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**In vivo and in vitro uptake of (3h) Estradiol
in brain and pituitary of the female
Monogolian gerbil, *Meriones unguiculatus***

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IN VIVO AND IN VITRO UPTAKE OF (³H)ESTRADIOL IN
BRAIN AND PITUITARY OF THE FEMALE MONGOLIAN
GERBIL, MERIONES UNGUICULATUS

A Thesis Presented

By

JANET M. GRAY

Submitted to the Graduate School of the
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of the requirements for the degree of

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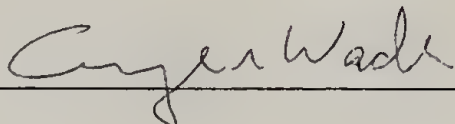
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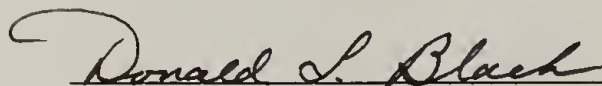
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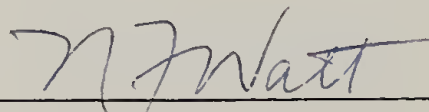
George N. Wade, Chairperson of Committee



Melinda A. Novak, Member



Donald L. Black, Member



Norman F. Watt, Department Head
Psychology

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ABSTRACT

Following injection of (^3H)estradiol in ovariectomized gerbils, the highest concentration of radioactivity was found in cell nuclei from the pituitary, followed by preoptic area, hypothalamus, amygdala and midbrain, with very low levels in the olfactory bulbs and cerebral cortex. Cell nuclear binding was significantly reduced by pretreatment with unlabeled estradiol and nafoxidine but not by progesterone, 5α -dihydrotestosterone or cortisol. The time course of estradiol binding in whole homogenate and cell nuclear fractions of brain and pituitary was determined by sacrificing animals .25, 1, 3, 6 or 12 hours after injection of (^3H)estradiol and measuring hormone uptake into the respective cell components. While whole homogenate radioactivity was highest at .25 hour, cell nuclear binding peaked at one hour. Radioactivity was still present in cell nuclei from pituitary, hypothalamus-preoptic area and amygdala 12 hours after (^3H)injection, although at levels only 2% of one hour peak levels.

Macromolecular binding of (^3H)estradiol was found in the cytoplasmic fraction of pooled hypothalamus-preoptic area-amygdala. Scatchard analysis of this in vitro binding indicated a K_d of $5.41 \times 10^{-10}\text{M}$. This binding is inhibited more by unlabeled estradiol and the synthetic estrogen, R2858, than by progesterone, 5α -dihydrotestosterone or cortisol. Pronase almost totally inhibited binding while RNase and

DNase had little effect.

These data indicate that the estradiol binding in female gerbil brain and pituitary is similar to that of other species previously studied.

INTRODUCTION

The role of ovarian hormones in the induction and maintenance of female sexual behavior has been studied extensively over the past several decades (for a review, see Lisk, 1973). Using ovariectomized animals, estradiol and progesterone levels necessary for the induction of behavioral estrus and the duration of estrus have been determined for the rat (Boling and Blandau, 1939; Blