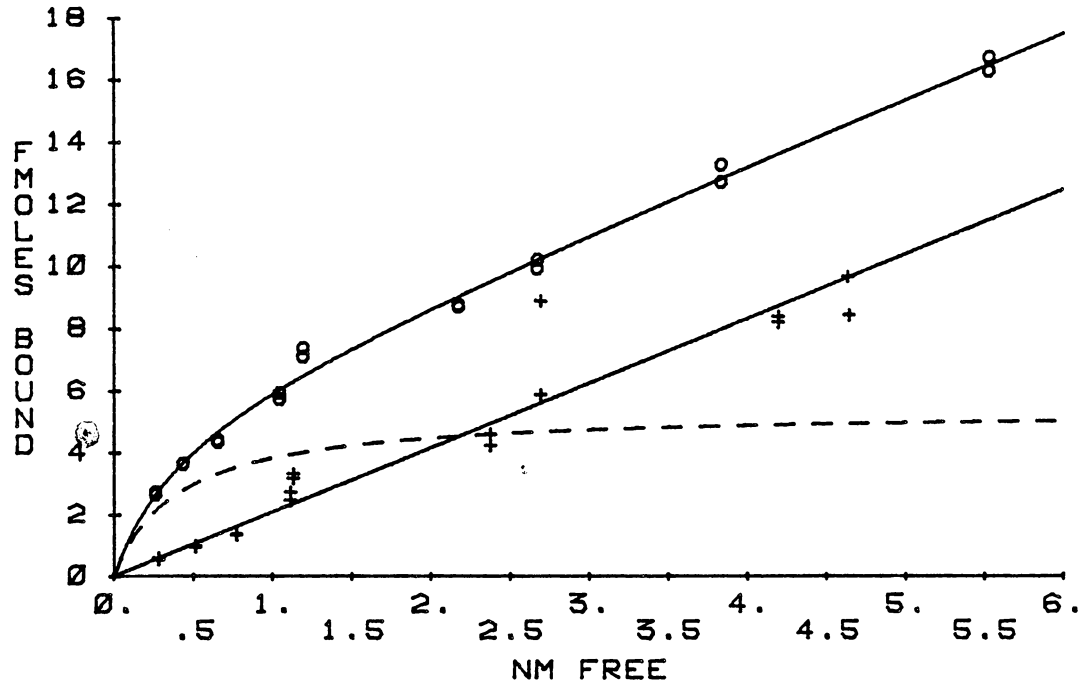
Figure II.2. Saturation analysis of diencephalic cytosol estradiol-receptors from 16-day, HM_2 line castrates.

A. Saturation curves of total (o—o), nonspecific (+—+), and specific (-----) ^{3}H-estradiol binding were constructed from a nine-point saturation analysis ranging from 0.26 to 5.56 nM ^{3}H-estradiol. The assays were conducted in duplicate using diencephalons from 40 male Japanese quail of the HM_2 line, subjected to a 3:21 light:dark photoperiod for 16 days. Total binding was determined in cytosols after 60 min of incubation at 25 C with ^{3}H-estradiol and 0.514 mg cytosol protein/tube (roughly 1.5 diencephalons/tube), followed by receptor precipitation with protamine sulfate. Nonspecific binding was determined in a parallel set of duplicate assay tubes, containing both ^{3}H-estradiol and a 100-fold molar excess of diethylstilbestrol. Specific binding curves were obtained by subtracting the simple least-squares linear regression of nonspecific binding, $y=2.08x$, from Marquardt's nonlinear least-squares regression of total binding, $y = 2.08x + \frac{5.35x}{x+.40}$.

B. Scatchard analysis of the specific binding curve from Part A estimated the receptor binding parameters at, $K_d=0.40 \pm .06$ nM**, and $B_{max}=5.35 \pm .20$ fmoles/tube** = $10.4 \pm .4$ fmoles/mg protein.

**Significant ($P \leq .001$).

(A)


FMOLES BOUND
0
2
4
6
8
10
12
14
16
18
0.
.5
1.
1.5
2.
2.5
3.
3.5
4.
4.5
5.
5.5
6.
NM FREE

(B)

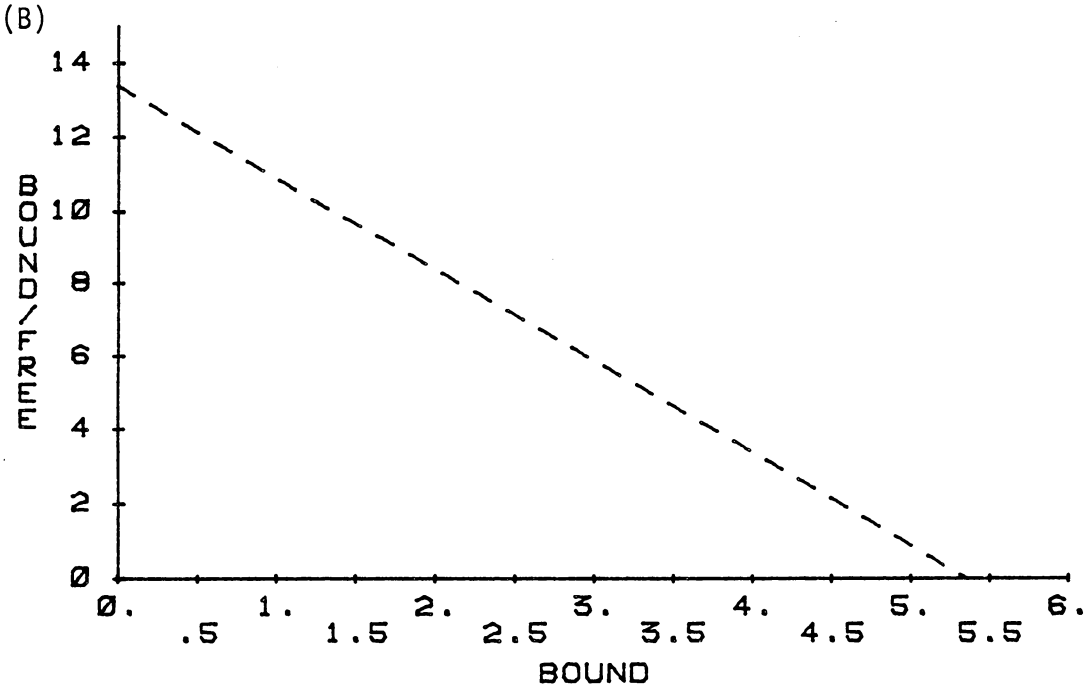
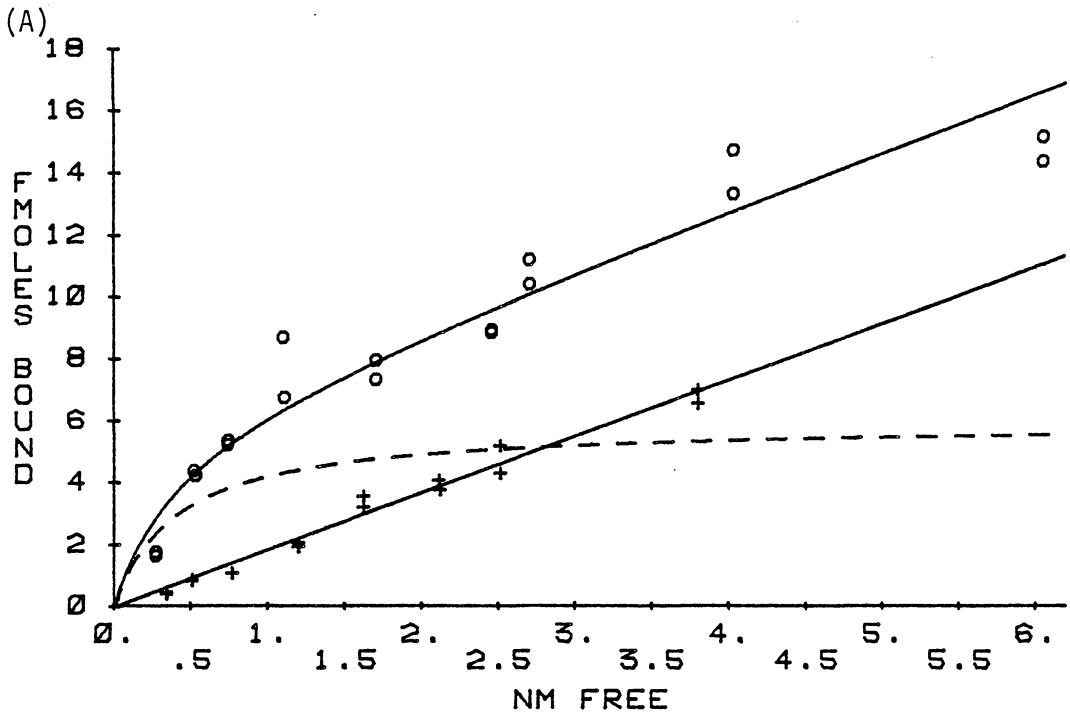

BOUND\FREE
0
2
4
6
8
10
12
14
0.
.5
1.
1.5
2.
2.5
3.
3.5
4.
4.5
5.
5.5
6.
BOUND

Figure II.3. Saturation analysis of diencephalic cytosol estradiol-receptors from 15-day, LM_2 line castrates.

A. Saturation curves of total (o——o), nonspecific (+——+), and specific (-----) ^{3}H-estradiol binding were constructed from a nine-point saturation analysis ranging from 0.28 to 6.09 n$\underline{M}$ ^{3}H-estradiol. The assays were conducted in duplicate using diencephalons from 40 male Japanese quail of the LM_2 line, subjected to a 3:21 light:dark photoperiod for 15 days. Total binding was determined in cytosols after 60 min of incubation at 25 C with ^{3}H-estradiol and 0.624 mg cytosol protein/tube (roughly 1.5 diencephalons/tube), followed by receptor precipitation with protamine sulfate. Nonspecific binding was determined in a parallel set of duplicate assay tubes, containing both ^{3}H-estradiol and a 100-fold molar excess of diethylstilbestrol. Specific binding curves were obtained by subtracting the simple least-squares linear regression of nonspecific binding, y=1.83x, from Marquardt's nonlinear least-squares regression of total binding, $y = 1.83x + \frac{5.92x}{x+.41}$.

B. Scatchard analysis of the specific binding curve from Part A estimated the receptor binding parameters at, K_d=0.41 $\pm$ .20 n$\underline{M}$*, and B_{max}=5.92 $\pm$ .65 fmoles/tube** = 9.5 $\pm$ 1.0 fmoles/mg protein.

*Significant ($P \leq .06$).
**Significant ($P \leq .001$).


(A)
FMOLES BOUND
0
2
4
6
8
10
12
14
16
18
0.
.5
1.
1.5
2.
2.5
3.
3.5
4.
4.5
5.
5.5
6.
NM FREE

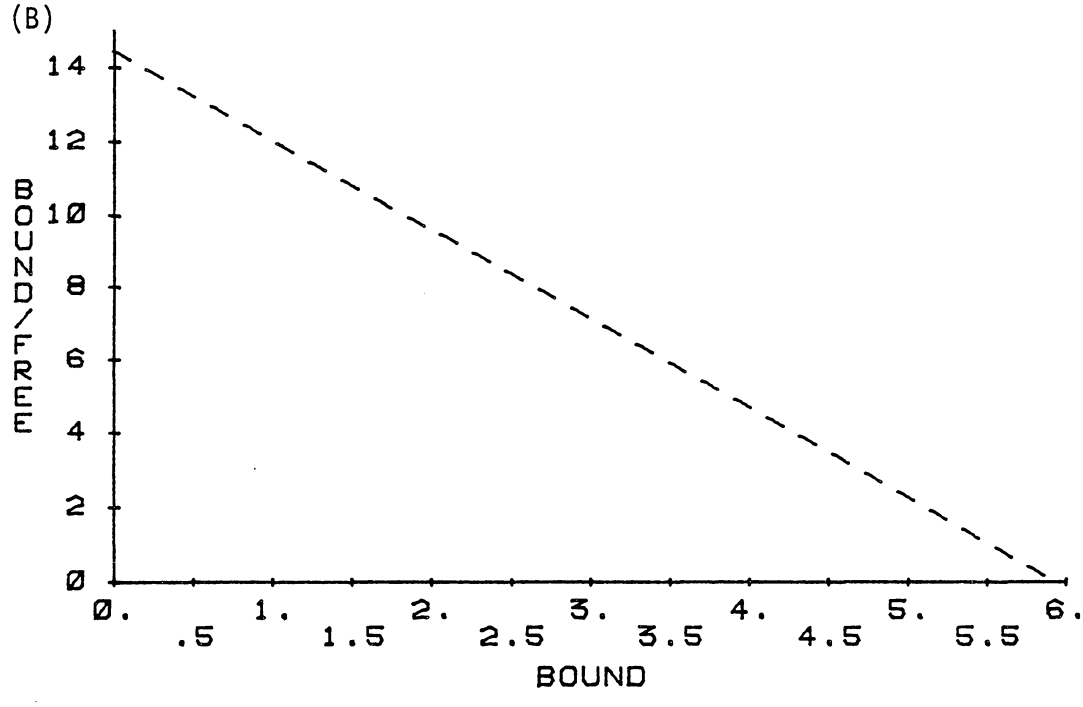
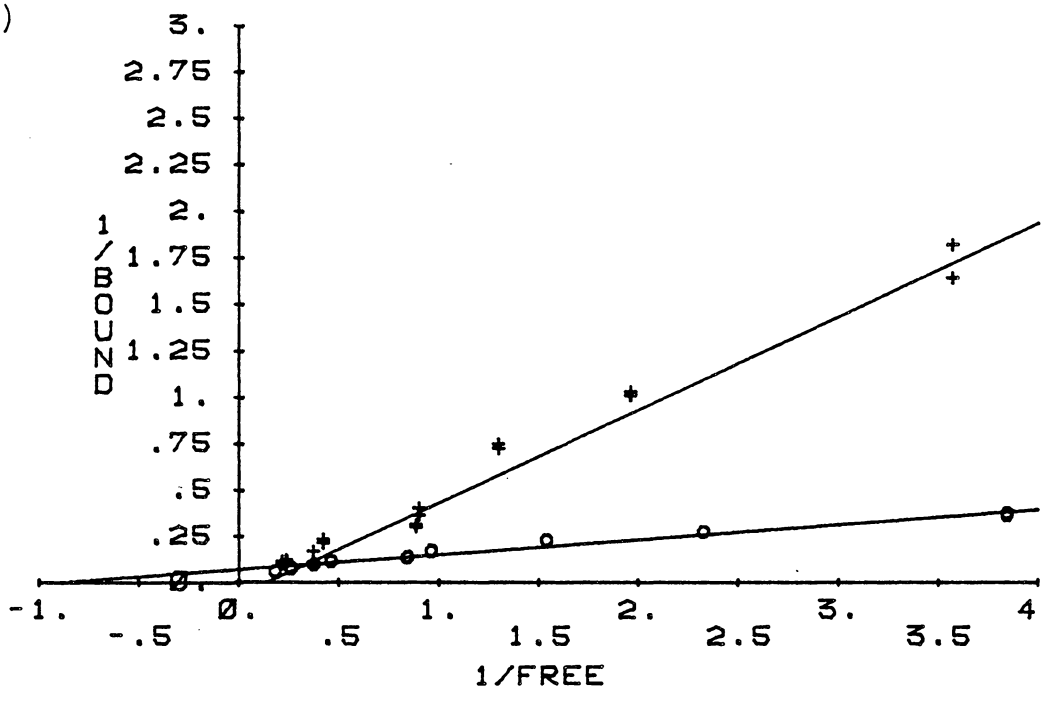

(B)
BOUND/FREE
0
2
4
6
8
10
12
14
0.
.5
1.
1.5
2.
2.5
3.
3.5
4.
4.5
5.
5.5
6.
BOUND

Figure II.4. Double reciprocal plots of total and nonspecific estradiol binding in diencephalic cytosols from (a) 16-day, HM_2 and (B) 15-day, LM_2 line castrates.

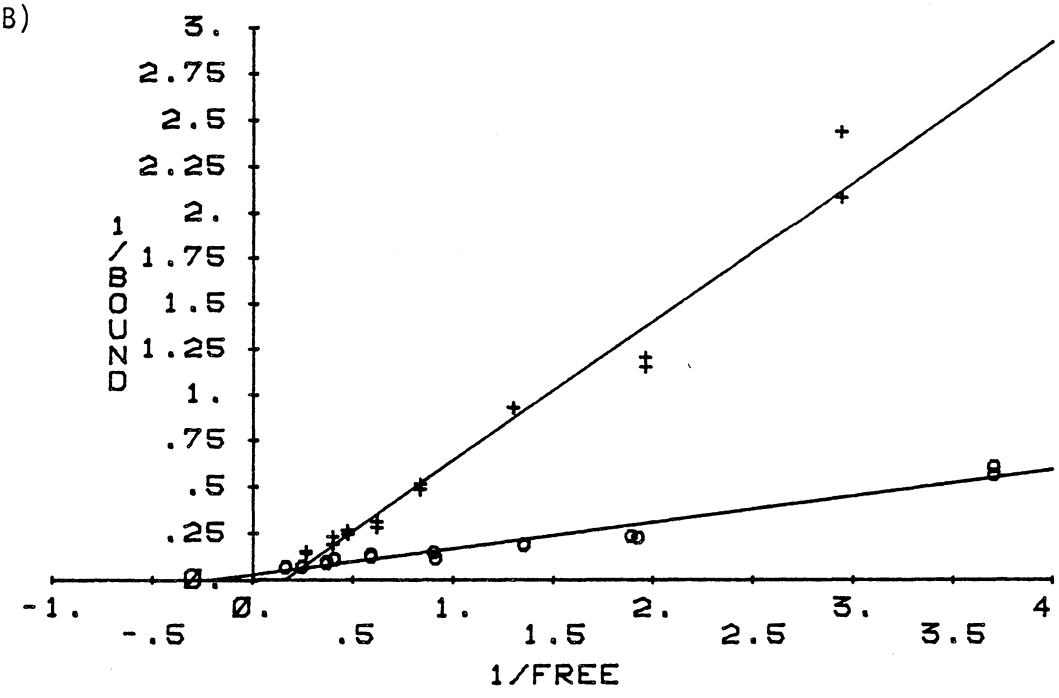
Double reciprocal plots of the total (o—o) and nonspecific (+—+) binding data from Figure II.2 (A) and Figure II.3 (B) were constructed to confirm the use of diethylstilbestrol (DES) as a competitive inhibitor of ^{3}H-estradiol binding. Lines were drawn by simple least-squares linear regression, resulting in R^2 values of 0.96 (A) and 0.95 (B) for total binding (^{3}H-estradiol), and 0.98 (A) and 0.97 (B) for nonspecific binding (^{3}H-estradiol + DES). In both Plots A and B, the presence of DES increased the apparent K_d but did not appreciably affect B_{max}, thereby indicating the competitive, i.e., mutually exclusive, nature of ^{3}H-estradiol or DES binding (Clark and Peck, 1980).[1]

[1]The reciprocal of the intercept on the ordinate represents B_{max} whereas the slope represents K_d/B_{max}.

(A)


3.
2.75
2.5
2.25
2.
1.75
1.5
1.25
1.
.75
.5
.25
Ø.
1/BOUND
-1.
-.5
Ø.
.5
1.
1.5
2.
2.5
3.
3.5
4.
1/FREE

(B)


3.
2.75
2.5
2.25
2.
1.75
1.5
1.25
1.
.75
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.25
Ø.
1/BOUND
-1.
-.5
Ø.
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1.
1.5
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2.5
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3.5
4.
1/FREE

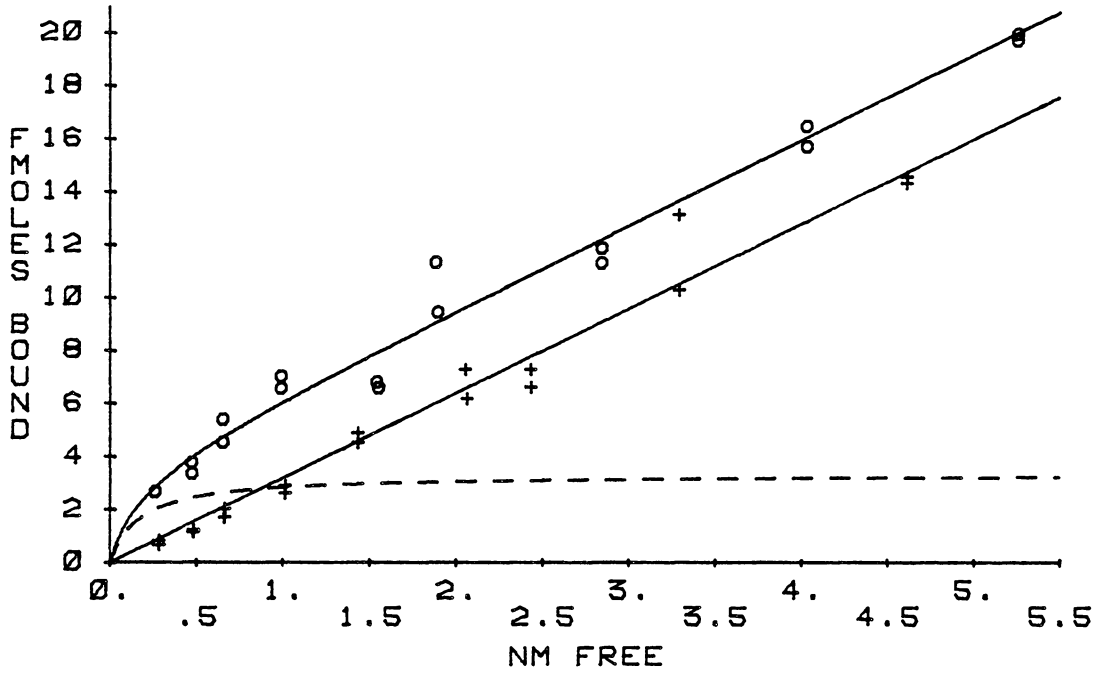
Figure II.5. Saturation analysis of diencephalic cytosol estradiol-receptors from 24-day, LM_1 line castrates.

A. Saturation curves of total (o—o), nonspecific (+—+), and specific (-----) ^{3}H-estradiol binding were constructed from a nine-point saturation analysis ranging from 0.26 to 5.30 nM ^{3}H-estradiol. The assays were conducted in duplicate using diencephalons from 40 male Japanese quail of the LM_1 line, subjected to a 3:21 light:dark photoperiod for 24 days. Total binding was determined in cytosols after 60 min of incubation at 25 C with ^{3}H-estradiol and 0.710 mg cytosol protein/tube (roughly 1.5 diencephalons/tube), followed by receptor precipitation with protamine sulfate. Nonspecific binding was determined in a parallel set of duplicate assay tubes, containing both ^{3}H-estradiol and a 100-fold molar excess of diethylstilbestrol. Specific binding curves were obtained by subtracting the simple least-squares linear regression of nonspecific binding, $y=3.2x$, from Marquardt's nonlinear least-squares regression of total binding, $y = 3.2x + \frac{3.31x}{x+.17}$.

B. Scatchard analysis of the specific binding curve from Part A estimated the receptor binding parameters at, K_d=0.17 $\pm$ .13 nM*, and B_{max}=3.31 $\pm$ .38 fmoles/tube** = 4.7 $\pm$ fmoles/mg protein.

*Nonsignificant (P>.1).
**Significant (P$\leq$.001).

(A)


FMOLES BOUND
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16
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2.
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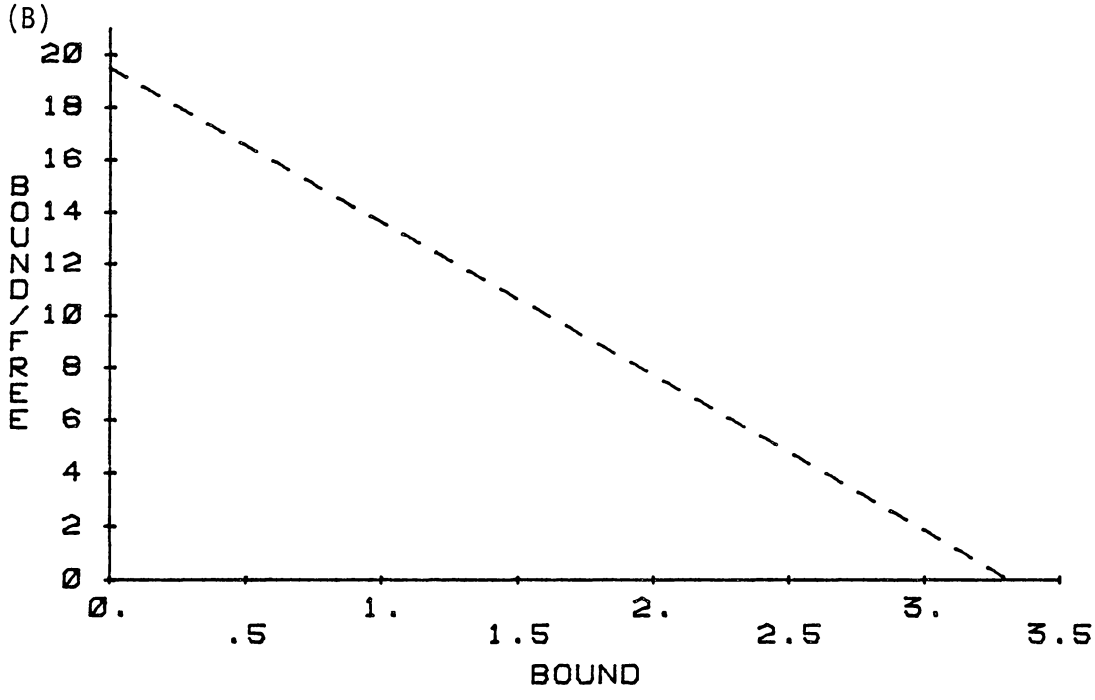
3.
3.5
4.
4.5
5.
5.5
NM FREE

(B)


BOUND/FREE
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.5
1.
1.5
2.
2.5
3.
3.5
BOUND

Figure II.6. Saturation analysis of diencephalic cytosol estradiol-receptors from 24-day, HM_1 line castrates.

Saturation curves of total (o——o) and nonspecific (+——+) ^{3}H-estradiol binding were constructed from a nine-point saturation analysis ranging from 0.22 to 4.27 nM ^{3}H-estradiol. The assays were conducted in duplicate using diencephalons from 40 male Japanese quail of the HM_1 line, subjected to a 3:21 light:dark photoperiod for 24 days. Total binding was determined in cytosols after 60 min of incubation at 25 C with ^{3}H-estradiol and 0.577 mg cytosol protein/tube (roughly 1.5 diencephalons/tube), followed by receptor precipitation with protamine sulfate. Nonspecific binding was determined in a parallel set of duplicate assay tubes, containing both ^{3}H-estradiol and a 100-fold molar excess of diethylstilbestrol. No specific binding was obtained upon subtraction of the nonspecific binding component from the total.

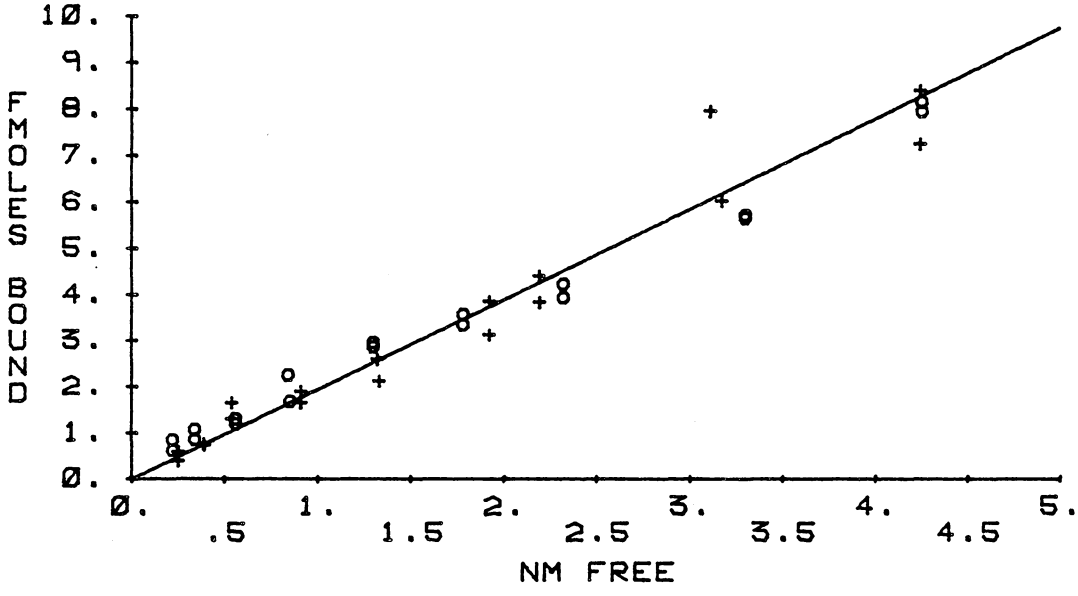
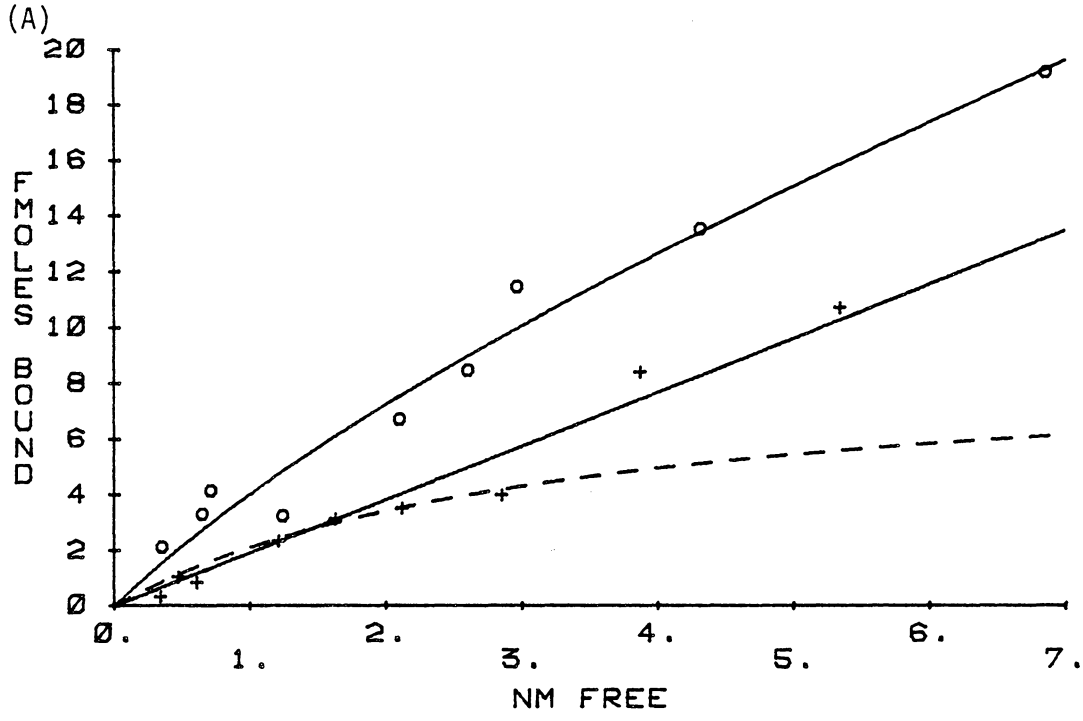

10.
9.
8.
7.
6.
5.
4.
3.
2.
1.
0.
0.
.5
1.
1.5
2.
2.5
3.
3.5
4.
4.5
5.
NM FREE

Figure II.7. Saturation analysis of diencephalic cytosol estradiol-receptors from 30-day, HM_2 line castrates.

A. Saturation curves of total (o—o), nonspecific (+—+), and specific (-----) ^{3}H-estradiol binding were constructed from a nine-point saturation analysis ranging from 0.35 to 6.88 nM ^{3}H-estradiol. The assays were conducted in duplicate using diencephalons from 22 male Japanese quail of the HM_2 line, subjected to a 3:21 light:dark photoperiod for 24 days. Total binding was determined in cytosols after 60 min of incubation at 25 C with ^{3}H-estradiol and 0.398 mg cytosol protein/tube (roughly 1.5 diencephalons/tube), followed by receptor precipitation with protamine sulfate. Nonspecific binding was determined in a parallel set of duplicate assay tubes, containing both ^{3}H-estradiol and a 100-fold molar excess of diethylstilbestrol. Specific binding curves were obtained by subtracting the simple least-squares linear regression of nonspecific binding, y=1.93x, binding Marquardt's nonlinear least-squares regression of total binding, $y = 1.93x + \frac{9.03x}{x+3.27}$.

B. Scatchard analysis of the specific binding curve from Part A estimated the receptor binding parameters at, K_d=3.27 $\pm$ 2.24 nM*, and B_{max}=9.03 $\pm$ 3.09 fmoles/tube* = 22.7 fmoles/mg protein.

*Nonsignificant (P>.1).


(A)
FMOLES BOUND
20
18
16
14
12
10
8
6
4
2
0
0.
1.
2.
3.
4.
5.
6.
7.
NM FREE

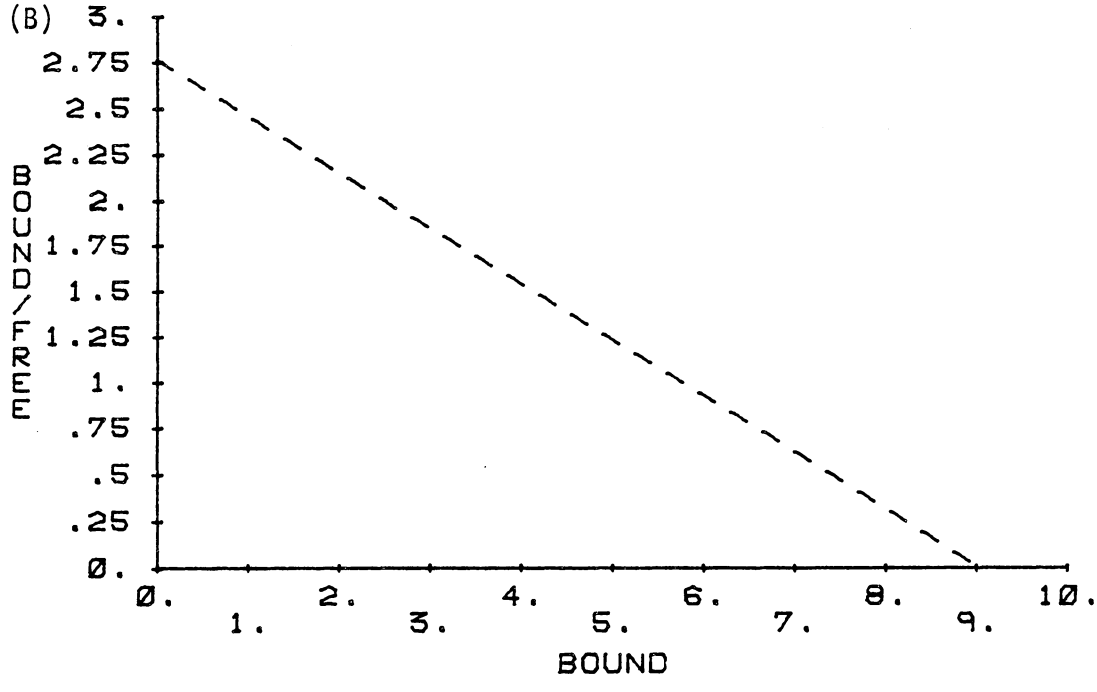
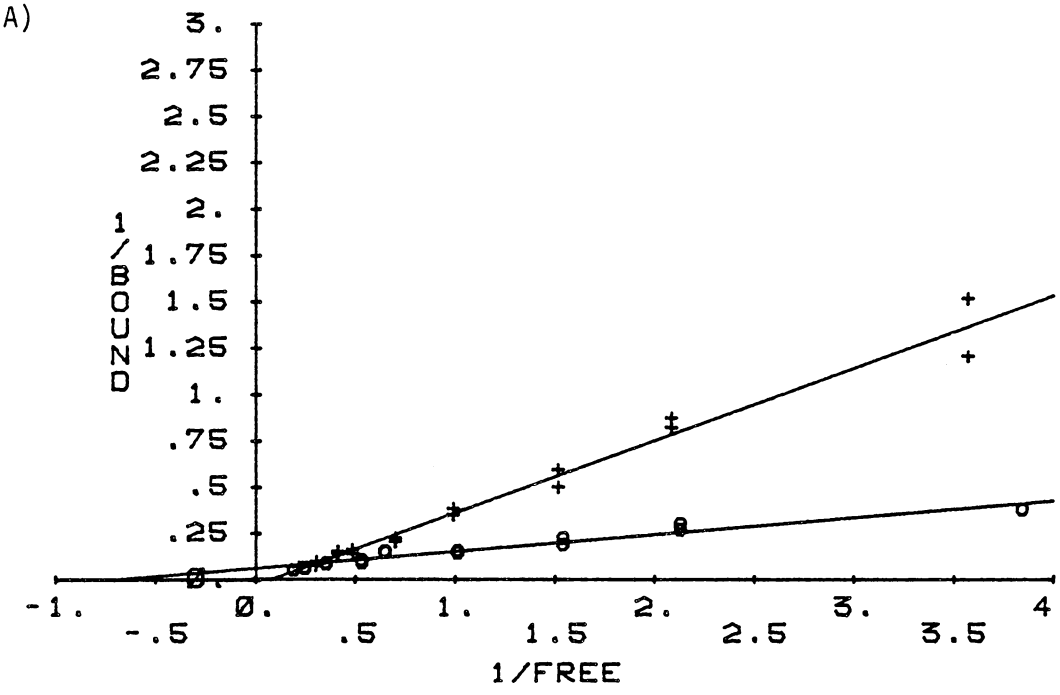

(B)
BOUND/FREE
3.
2.75
2.5
2.25
2.
1.75
1.5
1.25
1.
.75
.5
.25
0.
0.
1.
2.
3.
4.
5.
6.
7.
8.
9.
10.
BOUND

Figure II.8. Double reciprocal plots of total and nonspecific estradiol binding in diencephalic cytosols from (a) 24-day, LM_1 and (B) 30-day, HM_2 line castrates.

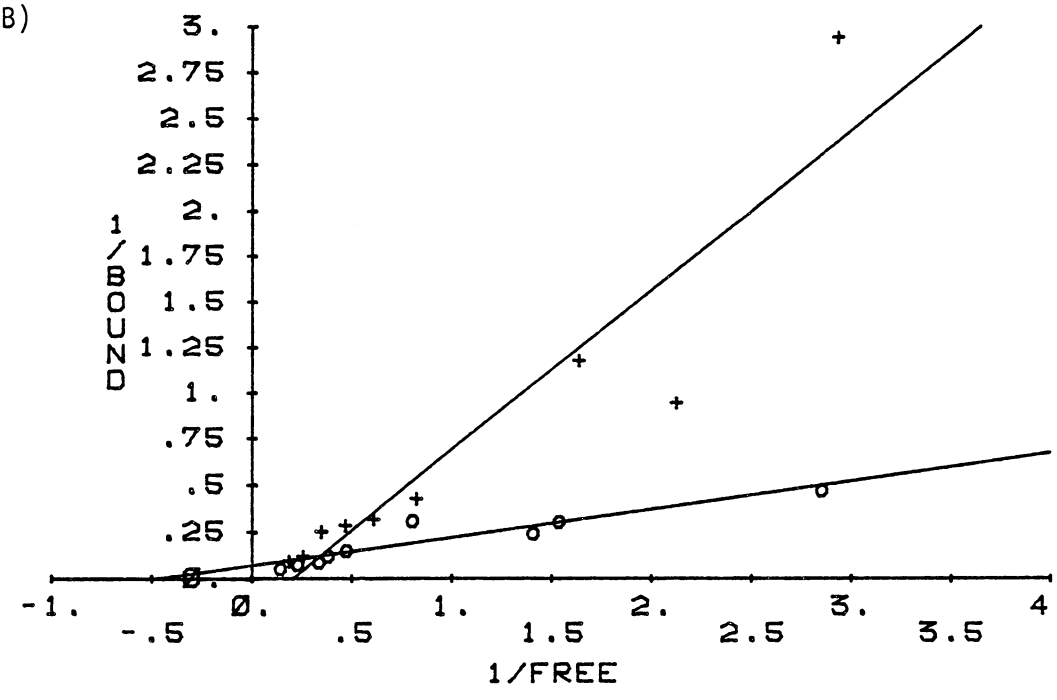
Double reciprocal plots of the total (o——o) and nonspecific (+——+) binding data from Figure II.5 (A) and Figure II.6 (B) were constructed to confirm the use of diethylstilbestrol (DES) as a competitive inhibitor of ^{3}H-estradiol binding. Lines were drawn by simple least-squares linear regression, resulting in R^2 values of 0.95 (A) and 0.88 (B) for total binding (^{3}H-estradiol), and 0.98 (A) and 0.86 (B) for nonspecific binding (^{3}H-estradiol + DES). In both Plots A and B, the presence of DES increased the apparent K_d but did not appreciably affect B_{max}, thereby indicating the competitive, i.e., mutually exclusive, nature of ^{3}H-estradiol or DES binding (Clark and Peck, 1980).[1]

[1]The reciprocal of the intercept on the ordinate represents B_{max} whereas the slope represents K_d/B_{max}.

(A)


3.
2.75
2.5
2.25
2.
1.75
1.5
1.25
1.
.75
.5
.25
0.
1/BOUND
-1.
-.5
0.
.5
1.
1.5
2.
2.5
3.
3.5
4.
1/FREE

(B)


3.
2.75
2.5
2.25
2.
1.75
1.5
1.25
1.
.75
.5
.25
0.
1/BOUND
-1.
-.5
0.
.5
1.
1.5
2.
2.5
3.
3.5
4.
1/FREE

GENERAL DISCUSSION

The results of Experiments I and II show that line differences in mating frequency depend upon a mechanism other than (1) rate of testosterone aromatization or (2) nature of the cytoplasmic estradiol-receptor interaction. In view of the current literature and the results of Experiment I, an alternative mechanism was proposed to involve the reduction of testosterone to 5α- versus 5β-dihydrotestosterone (Experiment I). In addition, in Experiment II, reference was made to a possible regulatory mechanism based on the dynamic relationships between cytoplasmic and nuclear estrogen binding. An analysis of these two possibilities in combination with a comprehensive review of the literature, suggests an integrated system consisting of 5α-dihydro-testosterone in cross-reaction with estradiol receptors. The model, discussed below with reference to the literature, implies a facilitory role for 5α-dihydrotestosterone in the recyclization of nuclear estradiol receptors.[1]

The initiation of any receptor-mediated response requires receptor association with nuclear genetic material (Clark and Peck, 1980). According to receptor theory, only steroid-bound cytoplasmic receptors may become activated for nuclear entry. Given the short half-life of the nuclear hypothalamic estradiol receptor (Kelner et al., 1981), it

[1]Note that this model has been constructed entirely by extrapolation of the literature and, therefore, should be regarded as purely speculative.

follows that any repetitive estradiol-induced behavioral response such as the HM line quail copulatory act, must require continued availability, i.e., replenishment of cytoplasmic receptors. A study of the dynamic depletion and replenishment patterns of cytoplasmic receptors in rat hypothalamus has revealed that replenishment results both from de novo synthesis and recyclization of nuclear receptors (Cidlowski and Muldoon, 1976).

In their study, Cidlowski and Muldoon (1976) noted that replenishment of cytosolic estradiol-receptor pools was more pronounced in mature male rat hypothalami than in intact or ovariectomized females or prepubertal males. Therefore, they suggested that androgens might be responsible for the enhanced replenishment pattern of the male. Furthermore, it was concluded that because the replenishment patterns of both sexes were equally affected by cycloheximide (an inhibitor of protein synthesis), the enhanced replenishment among males did not appear to result from increased de novo synthesis. Instead, the male replenishment pattern was attributed to more efficient recyclization of nuclear receptors. In other words, androgens were considered to have a priming effect on estradiol-receptor recyclization. This hypothesis is strengthened by the observation that treatment of ovariectomized females with 5α-dihydrotestosterone effectively reduced cytoplasmic receptor depletion (Cidlowski and Muldoon, 1976). Moreover, 5α-dihydrotestosterone has been found to cross-react with estradiol-receptors with significantly reduced affinity to suggest a shorter half-life for the

nuclear 5α-dihydrotestosterone-receptor complex (ibid). Thus, a facilitory role for 5α-dihydrotestosterone in the recyclization of nuclear estradiol receptors was implied.

The importance of estradiol in the induction of copulation in male quail has been established (Adkins, 1977 ; Adkins and Pniewski, 1978; Adkins, 1978; Balander, 1978; Adkins et al., 1980; Steimer and Hutchison, 1980; Adkins-Regan, 1981). That estradiol binding to hypothalamic-preoptic area neurons is a necessary prerequisite for the expression of masculine sexual behavior is also consistent with a mechanism involving dihydrotestosterone enhancement of estradiol-receptor recyclization. Perhaps a simplified model of dynamic receptor-steroid interaction might then be described as follows:

> Cytoplasmic pools of estradiol (E) and 5α-dihydrotestosterone (D) are maintained via nonspecifically bound steroid at equilibrium with cytoplasmic receptors (R_c). Steroid-bound specific cytoplasmic receptors (ER_c, DR_c) undergo translocation to the nucleus (ER_n, DR_n) where they compete for a "limited" number of binding sites on the genome. Those ER_n complexes not attached to genomic sites are degraded in the nucleus whereas the DR_n complexes rapidly reenter the cytoplasm. In the cytoplasm, free R_c resulting from the degradation of DR_c, is equilibrated with estradiol and dihydrotestosterone, thereby elevating the R_c pool and providing greater opportunity for effective association of ER with chromatin. Over a period of time, enhanced E receptor recyclization results in the repetitive copulatory pattern that distinguishes the HM from the LM phenotype.

Though not indicated, it is assumed that receptors maintain a steady state of steroid exchange, and that some nuclear estradiol-receptor complexes may reenter the cytoplasm and be recycled. Also, as an alternative to the interpretation of a limited number of genomic binding sites, the concept of heterogeneous nuclear RNA (hnRNA) might also be applicable. Heterogeneous RNA is composed of a number of RNAs including mRNA. However, a great deal of hnRNA is rapidly degraded in the nucleus without prior cleavage of mRNA sequences (Lehninger, 1975). Therefore, transcription of large amounts of presumably "nonsensical" RNA strands (hnRNA) may function as an overflow mechanism in the presence of limited gene resources. Though the complete role of hnRNA is yet to be determined, it has been implicated in the regulation of gene expression (Lehninger, 1975).

With regard to mating line chickens, McCollum et al. (1971) suggested that variations in neural thresholds were primarily responsible for phenotypic differences in mating behavior whereas absolute hormone levels were secondarily involved. Similarly, the proposed model depends primarily upon a genetic threshold for 5α-reductase activity and secondarily on the presence of a stimulatory blood level of estradiol. In the selected lines, a differential ratio of 5α to 5β-reductase activity might mediate the degree of behavioral responsiveness to estradiol.

Though insufficient evidence is available to confirm the proposed mechanism for the mediation of sexual behavior in the selected lines

of Japanese quail, additional inferences can be made from studies with rodents. For example, Fox (1975) detected a receptor in mouse hypothalamus that was stereo-specific for both estrogens and androgens. Based on these findings, he suggested that sex steroids functioned via a hypothalamic mechanism which detected relative concentrations of androgens and estrogens rather than the absolute levels of either hormone. Also in rats, the synergistic action of 5α-dihydrotestosterone and estradiol on the expression of masculine sexual behavior has been described (Baum and Vreeburg, 1973; Larsson et al., 1973; Lodder and Baum, 1977). According to the quail model, cross-reaction of 5α-dihydro testosterone with estradiol-receptors would enhance cytosolic estradiol replenishment and ultimately increase estradiol-induced copulation. In support of this hypothesis, it is of relevance to note that the degree of estradiol responsiveness of female rats pretreated with anti-estrogens has been correlated with the level of replenishment of uterine cytosol receptors following the anti-estrogen treatment (Ferguson and Katzenellenbogen, 1977).

SUMMARY

The neuroendocrinology of masculine sexual behavior was examined in Japanese quail selected bi-directionally for adult mating frequency. Experiments were conducted with brain tissues from functionally castrated, High (HM) and Low Mating (LM) line quail to compare (1) the in vivo aromatization of an intramuscular injection of ^{3}H-testosterone and (2) cytosolic estradiol-binding by in vitro saturation analyses.

In Experiment I, after one hour of in vivo incubation with ^{3}H-testosterone, all radioactivity recovered in brain tissues was in the form of testosterone, dihydrotestosterone, or estradiol. Neither the total ^{3}H nor ^{3}H-testosterone metabolite radioactivity differed upon comparison of the two genetic lines. Of all ^{3}H-testosterone metabolite radioactivity, ^{3}H-estradiol represented 45 $\pm$ 6 % in the HM line and 46 $\pm$ 6% in the LM line, indicating that the line difference in mating frequency was not due to a corresponding difference in aromatase activity. Inasmuch as both the HM and LM line birds actively converted testosterone to estradiol, these results implicated a neural mechanism involving estradiol-receptor interactions. This possibility was investigated in Experiment II.

Using a single-class binding site model in Experiment II, the apparent dissociation constant (K_d) for the estradiol-receptor interaction in the HM line was 0.40 $\pm$ .06 nM and the number of specific estradiol binding sites (B_{max}) was 10.4 $\pm$ .4 fmoles/mg cytosol protein. For the LM line, an apparent K_d of 0.41 $\pm$ .20 nM and a B_{max} of 9.5 $\pm$ 1.0

fmoles/mg cytosol protein were obtained. The similarity between corresponding binding parameters for the divergent mating lines indicated that the number and/or estradiol-binding affinity of a single class of cytosol receptors was also not responsible for the line difference in mating frequency. Though these results confirm that interaction of estradiol with cytosol receptors was not the limiting neuroendocrinological mechanism in the differential expression of masculine sexual behavior, the possible involvement of dynamic interrelationships between cytoplasmic and nuclear estrogen binding has not yet been determined.

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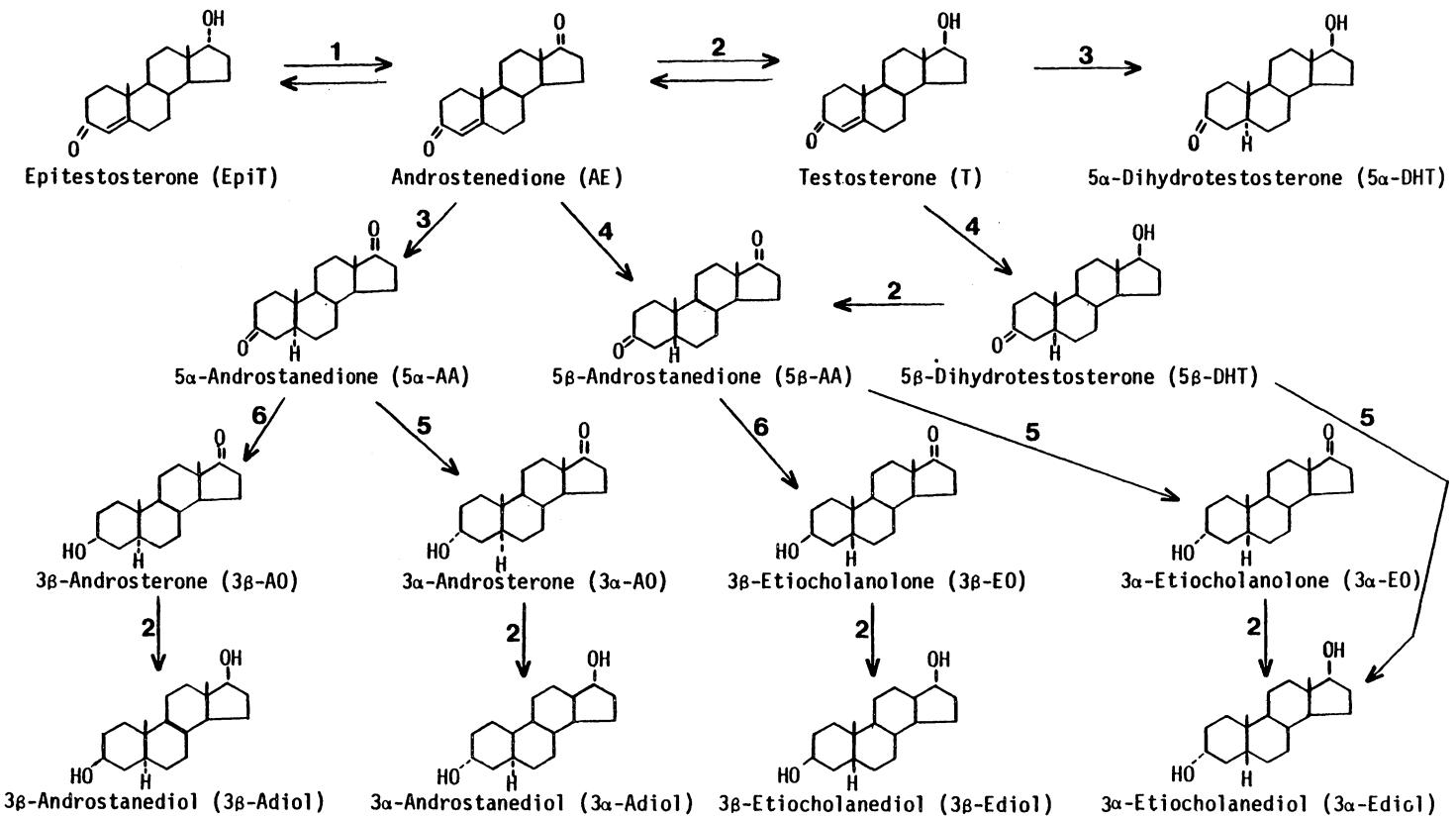
APPENDIX A

Chemical Reactions Describing the Metabolism of Androgens to Estrogens



1. 17α-Hydroxy-dehydrogenase
2. 17β-Hydroxy-dehydrogenase
3. 5α-Reductase
4. 5β-Reductase
5. 3α-Hydroxy-dehydrogenase
6. 3β-Hydroxy-dehydrogenase

Figure A.1. Metabolism of androgens (adapted from Heftman, 1970).

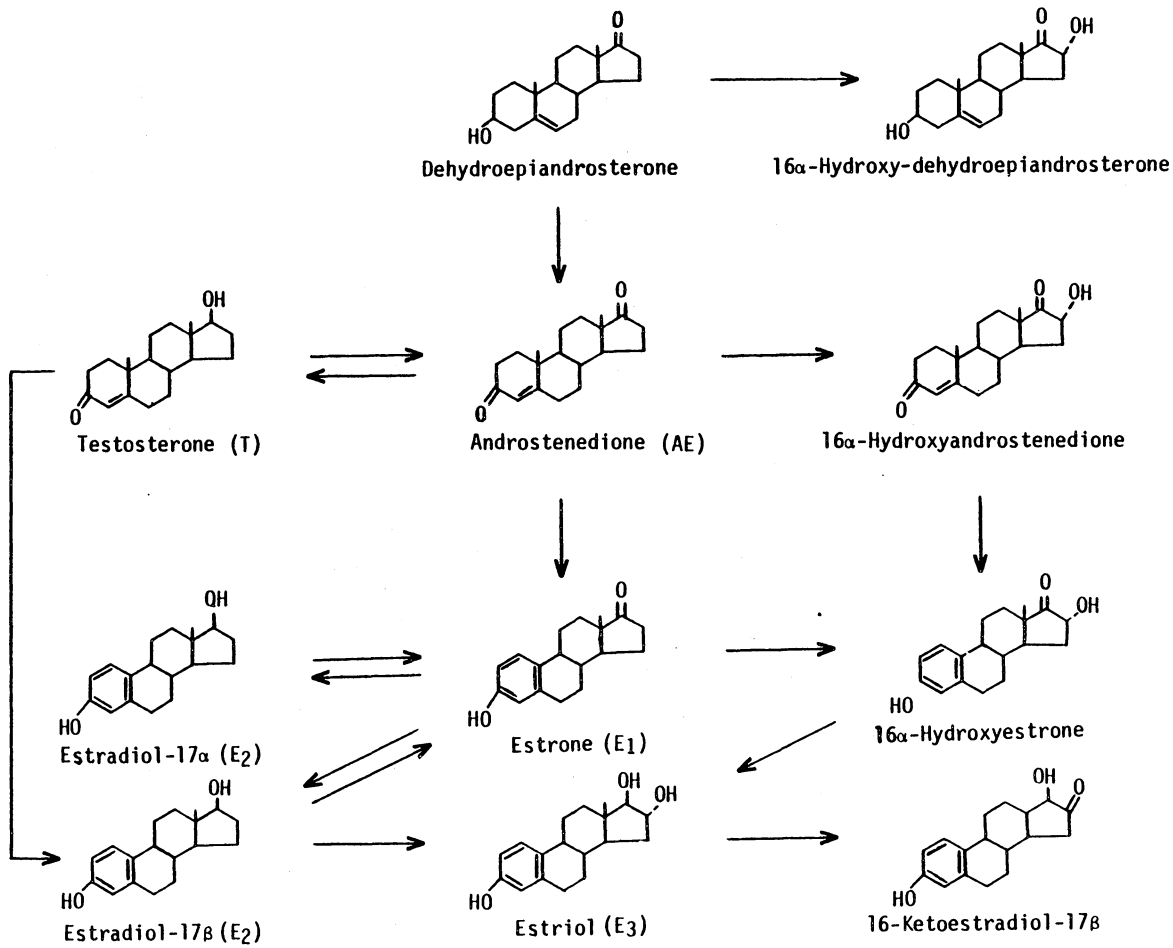


Figure A.2. Biosynthesis of the principal estrogens (adapted from Heftman, 1970).

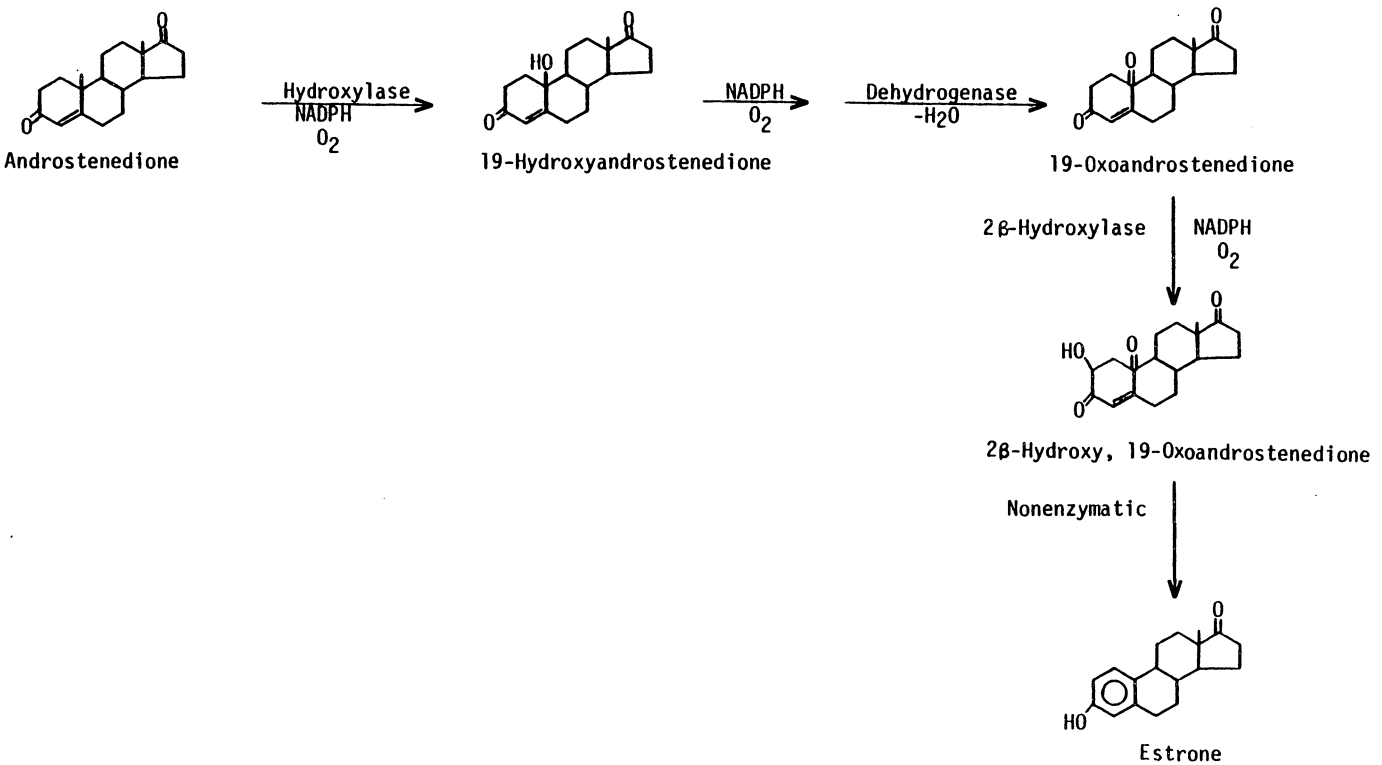


Figure A.3. Mechanism of aromatization of androgens (adapted from Thompson and Siiteri, 1973).

APPENDIX B

Analysis of Variance Tables for Experiment I

Table B.1. Single degree of freedom contrasts for picomoles of radioactivity recovered per milligram brain protein in Experiment I.[1]

(A) Total picomoles/mg protein.

Source	d.f.	SS	MS	F value
Model	3	7,470	2,490	1.94
HM_1 vs. LM_1	1	1,300	1,300	1.01
HM_2 vs. LM_2	1	840	840	.65
HM vs. LM	1	25	25	.02
Error	12	15,406	1,284	
Total	15	22,876		

(B) Metabolic picomoles/mg protein.

Source	d.f.	SS	MS	F value
Model	3	1,915	638	1.54
HM_1 vs. LM_1	1	98	98	.23
HM_2 vs. LM_2	1	276	276	.67
HM vs. LM	1	23	23	.06
Error	12	4,961		
Total	15	6,876		

[1]All F values were nonsignificant.

Table B.2. Single degree of freedom contrasts for picomoles of radioactivity recovered per microgram brain DNA in Experiment I.[1]

(A) Total picomoles/µg DNA.

Source	d.f.	SS	MS	F value
Model	3	6.69	2.23	2.51
HM_1 vs. LM_1	1	.46	.46	.52
HM_2 vs. LM_2	1	2.09	2.09	2.35
HM vs. LM	1	.29	.29	.32
Error	12	10.71	.89	
Total	15	17.40		

(B) Metabolic picomoles/µg DNA.

Source	d.f.	SS	MS	F value
Model	3	1.83	.61	2.44
HM_1 vs. LM_1	1	.02	.02	.08
HM_2 vs. LM_2	1	.72	.72	2.88
HM vs. LM	1	.26	.26	1.04
Error	12	3.04	.25	
Total	15	4.87		

[1]All F values were nonsignificant.

Table B.3. Single degree of freedom contrasts for ^{3}H-estradiol as a percentage of all recovered metabolite radioactivity in Experiment I.[1]

Source	d.f.	SS	MS	F value
Model	3	.12	.04	1.33
HM_1 vs. LM_1	1	.009	.009	.30
HM_2 vs. LM_2	1	.003	.003	.10
HM vs. LM	1	.002	.002	.06
Error	12	.31	.03	
Total	15	.43		

[1]All F values were nonsignificant.

APPENDIX C

Determination of the Appropriate Incubation Conditions for Measuring Diencephalic Cytosol Estradiol-Receptors in Japanese Quail

Principles of Steroid-Receptor Measurement

A number of standard biochemical techniques have been adapted to the study of semi-purified and purified receptor preparations. A review and tutorial of the various techniques used for measuring both cytosolic and nuclear receptors has been published by Clark and Peck (1979). In general, these techniques involve the incubation of receptor with radiolabeled ligand, followed by separation of the bound and unbound fractions by adsorption of one fraction to some other compound. The amount of bound receptor is then indirectly determined by quantitation of the bound radioactive ligand. Regardless of the separation technique which is used, it is recommended that analyses be carried out by incubating receptor over a wide range of ligand concentrations (Clark and Peck, 1979). This is because receptor sites may display "heterogeneity with respect to stability" (ibid). That is, receptor sites are physically more stable under saturating ligand conditions than they are under sub-saturating conditions. Therefore, an accurate assessment of binding can be obtained only when the conditions of maximum receptor stability have been determined.

Receptor assays are normally conducted over a wide range of hormone concentrations extending from subsaturation to saturation (saturation analyses) and the data are plotted as a function of the amount of free or unbound hormone remaining (saturation curve). If saturation analyses are carried out using the incubation conditions appropriate for maximum receptor stability, estimates of receptor binding parameters may be

obtained by subjecting the data to Scatchard Analysis (Scatchard, 1949). Scatchard Analysis involves the transformation of one or more saturation curves so that the ratio of bound/free hormone is plotted as a function of the amount of bound hormone (Scatchard Curve). The slope of the Scatchard Curve approximates the affinity constant (K_a) for the receptor-hormone complex, and the intercept on the abcissa approximates the number of binding sites. To obtain accurate estimates of the receptor-hormone binding parameters, the data must be corrected for nonspecific binding (Clark and Peck, 1979). This is done by conducting competition studies (Clark and Peck, 1979) or by various statistical and mathematical methods (Rosenthal, 1967; Feldman, 1972; Richard-Foy et al., 1978; Munson and Rodbard, 1980).

The simplest and most common method of correcting for nonspecific binding is the competition study. It requires that saturation analyses be conducted both in the presence and absence of a competitive inhibitor. For example, to measure estrogen receptor, saturation analysis often includes the competitive inhibitor, diethylstilbestrol (DES). As a competitive inhibitor, diethylstilbestrol competes for binding sites in a mutually exclusive manner (Clark and Peck, 1979). The rationale for including a competitive inhibitor in receptor studies is based upon the relative natures of the specific and nonspecific receptor species. Under in vitro equilibrium conditions, the specific binding sites, having a much higher affinity for ligand than the nonspecific sites, will effectively exchange ligand which is present

at high concentration. When an excess of unlabeled competitive inhibitor is included at incubation, it will be (effectively) exchanged among the higher affinity or specific sites, whereas the radiolabeled ligand will bind to the lower affinity or nonspecific sites. Therefore, in the estrogen receptor example, assays are conducted in parallel, with one set of assay tubes containing receptor plus graded levels of radioactive estradiol, and the other set containing a 100-fold molar excess of diethylstilbestrol, as well. The data obtained from the tubes containing diethylstilbestrol represent nonspecific binding whereas those without diethylstilbestrol represent total binding (specific + nonspecific). The amount of specific binding is assumed to be the difference between the total and nonspecific binding. Data on specific binding, which is obtained in this manner, is then subjected to Scatchard Analysis.

Overview

Initital attempts to measure specific, high-affinity estradiol receptors in cytosols from quail diencephalons involved three different assay methods. These were the charcoal adsorption procedure described by Clark and Peck (1980), and the protamine sulfate precipitation methods described by Chamness et al. (1975) and Clark and Peck (1980). All three of these procedures were exchange assays developed for measuring estradiol binding in rat tissues.[1] When applied to quail diencephalon, in the present study, however, they were unsuccessful. As a result, virtually no specific binding was detected whereas high levels of nonspecific, low-affinity binding were apparent.

In order to obtain preliminary estimates of specific binding in cytosols from quail diencephalon, it was necessary to conform to several laboratory practices while using the basic protamine sulfate precipitation method of Clark and Peck (K. L. Kelner, personal communication). First, it was necessary to mix the ^{3}H-estradiol buffer solutions immediately prior to their use because ^{3}H-estradiol was not stable in aqueous solution. Second, to avoid enzymatic degradation of receptors, in vitro, it was necessary to obtain all diencephalons before homogenizing any of the tissues. Third, it was necessary to add "wash buffer" to the top of the assay tube during the pellet washing step.

[1]Steroid exchange assays are conducted at elevated temperature (typically 30 C) to invoke exchange of any endogenously bound steroid for ^{3}H-steroid. In this manner both occupied and unoccupied receptor are detected.

This latter step was designed to remove any residual unbound estradiol from the protamine-sulfate-protein pellet containing bound receptors. By adding "wash buffer" to the top of the assay tube, physical trapping of free steroids within the pellet was less apt to occur. Once these practices were incorporated into the general assay procedure described below, and preliminary estimates of specific binding were obtained, it was then possible to establish the incubation conditions for maximizing specific binding of ^{3}H-estradiol.

Procedures for obtaining maximum ^{3}H-estradiol binding by specific receptors were determined in a series of experiments with diencephalons from Japanese quail. As a qualitative control for the assay procedure, two experiments also included assays for estradiol receptors in cytosols from rat uteri. After the initial detection of specific binding sites in quail diencephalons, the assay procedure was successively modified to reveal the range of ^{3}H-estradiol required for <u>in vitro</u> saturation of specific binding sites, as well as to define the conditions for minimizing their degradation during incubation with free ^{3}H-estradiol.

General Assay Procedure

The following procedure pertains to Experiments 1-7 of Appendix C. Additional information is included in the description of the individual experiment. Note that a temperature of 0-4 C was maintained for Steps 1-4 and 6-10.

Step 1.

For studies involving quail, diencephalons were pooled (7/6 ml in Experiments 1-3; 2/ml in Experiments 4-7) and homogenized in ice-cold Tris EDTA buffer (pH 7.4 at 2 C) with 10 strokes of a Kontes ground-glass homogenizer. For studies involving rats, each uterus was minced in 2 ml ice-cold Tris-EDTA buffer (pH 7.4 at 2 C), then homogenized in a total of 4 ml buffer with a Kontes ground-glass homogenizer.

Step 2.

In Experiments 1 and 4-7, the combined rat or quail homogenates were centrifuged at 31,800 x g for 30 min at 0-4 centigrade. The high-speed supernatant (containing cytosol) was diluted with ice-cold Tris-EDTA buffer (pH 7.4 at 2 C).

In Experiments 2-3, the rat or quail homogenates were diluted to 20 ml or 14 ml, respectively. One ml aliquots were then layered onto 1.2 M sucrose pads and centrifuged in a swinging bucket rotor at 6,900 x g for 45 min at 0-4 C to sediment nucler receptors. The top layer (875 µl, containing crude cytosol) was removed from each tube, pooled, and

recentrifuged at 31,800 x g for 30 min at 0-4 centigrade. The pellet was recovered for nuclear receptor studies not covered here.

Step 3.

While the homogenates were being centrifuged, solutions of (2,4,6,7,16,17-^{3}H)-estradiol (147 Ci/mmol, Amersham, Arlington Hts, IL) were prepared in ice-cold Tris-EDTA buffer (pH 7.4 at temperature of incubation) along with parallel ^{3}H-estradiol solutions containing a 100-fold molar excess of diethylstilbestrol (DES). The stock solutions of both hormones were previously prepared in absolute ethanol, and the ^{3}H-estradiol stock solution was checked for purity before use.

Step 4.

An aliquot of the diluted cytosol (800 µl in Experiments 1-3; 250 µl in Experiments 4-7) was added to each 12 x 75 mm assay tube containing 200 µl ^{3}H-estradiol solution.

Step 5.

After mixing, the tubes were incubated in a water bath for the specified time and temperature.

Step 6.

If the incubation temperature was above 0-4 C, incubation was terminated by chilling the assay tubes in ice-water for 10 min.

Step 7.

To each assay tube, a volume of ice-cold protamine sulfate (1 mg/ml buffer), equal to the ^{3}H-estradiol plus cytosol volume, was added. The contents of the tubes were mixed and allowed to stand for 15 min to precipitate proteins.

Step 8.

Each tube was diluted to capacity with ice-cold wash buffer (Tris-EDTA + 1% Tween-80) and centrifuged for 15 min at 5,000 x g at 0-4 C, using a swinging bucket rotor.

Step 9.

The supernatant (containing unbound ^{3}H-estradiol) was decanted and an additional 2 ml ice-cold wash buffer was added to the tube. The pellet was washed by mixing and recentrifuged as in Step 8.

Step 10.

The supernatant was decanted and all residual fluid drained by inverting the tube on a paper towel.

Step 11.

Bound ^{3}H-estradiol was eluted from the protamine-sulfate-protein pellet by incubation with 2 ml scintillation fluid (toluene:dioxane: methanol:napthalene:PPO:POPOP 36.2:35:21.7:7.3:4.6:0.08 wt/vol) for 15 min at 40 C, followed by mixing.

Step 12.

The scintillation fluid, containing the eluted ^{3}H-estradiol and pellet, was poured into mini-scintillation vials. An additional 3 ml of scintillation fluid was used to rinse the tube and was added to the mini-vial.

Step 13.

Vials were counted for ^{3}H for 20 min in a Beckman LS-133 scintillation counter at 29% efficiency.

Results

The first experiment was conducted to obtain preliminary estimates of estradiol binding in quail diencephalon and rat uterus. The quail represented a mixture of the High (HM) and Low Mating (LM) lines and been subjected to a 3:21 light:dark photoperiod for three days. The rats were six weeks of age and were, therefore, sexually immature. The experiment consisted of point assays conducted in triplicate. For these assays, cytosols from seven quail diencephalons and two rat uteri were diluted to 14 and 11 ml, respectively. Total binding was determined in cytosols after 90 min of incubation at 30 C with 5.76 nM ^{3}H-estradiol and roughly 0.05 rat uterus or 1.0 quail diencephalon/tube, followed by receptor precipitation with protamine sulfate. Nonspecific binding was determined in a parallel set of assay tubes, containing both ^{3}H-estradiol and a 100-fold molar excess of diethylstilbestrol. Specific binding, estimated as the difference between total and nonspecific binding, was $(6 \pm .3) \times 10^{2}$ fmoles/tube or 0.6 nM for the quail cytosols, and $(7 \pm .1) \times 10^{2}$ fmoles or 0.7 nM for the rat cytosols.

The six remaining experiments consisted of five saturation analyses (Experiments 2-6) and one set of point assays (Experiment 7). Experiments 2-5 were conducted to determine the range of ^{3}H-estradiol required for *in vitro* saturation of specific binding sites, and Experiments 6-7 were conducted to define the appropriate incubation conditions. Results obtained from these experiments are shown in Figures C.1-C.7. In all experiments, total binding was determined in

cytosols after incubation with ^{3}H-estradiol, followed by receptor precipitation with protamine sulfate. Nonspecific binding was determined in a parallel set of assay tubes, containing both ^{3}H-estradiol and a 100-fold molar excess of diethylstilbestrol. Specific binding was estimated as the difference between total and nonspecific binding points. Detailed specifications not listed in the General Assay Procedure above have been included in the descriptions below.

In Experiment 2, saturation curves were constructed from a six-point saturation analysis ranging from 0.10 to 1.71 n$\underline{M}$ ^{3}H-estradiol (Part A of Figure C.1). The assays were conducted using a uterus from a six-week old, sexually immature rat. Binding was determined in cytosols after 90 min of incubation at 30 C with ^{3}H-estradiol and roughly 0.05 uterus per tube. Scatchard analysis of the specific binding data points from Part A estimated the receptor binding parameters at, K_d=2.26 n$\underline{M}$, and B_{max}=1,451 fmoles/uterus (r=-0.6; Part B of Figure C.1).

In Experiment 3, saturation curves were constructed from a six-point saturation analysis ranging from 0.13 to 3.59 n$\underline{M}$ ^{3}H-estradiol (Part A of Figure C.2). The assays were conducted using diencephalons from seven male Japanese quail of the HM and LM lines, subjected to a 3:21 light:dark photoperiod for four days. Binding was determined in cytosols after 90 min of incubation at 30 C with ^{3}H-estradiol and roughly 0.5 diencephalon/tube. In contrast to Figure C.1, the curves show that at all points, nonspecific binding represented the larger

portion of total binding, and that saturation of specific binding sites was not achieved. Scatchard analysis of the specifc binding data points from Part A estimated the receptor binding parameters at, K_d=4.44 nM, and B_{max}=9.2 fmoles/diencephalon (r=-0.3; Part B of Figure C.2). The Scatchard curve was considered to be invalid because all points were beyond the sensitivity of the assay, that is, less than two femtomoles of receptor/tube (Kelner et al., 1980). This indicated that either too little receptor was present in each assay tube, and/or that too much receptor was being degraded during incubation.

In Experiment 4, saturation curves were constructed from a seven-point saturation analysis ranging from 0.01 to 1.51 nM ^{3}H-estradiol (Part A of Figure C.3). The assays were conducted in duplicate using diencephalons from 28 male Japanese quail of the HM_1 line, subjected to a 3:21 light:dark photoperiod for 12 days. Binding was determined in cytosols after 90 min of incubation at 30 C with ^{3}H-estradiol and roughly 1.4 diencephalons/tube. Although it appeared that the percentage of nonspecific binding was reduced by concentrating 2.8 x the receptor/tube that was used in Experiment 3, saturation of specific binding sites was not achieved within the range of ^{3}H-estradiol studied. In addition, Scatchard analysis of the specific binding data points from Part A failed to estimate the receptor binding parameters (Part B of Figure C.3). All but two points represented binding of less than two femtomoles of receptor/tube and, therefore, were beyond sensitivity of the assay.

In Experiment 5, saturation curves were constructed from a seven-point saturation analysis ranging from 0.29 to 6.23 nM ^{3}H-estradiol (Figure C.4). The assays were conducted using diencephalons from 29 male Japanese quail of the LM_1 line, subjected to a 3:21 light:dark photoperiod for 19 days. Binding was determined in cytosols after 90 min of incubation at 30 C with ^{3}H-estradiol and roughly 1.4 diencephalons/tube. In comparison to Figure C.3, saturation of specific binding sites was achieved, although the percentage of nonspecific binding was high. For this reason, Scatchard analysis of the specific binding points from Part A failed to estimate the receptor binding parameters (r=-0.2; Part B of Figure C.4). As in Figures C.2 and C.3, a large proportion of the plotted points represented binding of less than two femtomoles of receptor/tube, the minimum sensitivity of the assay. These results indicated that an inordinate amount of receptor was being degraded during incubation at 30 C for 90 minutes.

In Experiment 6, saturation curves were constructed from a seven-point saturation analysis ranging from 0.29 to 6.23 nM ^{3}H-estradiol (Part A of Figure C.5). The assays were started simultaneously with Experiment 5, using the same pool of diencephalons. Binding was determined in cytosols after overnight incubation at 4 C with ^{3}H-estradiol and roughly 1.4 diencephalons/tube. Overnight incubation at 4 C was selected because these are the conditions recommended for measuring the very thermolabile progesterone-receptor species (Clark and Peck, 1980), and the application of these incubation conditions to

the quail estradiol binding system was expected to provide an indication of the degree of thermolability of the quail receptor. That is, if the quail estradiol-receptor is as thermolabile as the progesterone-receptor, then overnight incubation at 4 C should also be appropriate for measuring the quail receptor. As in Figure C.4, saturation of specific binding sites was achieved although the percentage of nonspecific binding was high.

Again, Scatchard analysis of the specific binding points from Part A failed to estimate the receptor binding parameters (Part B of Figure C.5). Instead, the plotted points formed a "hump" which was considered to be indicative of nonequilibrium incubation conditions (Clark and Peck, 1980). These results suggested, that, although the quail estradiol-receptor appeared to be more thermolabile than the rat estradiol-receptor studied in Experiment 2, incubation at 4 C was not appropriate for measuring quail estradiol-receptor. Therefore, it was concluded that the optimum temperature for incubation of quail estradiol-receptors would be between 4 and 30 C. According to receptor assay theory, it should also be possible to obtain equilibration of quail receptor by incubation at 4 C for an extended period of time (Schrader and O'Malley, 1980). Nevertheless, to assure measurement of total receptor (occupied and unoccupied), it would be necessary to invoke steroidal exchange by incubating receptor at elevated temperature. This method of receptor assay would compensate for any endogenously bound estradiol that resulted from a basal level of adrenal

steroidogenesis. Based on the literature concerning rat hypothalamic estradiol-receptors, it was felt that the incubation conditions appropriate for measuring quail estradiol-receptors would be similar to the 30 C, 90 min scheme.

Experiment 7 was conducted to compare specific binding by quail diencephalic cytosol estradiol-receptors subjected to nine different incubation time x temperature combinations. Estimates of specific binding were obtained from point assays conducted in duplicate using a 3 x 3 (time x temperature) factorial arrangement of incubation treatments. The assays were conducted with diencephalons from 40 male Japanese quail of the HM_1 and LM_1 lines, subjected to a 3:21 light:dark photoperiod for 14 days. Binding was determined in cytosols after 60, 90, or 120 min of incubation at 25, 30, or 35 C with 3.14 nM ^{3}H-estradiol and roughly 1.5 diencephalons/tube. The data in Figure C.6, plotted in Part A within time treatments, and in Part B, within temperature treatments, show that maximum specific binding was achieved after 60 min of incubation at 25 centigrade. An alternate expression of these data is presented in Figure C.7, a comparison of total and nonspecific binding as a percentage of free ^{3}H-estradiol remaining after incubation. The latter figure indicates that nonspecific binding was at a minimum relative to total binding after 60 min of incubation at 25 centigrade.

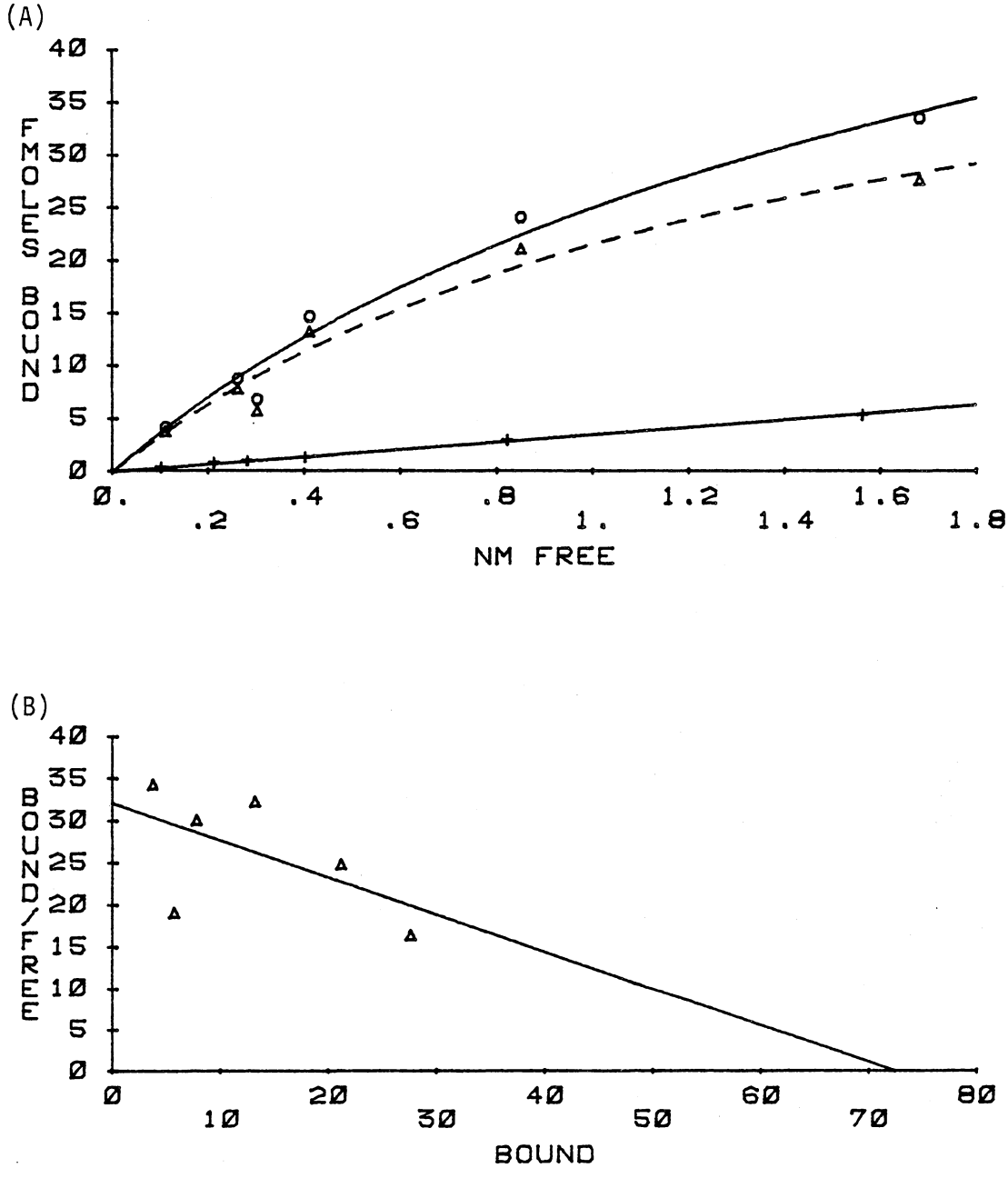


Figure C.1. Experiment 2: Saturation analysis of rat uterine cytosol estradiol-receptor. (A) Saturation curves of total (o—o), nonspecific (+—+), and specific (Δ--Δ) binding; (B) Scatchard analysis of the specific binding data points from Part A.

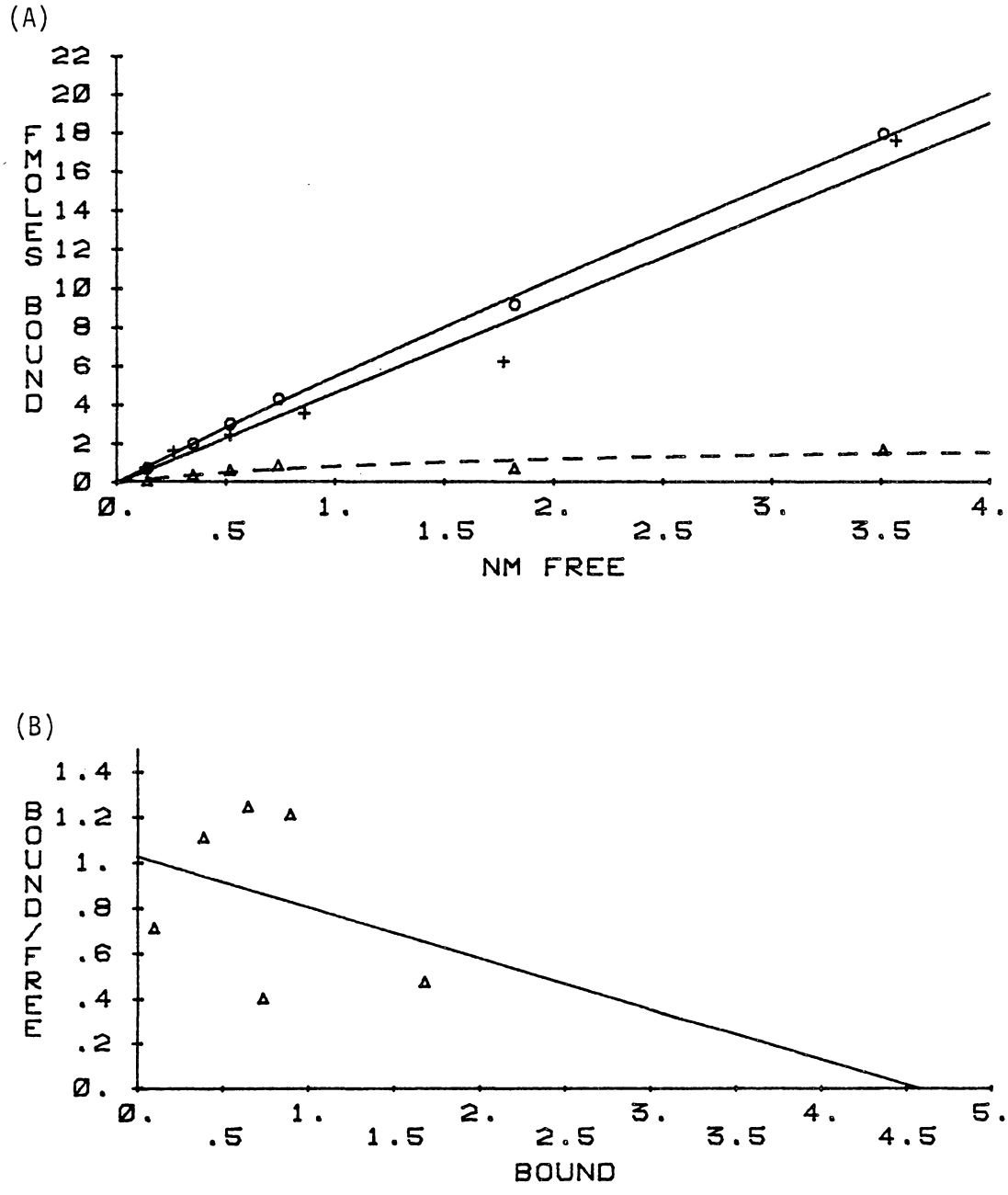


Figure C.2. Experiment 3: Saturation analysis of quail diencephalic cytosol estradiol-receptor. (A) Saturation curves of total (o—o), nonspecific (+—+), and specific (Δ--Δ) binding; (B) Scatchard analysis of the specific binding data points from Part A.

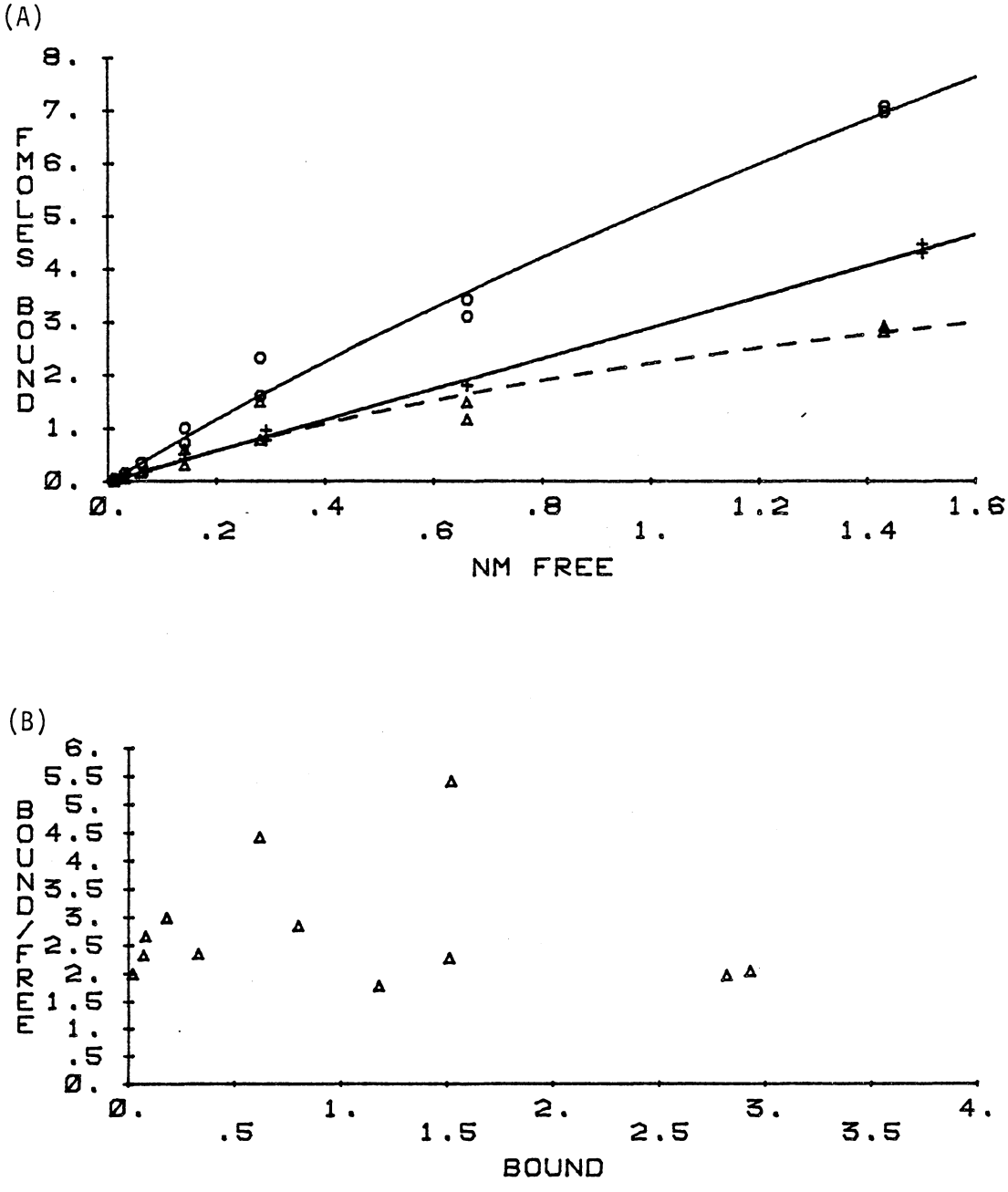


Figure C.3. Experiment 4: Saturation analysis of quail diencephalic cytosol estradiol-receptor. (A) Saturation curves of total (o—o), nonspecific (+—+), and specific (Δ--Δ) binding; (B) Scatchard analysis of the specific binding data points from Part A.

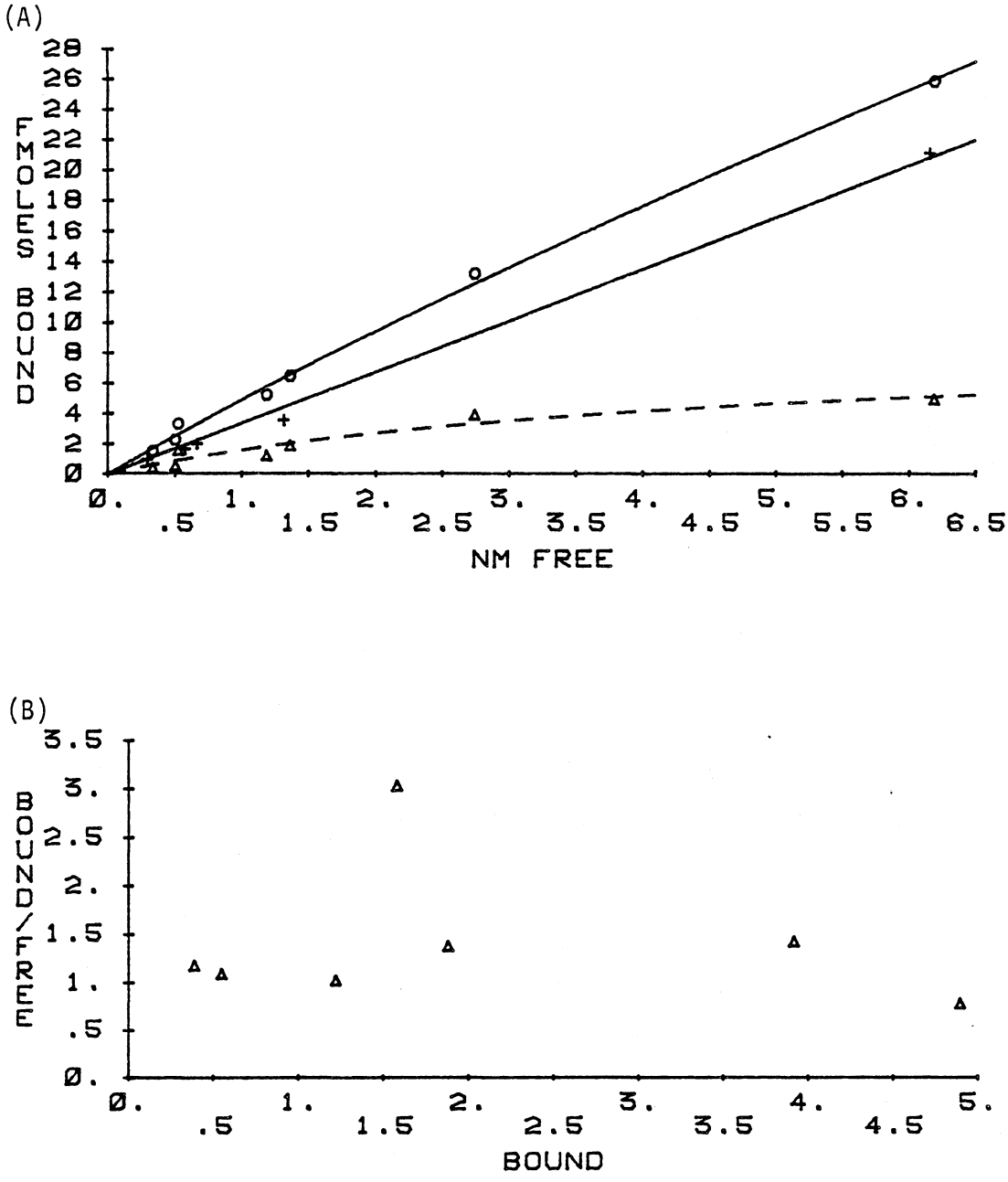


Figure C.4. Experiment 5: Saturation analysis of quail diencephalic cytosol estradiol-receptor. (A) Saturation curves of total (o—o), nonspecific (+—+), and specific (Δ--Δ) binding; (B) Scatchard analysis of the specific binding data points from Part A.

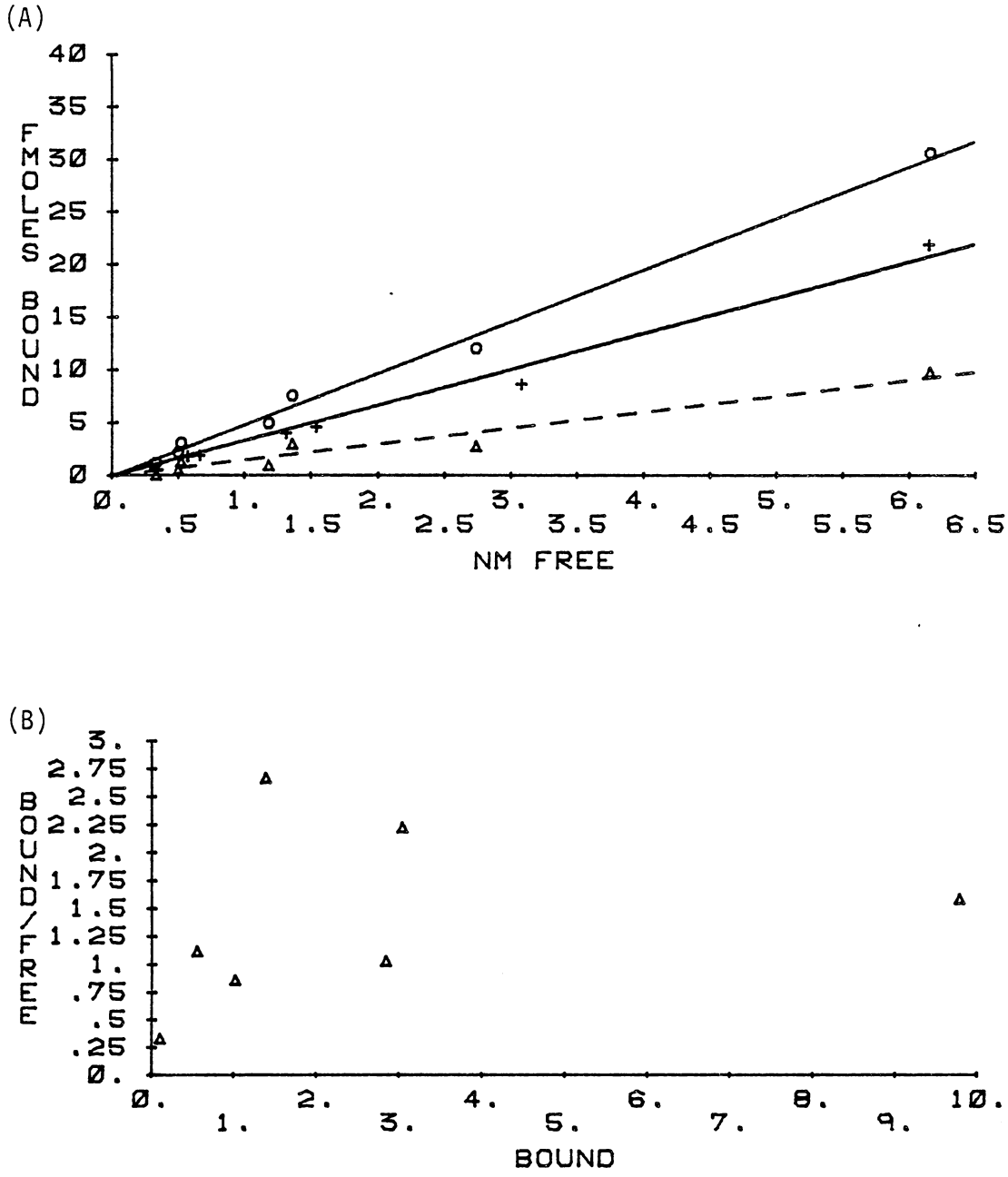


Figure C.5. Experiment 6: Saturation analysis of quail diencephalic cytosol estradiol-receptor. (A) Saturation curves of total (o—o), nonspecific (+—+), and specific (Δ--Δ) binding; (B) Scatchard analysis of the specific binding data points from Part A.

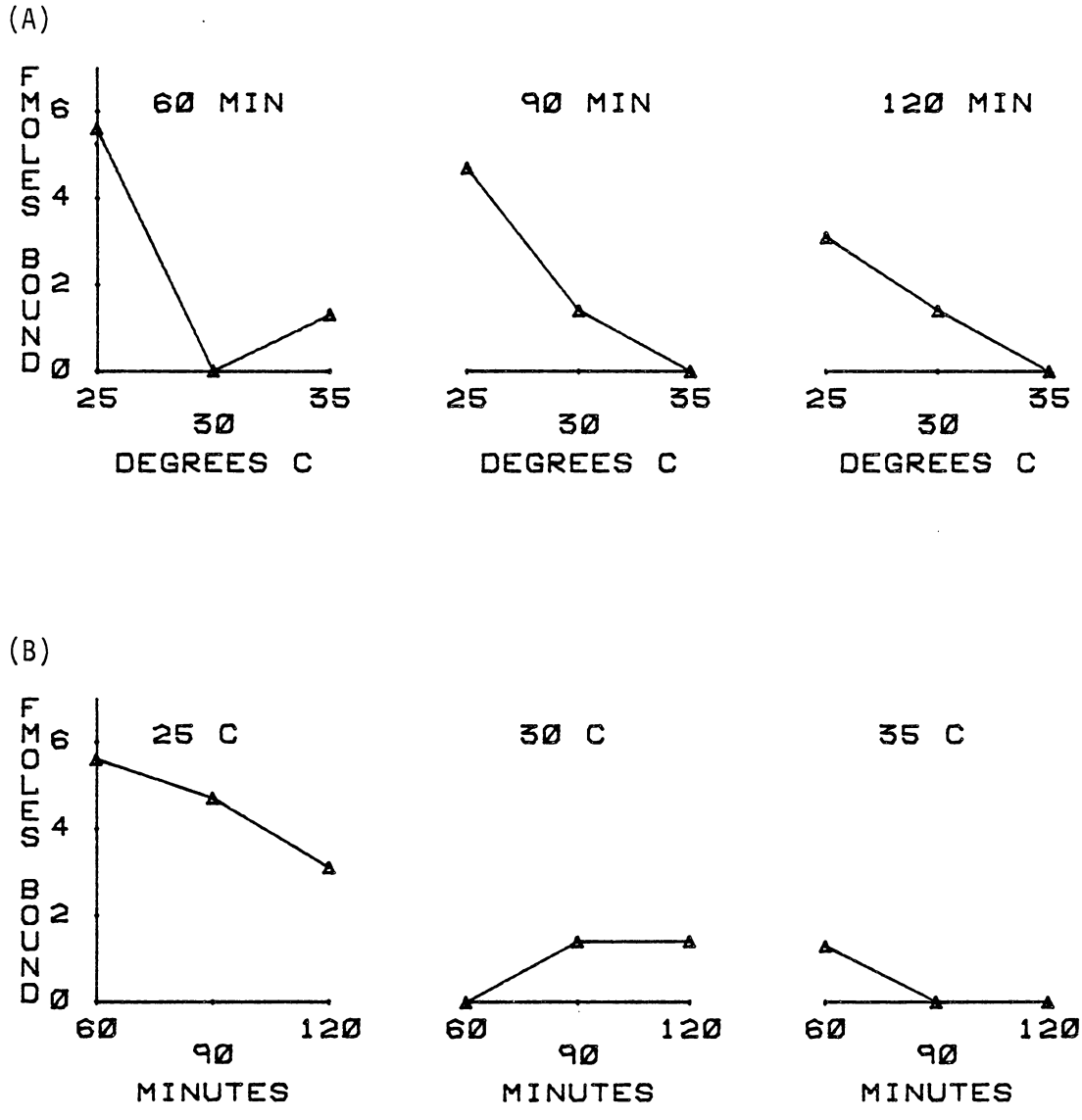


Figure C.6. Experiment 7: 1. A comparison of specific binding by quail diencephalic estradiol-receptors subjected to nine different incubation time x temperature combinations. (A) Data plotted within time treatments; (B) data plotted within temperature treatments.

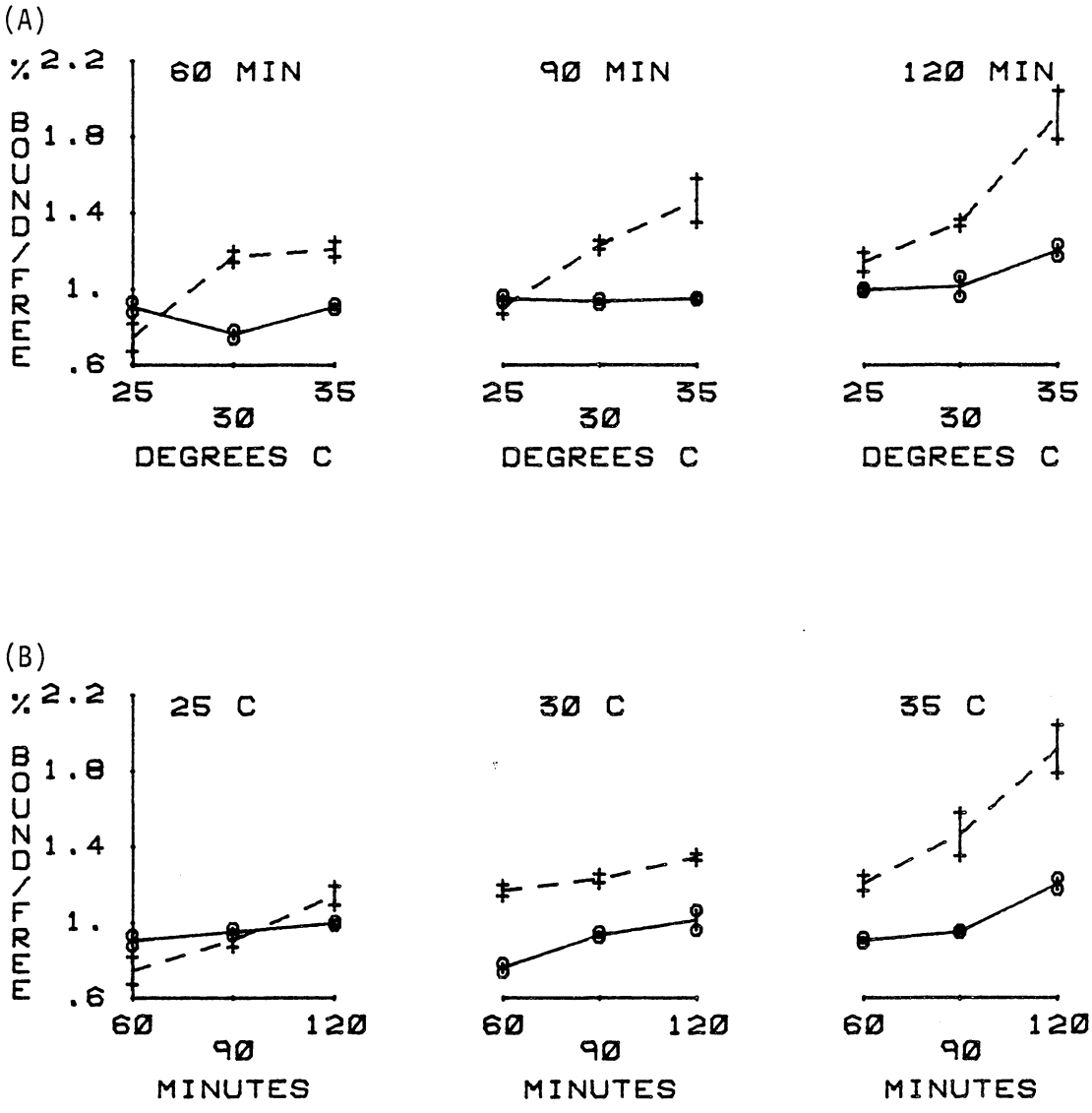


Figure C.7. 2. A comparison of total (o—o) and nonspecific (+—+) binding as a percent of free ^{3}H-estradiol remaining after incubation of quail diencephalic cytosols with nine different incubation time x temperature combinations. (A) Data plotted within time treatments; (B) data plotted within temperature treatments.

APPENDIX D

Validation of a Nonlinear Least-Squares Method for Estimating Receptor Binding Parameters

The literature contains three basic approaches to receptor parameter estimation by Scatchard analysis; the graphical, simple linear regression, and nonlinear curve fitting methods (Munson and Rodbard, 1980; Munck, 1976). In both the graphical and simple linear procedures, point estimates of specific binding are obtained from the differences between total and nonspecific point measurements, and the specific binding points are plotted by the method of Scatchard (1949). In the older graphical method, the Scatchard curve is drawn by "eyeball" fitting a negatively sloped line to the plotted points. With the more common linear approach, the Scatchard curve is instead drawn by least-squares regression of the plotted points. Alternatively, the nonlinear curve fitting method fits a hyperbolic saturation curve to point measurements of total binding, and the parameter estimates are obtained directly as a result of nonlinear regression.

Problems inherent to the graphical and simple linear methods have been discussed by Munson and Rodbard (1980). In general, Scatchard transformation using both the simple linear and graphical methods often distorts any measurement errors within duplicate total and/or nonspecific assay points. Due to technical problems associated with receptor assays, total and nonspecific point values may be such that an occasional negative value results for specific binding (ibid). Though the technique of simple linear regression is widely used, Munson and Rodbard (1980) have shown that parameter esimates are often distorted "due to correlated, non-uniform errors in both of the coordinates of the Scatchard plot".

To validate use of the nonlinear procedure in the present study, the data were analyzed by both regression methods. Scatchard analyses obtained by nonlinear curve fitting in Experiment II have been reproduced in Figures D.1-D.4. with corresponding results of the simple linear techinque superimposed. As shown in the captions to each of these figures, measurement errors were magnified with the simple linear technique. Whereas significant parameter estimates could not be obtained for the data in Figure D.2 using the traditional linear technique, significant parameter estimates were obtained with the curvilinear technique ($P \leq .06$). However, in Figures D.3 and D.4, binding was sufficiently low to preclude parameter estimation by either technique. Thus, to further exemplify the effect of the curvilinear technique, data from Appendix C were reanalyzed by nonlinear regression.

The latter assays were conducted to determine equilibrium conditions for measuring quail estradiol receptor, and, for comparsion to published data on rats, the data in Appendix C had been analyzed only by the traditional linear technique. With exception of the rat uterine assay shown in Figure D.5, parameter estimates obtained by either technique were not significantly different from zero (Tables D.6-D.10). These results were expected because saturation curves for the rat uterine receptor were representative of equilibrium conditions whereas the quail saturation curves were not (Clark and Peck, 1980). These results have been summarized in Tables D.11 and D.12. In addition to the parameter estimates obtained with Marquardt's nonlinear method, those obtained with the Gauss-Newton and Steepest Descent methods were also included in Table D.11 for comparison.

The relationship between nonlinear parameter estimation in the Saturation coordinate system and receptor parameter estimation in the Scatchard coordinate system may be described as follows:[1]

Given the saturation curve on the right,

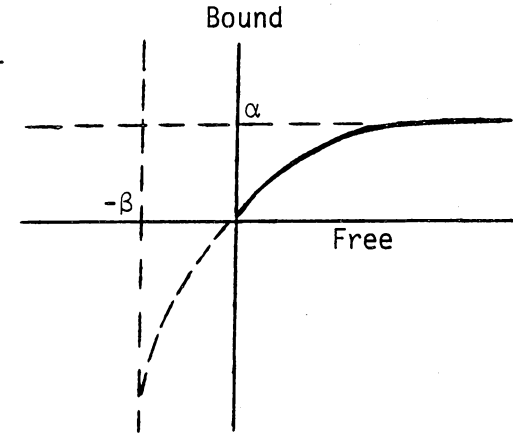
$$\text{Bound} = f(\text{Free}) = \frac{-\alpha\beta}{\text{Free} + \beta} + \alpha = \frac{\alpha\text{Free}}{\text{Free} + \beta}$$

then,

$$\text{Free} = \frac{-\alpha\beta}{\text{Bound} - \alpha} - \beta$$

$$= \frac{-\alpha\beta - \beta\text{Bound} + \alpha\beta}{\text{Bound} - \alpha}$$

$$= \frac{-\beta\text{Bound}}{\text{Bound} - \alpha}$$



therefore,

$$\frac{\text{Bound}}{\text{Free}} = \frac{\text{Bound}}{-\beta\text{Bound}/\text{Bound}-\alpha}$$

$$= \frac{\text{Bound} - \alpha}{-\beta}$$

$$= -\frac{1}{\beta}\text{Bound} + \frac{\alpha}{\beta}$$

thus, $\frac{\text{Bound}}{\text{Free}}$ is a linear function of Bound, with a slope of $-\frac{1}{\beta}$, an intercept on the ordinate of $\frac{\alpha}{\beta}$, and an intercept on the abcissa of α.

*This is the Scatchard equation.

[1]A detailed quantitative discussion may be found in Munck (1976).

Table D.1. Least-squares summary statistics for saturation analysis of diencephalic cytosol estradiol-receptors from the 16-day, HM_2 line castrates described in Figure II.2.

(A) Simple linear regression of nonspecific binding using the model $y = mx$.

Source	d.f.	SS	MS	F value	PR > F	R^2	Slope estimate ± SE
Regression	1	481.23	481.23	529.93	.0001	.97	2.08 ± .09
Residual	17	15.44	.91				
Total	18	496.67					

(B) Marquardt's nonlinear regression of total binding using the model $y = 2.08x + (\frac{\alpha x}{x + \beta})$

Source	d.f.	SS	MS	Parameter	Estimate ± SE	t value	PR > F
Regression	2	1490.19	745.10	α	5.35 ± .20	26.75	.001
Residual	16	2.10	.13	β	.40 ± .06	6.67	.001
Total							
Uncorrected	18	1492.29					
Corrected	17	334.69					

Table D.2. Least-squares summary statistics for saturation analysis of diencephalic cytosol estradiol-receptors from the 15-day, LM_2 line castrates described in Figure II.3.

(A) Simple linear regression of nonspecific binding using the model $y = mx$.

Source	d.f.	SS	MS	F value	PR > F	R^2	Slope estimate ± SE
Regression	1	202.59	202.59	2070.50	.0001	.99	1.83 ± .04
Residual	15	1.47	.10				
Total	16	204.06					

(B) Marquardt's nonlinear regression of total binding using the model $y = 1.83x + (\frac{\alpha x}{x + \beta})$

Source	d.f.	SS	MS	Parameter	Estimate ± SE	t value	PR > F
Regression	2	1542.63	771.32	α	5.92 ± .65	9.11	.001
Residual	16	22.71	1.42	β	.41 ± .20	2.05	.060
Total							
Uncorrected	18	1565.34					
Corrected	17	305.32					

Table D.3. Least-squares summary statistics for saturation analysis of diencephalic cytosol estradiol-receptors from the 24-day, LM_1 line castrates described in Figure II.5.

(A) Simple linear regression of nonspecific binding using the model $y = mx$.

Source	d.f.	SS	MS	F value	PR > F	R^2	Slope estimate ± SE
Regression	1	943.79	943.79	1539.12	.0001	.99	3.20 ± .08
Residual	17	10.42	.61				
Total	18	954.21					

(B) Marquardt's nonlinear regression of total binding using the model $y = 3.20x + (\frac{\alpha x}{x + \beta})$

Source	d.f.	SS	MS	Parameter	Estimate ± SE	t value	PR > F
Regression	2	2052.52	1026.26	α	3.31 ± .38	8.71	.001
Residual	16	12.02	.75	β	.17 ± .13	1.31	.211
Total							
Uncorrected	18	2064.54					
Corrected	17	547.83					

Table D.4. Least-squares summary statistics for saturation analysis of diencephalic cytosol estradiol-receptors from the 24-day, HM_1 line castrates as described in Figure II.6.

(A) Simple linear regression of nonspecific binding using the model $y = mx$.

Source	d.f.	SS	MS	F value	PR > F	R^2	Slope estimate ± SE
Regression	1	298.53	298.53	901.63	.0001	.98	1.95 ± .06
Residual	17	5.63	.33				
Total	18	304.16					

(B) Marquardt's nonlinear regression of total binding using the model $y = 3.20x + (\frac{\alpha x}{x + \beta})$

Source	d.f.	SS	MS	Parameter	Estimate ± SE	t value	PR > F
Regression	2	281.01	140.50	α	-.01 ± .05*	-	-
Residual	16	1.76	.11	β	-3.25 ± .21*	-	-
Total							
Uncorrected	18	282.77					
Corrected	17	93.82					

*Illogical parameter estimates.

Table D.5. Least-squares summary statistics for saturation analysis of diencephalic cytosol estradiol-receptors from the 30-day, HM_2 line castrates, as described in Figure II.7.

(A) Simple linear regression of nonspecific binding using the model $y = mx$.

Source	d.f.	SS	MS	F value	PR > F	R^2	Slope estimate ± SE
Regression	1	227.63	227.63	476.46	.0001	.98	1.93 ± .09
Residual	8	3.82	.48				
Total	9	231.45					

(B) Marquardt's nonlinear regression of total binding using the model $y = 1.93x + (\frac{\alpha x}{x + \beta})$

Source	d.f.	SS	MS	Parameter	Estimate ± SE	t value	PR > F
Regression	2	837.03	418.52	α	9.03 ± 3.09	2.92	.023
Residual	7	7.89	1.13	β	3.27 ± 2.24	1.46	.191
Total							
Uncorrected	9	844.92					
Corrected	8	263.63					

Table D.6. Least-squares summary statistics for saturation analysis of uterine cytosol estradiol-receptors from immature rat as described in Figure C.1.

(A) Simple linear regression of nonspecific binding using the model $y = mx$.

Source	d.f.	SS	MS	F value	PR > F	R^2	Slope estimate ± SE
Regression	1	41.29	41.29	4135.42	.0001	1.00	3.49 ± .05
Residual	5	.05	.01				
Total	6	41.34					

(B) Marquardt's nonlinear regression of total binding using the model $y = 3.49x + (\frac{\alpha x}{x + \beta})$

Source	d.f.	SS	MS	Parameter	Estimate ± SE	t value	PR > F
Regression	2	2040.95	1020.48	α	52.74 ± 11.91	4.43	.012
Residual	4	16.73	4.18	β	1.45 ± .55	2.64	.060
Total							
Uncorrected	6	2057.68					
Corrected	5	647.93					

Table D.7. Least-squares summary statistics for nonequilibrium saturation analysis of diencephalic cytosol estradiol-receptors from the 4-day, HM and LM line castrates, as described in Figure C.2.

(A) Simple linear regression of nonspecific binding using the model $y = mx$.

Source	d.f.	SS	MS	F value	PR > F	R^2	Slope estimate ± SE
Regression	1	364.35	364.35	344.58	.0001	.99	4.63 ± .25
Residual	5	5.29	1.06				
Total	6	369.64					

(B) Marquardt's nonlinear regression of total binding using the model $y = 4.63x + \left(\frac{\alpha x}{x + \beta}\right)$

Source	d.f.	SS	MS	Parameter	Estimate ± SE	t value	PR > F
Regression	2	438.02	219.01	α	2.16 ± .76	2.84	.048
Residual	4	.27	.07	β	1.57 ± 1.17	1.34	.265
Total							
Uncorrected	6	438.29					
Corrected	5	207.03					

Table D.8. Least-squares summary statistics for nonequilibrium saturation analysis of diencephalic cytosol estradiol-receptors from the 12-day, HM line castrates, as described in Figure C.3.

(A) Simple linear regression of nonspecific binding using the model $y = mx$.

Source	d.f.	SS	MS	F value	PR > F	R^2	Slope estimate ± SE
Regression	1	47.41	47.41	7466.60	.0001	1.00	2.91 ± .03
Residual	13	.08	.01				
Total	14	47.49					

(B) Marquardt's nonlinear regression of total binding using the model $y = 2.91x + (\frac{\alpha x}{x + \beta})$

Source	d.f.	SS	MS	Parameter	Estimate ± SE	t value	PR > F
Regression	2	129.74	64.89	α	6.95 ± 2.58	2.69	.021
Residual	12	.82	.07	β	2.12 ± 1.18	1.80	.098
Total							
Uncorrected	14	130.56					
Corrected	13	76.46					

Table D.9. Least-squares summary statistics for nonequilibrium saturation analysis of diencephalic cytosol estradiol-receptors from the 19-day, LM line castrates, as described in Figure C.4.

(A) Simple linear regression of nonspecific binding using the model $y = mx$.

Source	d.f.	SS	MS	F value	PR > F	R^2	Slope estimate ± SE
Regression	1	462.66	462.66	2011.87	.0001	1.00	3.38 ± .08
Residual	4	.92	.23				
Total	5	463.58					

(B) Marquardt's nonlinear regression of total binding using the model $y = 3.38x + \left(\frac{\alpha x}{x + \beta}\right)$

Source	d.f.	SS	MS	Parameter	Estimate ± SE	t value	PR > F
Regression	2	925.24	462.62	α	8.81 ± 2.52	3.50	.019
Residual	5	1.39	.28	β	4.55 ± 2.28	2.00	.103
Total							
Uncorrected	7	926.63					
Corrected	6	450.03					

Table D.10. Least-squares summary statistics for nonequilibrium saturation analysis of diencephalic cytosol estradiol-receptors from the 19-day, LM line castrates, as described in Figure C.5.

(A) Simple linear regression of nonspecific binding using the model $y = mx$.

Source	d.f.	SS	MS	F value	PR > F	R^2	Slope estimate ± SE
Regression	1	597.66	597.66	788.95	.0001	.99	3.38 ± .12
Residual	6	4.54	.76				
Total	7	602.20					

(B) Marquardt's nonlinear regression of total binding using the model $y = 3.38x + (\frac{\alpha x}{x + \beta})$

Source	d.f.	SS	MS	Parameter	Estimate ± SE	t value	PR > F
Regression	1	1181.22	1181.22	α	$(10.9 \pm .85)\times10^{10}$	NA	NA
Residual	6	4.06	.68	β	$(7.24 \pm 0)\times10^{10}$	NA	NA
Total							
Uncorrected	7	1185.28					
Corrected	6	635.61					

Table D.11. A comparison of the nonlinear least-squares parameter estimates obtained by the Marquardt, Gauss-Newton, and Steepest Descent Methods.[1]

	Parameter α			Parameter β		
Figure	Marquardt	Gauss-Newton	Steepest Descent*	Marquardt	Gauss-Newton	Steepest Descent*
II.2	5.35 ± .20	5.35 ± .20	5.37 ± .20	.40 ± .06	.40 ± .06	.40 ± .06
II.3	5.92 ± .65	5.92 ± .65	5.99 ± .66	.41 ± .20	.41 ± .20	.42 ± .20
II.5	3.31 ± .38	3.31 ± .38	3.31 ± .38	.17 ± .13	.17 ± .13	.17 ± .13
II.6	< 0	< 0	0	< 0	< 0	0
II.7	9.03 ± 3.09	9.03 ± 3.09	8.98 ± 3.06	3.27 ± 2.24	3.27 ± 2.24	3.23 ± 2.21
C.1	52.74 ± 11.91	52.74 ± 11.91	10.01 ± 8.69	1.45 ± .55	1.45 ± .55	.04 ± .28
C.2	2.16 ± .76	2.16 ± .76	2.15 ± .75	1.57 ± 1.17	1.57 ± 1.17	1.56 ± 1.16
C.3	6.95 ± 2.58	6.95 ± 2.58	6.55 ± 2.25	2.12 ± 1.18	2.12 ± 1.18	1.93 ± 1.02
C.4	8.81 ± 2.52	8.81 ± 2.52	8.76 ± 2.48	4.55 ± 2.28	4.55 ± 2.28	4.50 ± 2.24
C.5	$(10.9 \pm 0.85)\times10^{10}$	$2.17 \pm 0.17)\times10^{10}$*	---	$(7.24 \pm 0)\times10^{10}$	$(1.4 \pm 0)\times10^{10}$	---

[1]All algorithms were generated by SAS (Helwig and Council, 1979); where no values are presented, the algorithm failed to converge.

*Convergence assumed.

Table D.12. A comparison of the statistical significance of the nonlinear least-squares parameter estimates obtained by the Marquardt method.

		Parameter α			Parameter β		
Figure	df	Estimate ± SE	t value	PR > t	Estimate ± SE	t value	PR > t
II.2*	16	5.35± .20	26.75	.001	.40± .06	6.67	.001
II.3*	16	5.92± .65	9.11	.001	.41± .20	2.05	.060
II.5	16	3.31± .38	8.71	.001	.17± .13	1.31	.211
II.6	16	< 0	NA[1]	NA	< 0	NA	NA
II.7	7	9.03± 3.09	2.92	.023	3.27± 2.24	1.46	.191
C.1*	4	52.74±11.91	4.43	.012	1.45± .55	2.64	.060
C.2	4	2.16± .76	2.84	.048	1.57± 1.17	1.34	.265
C.3	14	6.95± 2.58	2.69	.021	2.12± 1.18	1.80	.098
C.4	5	8.81± 2.52	3.50	.019	4.55± 2.28	2.00	.103
C.5	6	$(10.9 \pm .85)\times10^{10}$	NA	NA	$(7.24\pm 0)\times10^{10}$	NA	NA

[1]NA = not applicable

*Both α and β are statistically significant ($P \leq .06$)

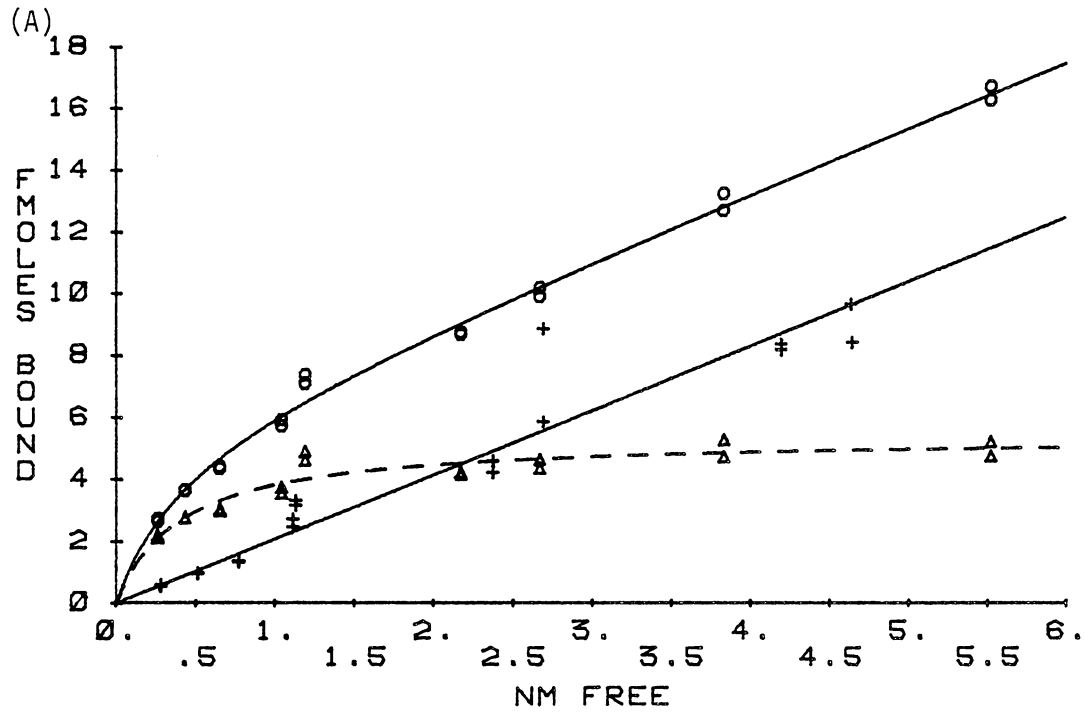
Figure D.1. Saturation analysis of diencephalic cytosol estradiol-receptors from 16-day, HM_2 line castrates: Nonlinear curve fitting versus the traditional simple least-squares method of analysis.[1]

A. Saturation curves of total (o——o), nonspecific (+——+), and specific (Δ---Δ) ^{3}H-estradiol binding were constructed from the curve fitting methods described in Appendix D. Points corresponding to the total and nonspecific binding curves were empirically derived whereas specific points were obtained by total and nonspecific point differences.

B. Scatchard analysis of specific binding was generated by the nonlinear curve fitting method (-----), or computed by the traditional method of simple least-squares regression of the plotted points (Δ——Δ). By either procedure, the parameter estimates of K_d and B_{max} were statistically significant, although the curvilinear procedure resulted in a 90% reduction in mean square error (MSE) as shown below.

Method	K_d	B_{max}	MSE	Significance level
Traditional	0.46	5.60	1.31	.05, r=-.89
Curvilinear	0.40	5.35	0.13	.001

[1]Data pertinent to the nonlinear curve-fitting method were obtained from Experiment II, Figure II.2


(A)
18
16
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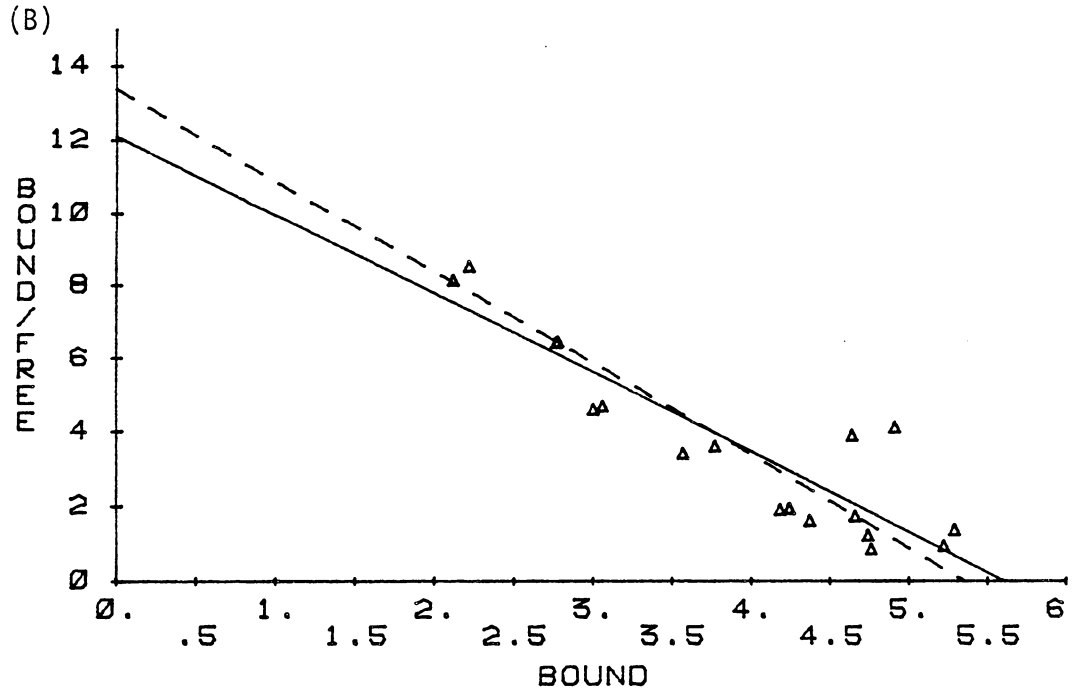
(B)
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BOUND

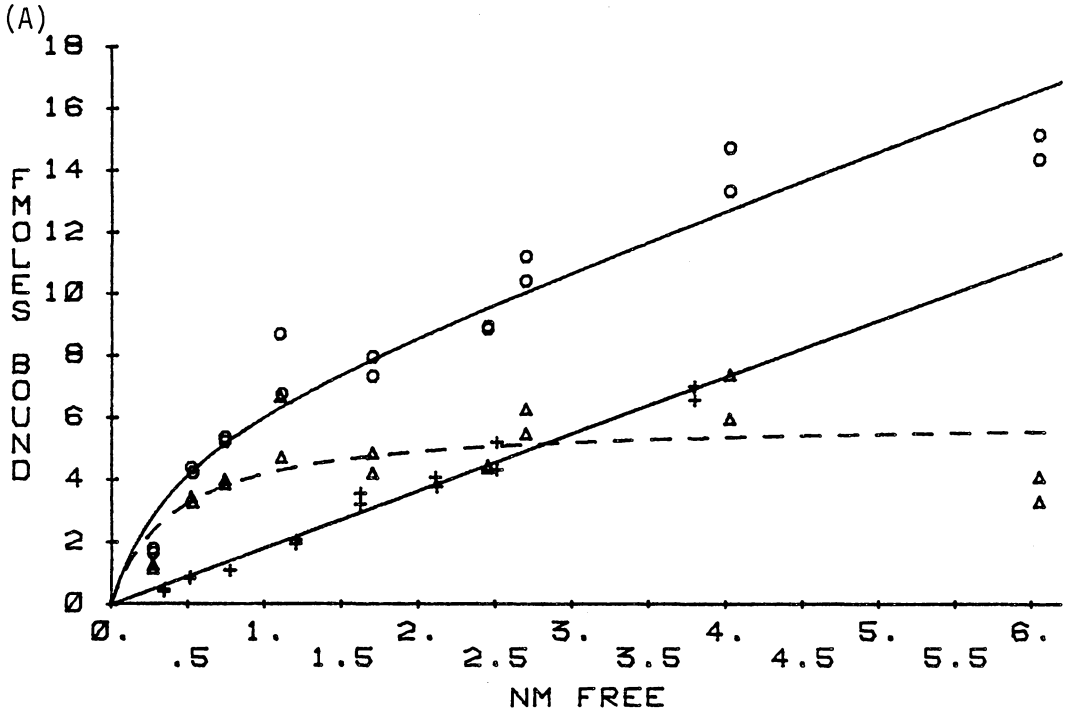
Figure D.2. Saturation analysis of diencephalic cytosol estradiol-receptors from 15-day, LM_2 line castrates: Nonlinear curve fitting versus the traditional simple least-squares method of analysis.[1]

A. Saturation curves of total (o——o), nonspecific (+——+), and specific (Δ---Δ) ^{3}H-estradiol binding were constructed from the curve fitting methods described in Appendix D. Points corresponding to the total and nonspecific binding curves were empirically derived whereas specific points were obtained by total and nonspecific point differences.

B. Scatchard analysis of specific binding was generated by the nonlinear curve fitting method (-----), or computed by the traditional method of simple least-squares regression of the plotted points (Δ——Δ). Significant parameter estimates for K_d and B_{max} were obtained only by the nonlinear curve fitting method. As shown below, a 63% reduction in mean square error (MSE) resulted from the curvilinear technique.

Method	K_d	B_{max}	MSE	Significance level
Traditional	2.98	14.38	3.81	nonsig, r=-.28
Curvilinear	0.41	5.92	1.42	.060, K_d; .001, B_{max}

[1]Data pertinent to the nonlinear curve-fitting method were obtained from Experiment II, Figure II.3


(A)
MOLES BOUND
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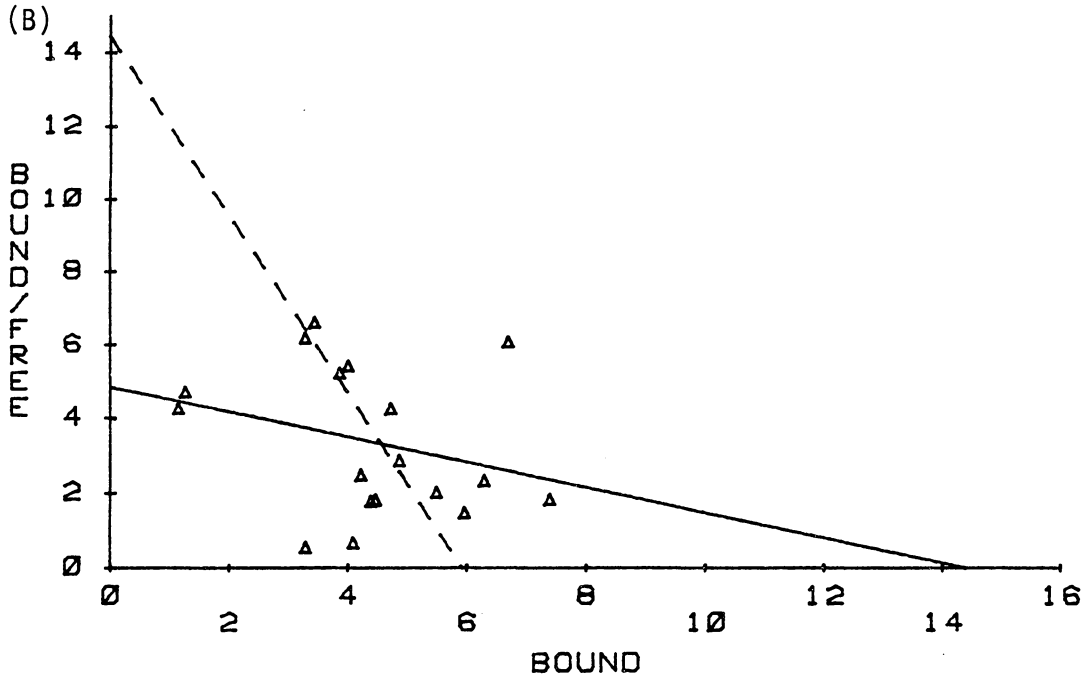
(B)
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16
BOUND

Figure D.3. Saturation analysis of diencephalic cytosol estradiol-receptors from 24-day, LM_1 line castrates: Nonlinear curve fitting versus the traditional simple least-squares method of analysis.[1]

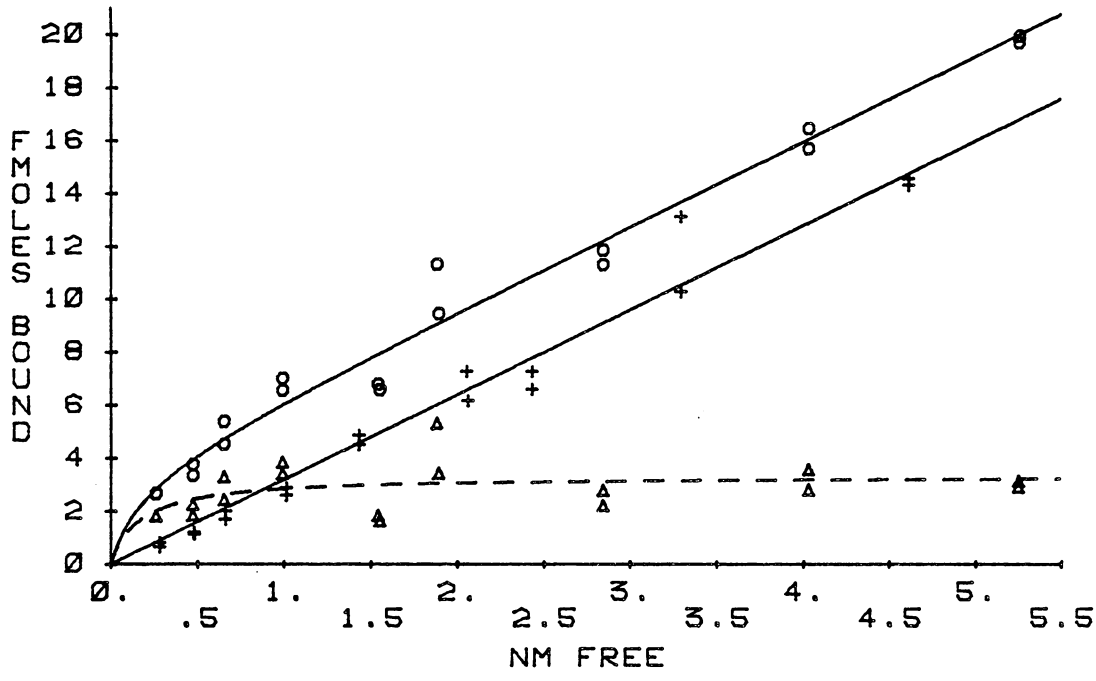
A. Saturation curves of total (o——o), nonspecific (+——+), and specific (Δ---Δ) ^{3}H-estradiol binding were constructed from the curve fitting methods described in Appendix D. Points corresponding to the total and nonspecific binding curves were empirically derived whereas specific points were obtained by total and nonspecific point differences.

B. Scatchard analysis of specific binding was generated by the nonlinear curve fitting method (-----), or computed by the traditional method of simple least-squares regression of the plotted points (Δ——Δ). Neither of the analysis methods resulted in statistically significant parameter estimates. The curvilinear technique did, however, result in an 85% reduction in mean square error (MSE) as shown below.

Method	K_d	B_{max}	MSE	Significance level
Traditional	2.27	9.20	4.97	nonsig, r=-.19
Curvilinear	0.17	3.31	0.75	nonsig

[1]Data pertinent to the nonlinear curve-fitting method were obtained from Experiment II, Figure II.5

(A)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(B)

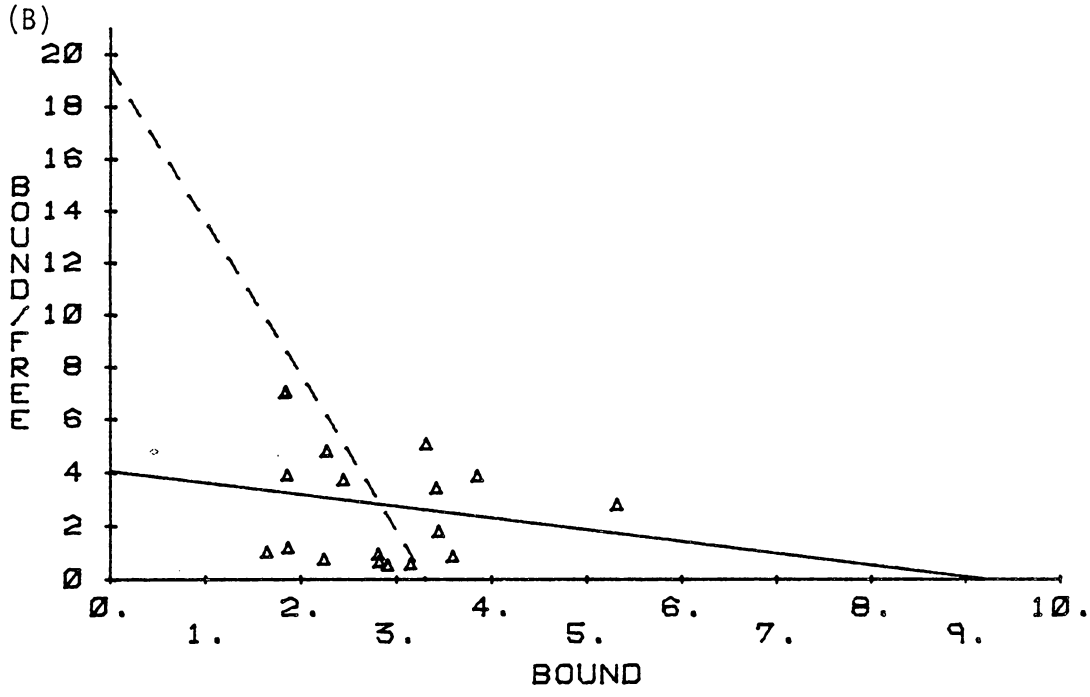
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Figure D.4. Saturation analysis of diencephalic cytosol estradiol-receptors from 30-day, HM_2 line castrates: Nonlinear curve fitting versus the traditional simple least-squares method of analysis.[1]

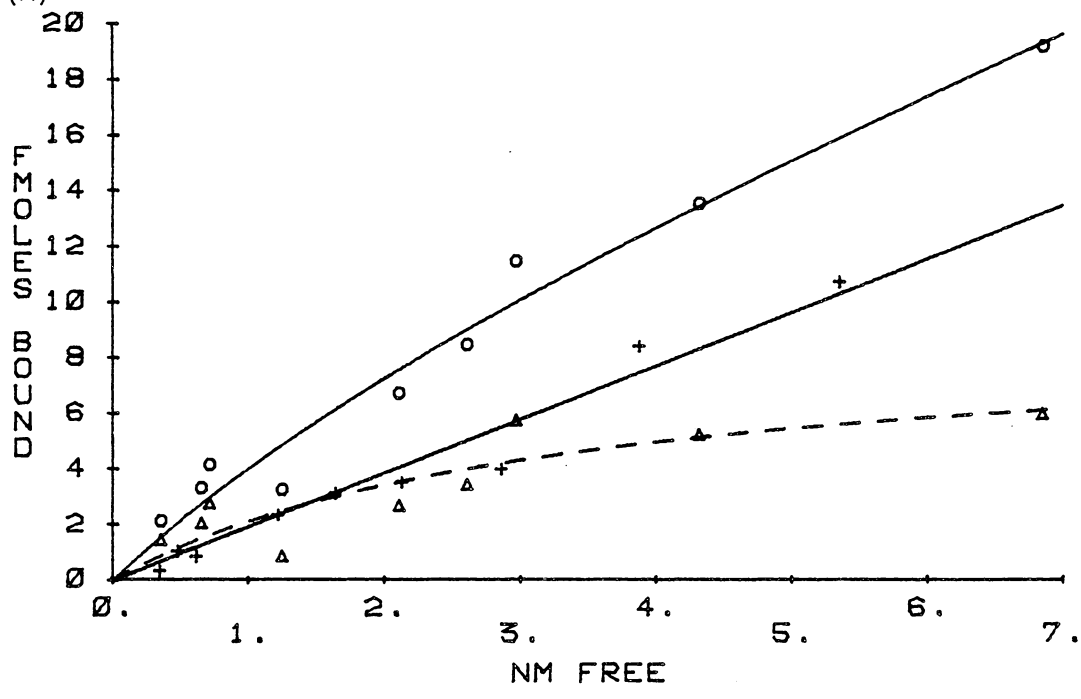
A. Saturation curves of total (o——o), nonspecific (+——+), and specific (Δ---Δ) ^{3}H-estradiol binding were constructed from the curve fitting methods described in Appendix D. Points corresponding to the total and nonspecific binding curves were empirically derived whereas specific points were obtained by total and nonspecific point differences.

B. Scatchard analysis of specific binding was generated by the nonlinear curve fitting method (-----), or computed by the traditional method of simple least-squares regression of the plotted points (Δ——Δ). Neither of the analysis methods resulted in statistically significant parameter estimates. The curvilinear technique did, however, result in an 36% reduction in mean square error (MSE) as shown below.

Method	K_d	B_{max}	MSE	Significance level
Traditional	3.72	11.04	1.76	nonsig, r=-.38
Curvilinear	3.27	9.03	1.13	nonsig

[1]Data pertinent to the nonlinear curve-fitting method were obtained from Experiment II, Figure II.7

(A)


FMOLES BOUND
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(B)

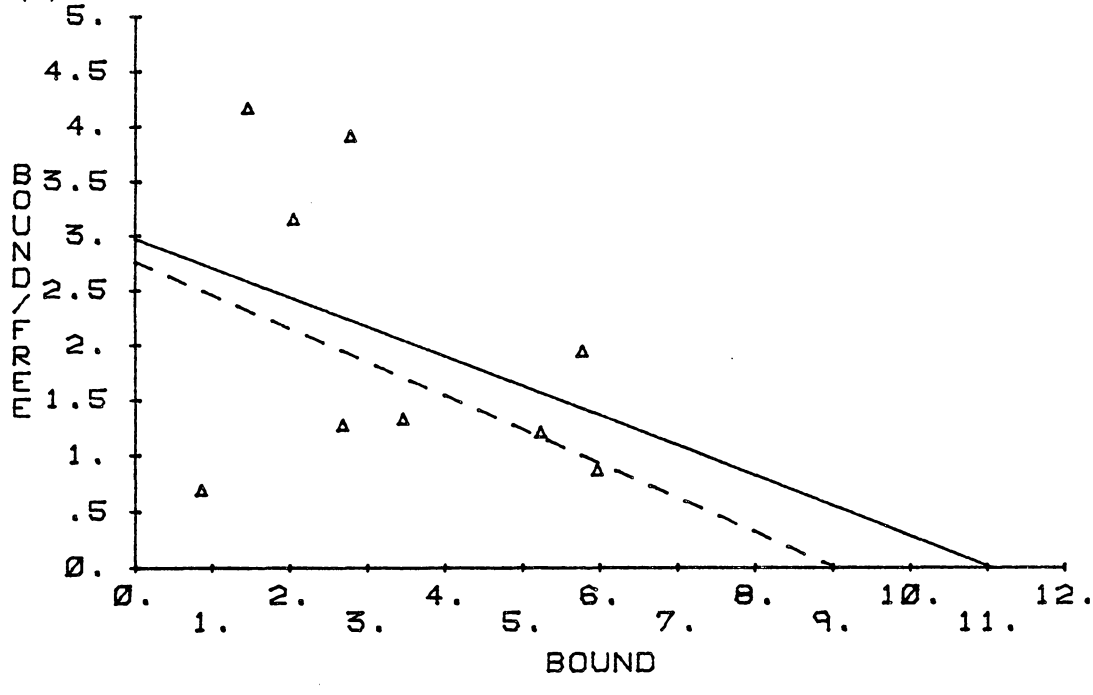
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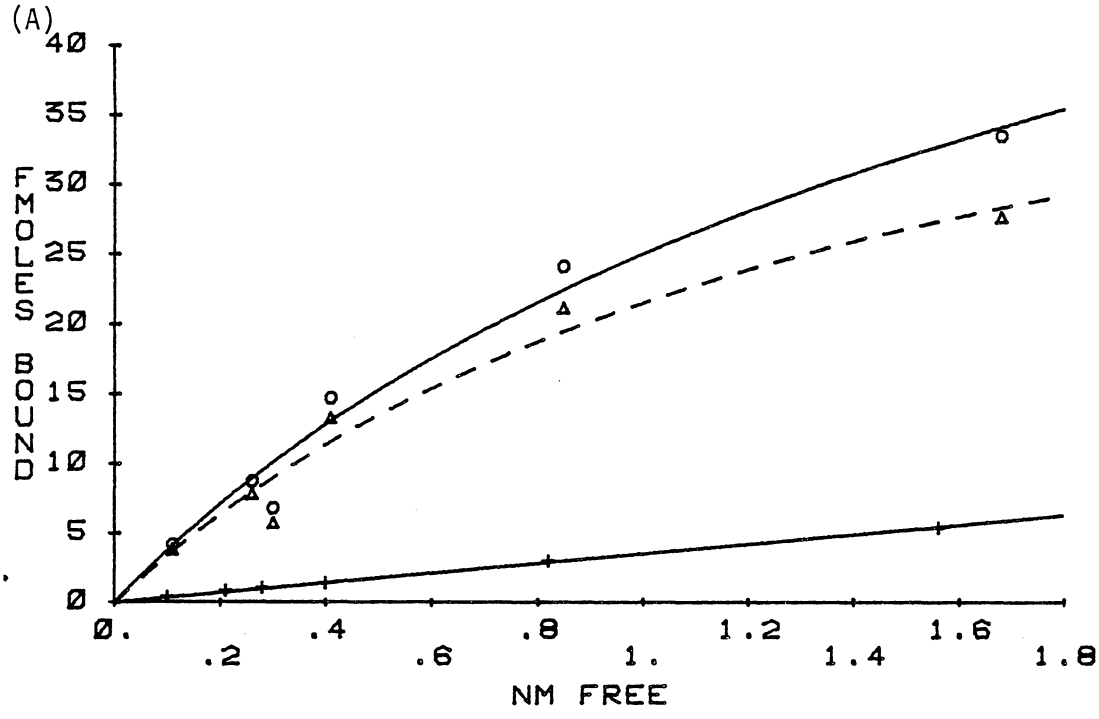
Figure D.5. Saturation analysis of uterine cytosol estradiol-receptors from immature rat: Nonlinear curve fitting versus the traditional simple least-squares method of analysis.[1]

A. Saturation curves of total (o——o), nonspecific (+——+), and specific (Δ---Δ) ^{3}H-estradiol binding were constructed from the curve fitting methods described in Appendix D. Points corresponding to the total and nonspecific binding curves were empirically derived whereas specific points were obtained by total and nonspecific point differences.

B. Scatchard analysis of specific binding was generated by the nonlinear curve fitting method (-----), or computed by the traditional method of simple least-squares regression of the plotted points (Δ——Δ). Significant parameter estimates for K_d and B_{max} were obtained only by the nonlinear curve fitting method. As shown below, a 91% reduction in mean square error (MSE) resulted from the curvilinear technique.

Method	K_d	B_{max}	MSE	Significance level
Traditional	2.26	72.53	44.97	nonsig, r=-.60
Curvilinear	1.45	52.74	4.18	.060, K_d; .012, B_{max}

[1]Data pertinent to the nonlinear curve-fitting method were obtained from Appendix Figure C.1.


(A)
FMOLES BOUND
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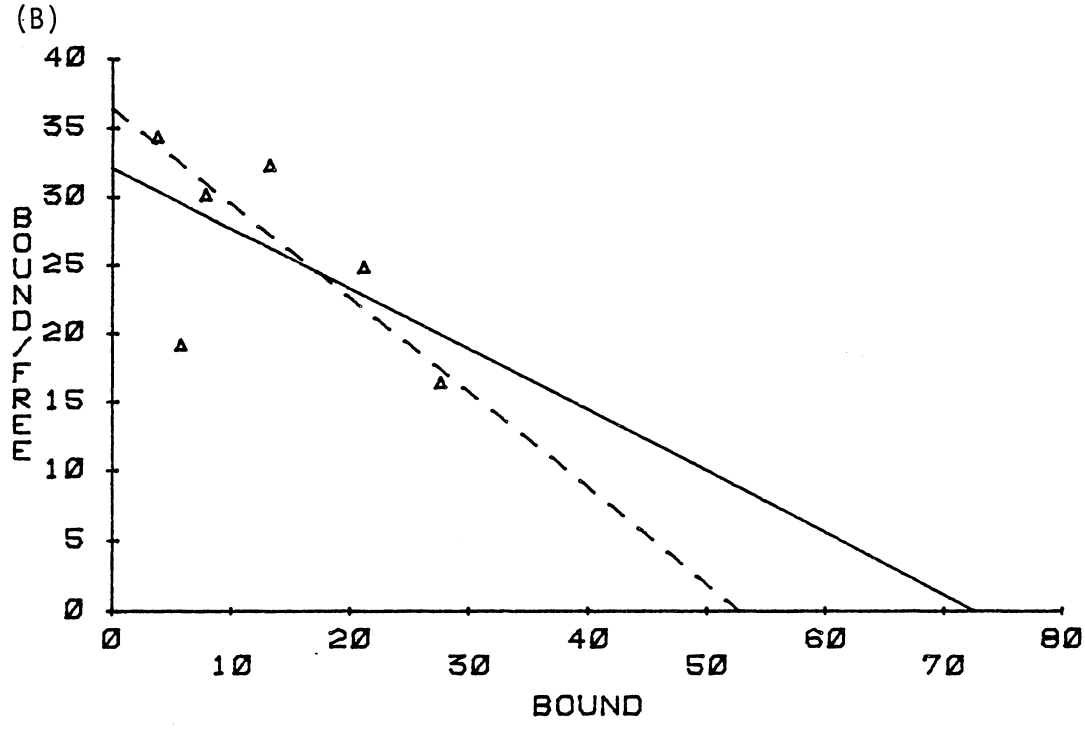
NM FREE


(B)
BOUND\FREE
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ANDROGEN AROMATIZATION AND CYTOSOL ESTRADIOL-RECEPTORS IN THE MEDIATION OF MASCULINE SEXUAL BEHAVIOR IN JAPANESE QUAIL

by

Martha Anne Cohen Parsons

(ABSTRACT)

The neuroendocrinology of masculine sexual behavior was examined in Japanese quail selected bi-directionally for adult mating frequency. Experiments were conducted with brain tissues from functionally castrated, High (HM) and Low Mating (LM) line quail to compare (1) the in vivo aromatization of ^{3}H-testosterone and (2) cytosolic estradiol-binding by in vitro saturation analyses.

After in vivo incubation with ^{3}H-testosterone, all radioactivity recovered in brain tissues was in the form of testosterone, dihydrotestosterone, or estradiol. Neither the total ^{3}H nor ^{3}H-testosterone metabolite radioactivity differed upon comparison of the two genetic lines. Of all ^{3}H-testosterone metabolite radioactivity, ^{3}H-estradiol represented 45 $\pm$ 6% in the HM line and 46 $\pm$ 6% in the LM line, indicating that the line difference in mating frequency was not due to a corresponding difference in aromatase activity. Inasmuch as both the HM and LM line birds actively converted testosterone to estradiol, these results implicated a neural mechanism involving estradiol-receptor interactions.

Estradiol-receptor binding parameters were estimated in subsequent experiments by fitting a hyperbolic saturation curve to point measurements of total binding. Using this single-class binding site model, the apparent dissociation constant (K_d) for the estradiol-receptor interaction in the HM line was 0.40 $\pm$ .06 nM and the number of specific estradiol binding sites (B_{max}) was 10.4 $\pm$ .4 fmoles/mg cytosol protein. For the LM line, an apparent K_d of 0.41 $\pm$ .20 nM and a B_{max} of 9.5 $\pm$ 1.0 fmoles/mg cytosol protein were obtained. The similarity between corresponding binding parameters for the divergent mating lines indicated that the number and/or estradiol-binding affinity of a single class of cytosol receptors was also not responsible for the line difference in mating frequency. Though these results confirm that interaction of estradiol with cytosol receptors was not the limiting neuroendocrinological mechanism in the differential expression of masculine sexual behavior in quail, the possible involvement of dynamic interrelationships between cytoplasmic and nuclear estrogen binding was discussed.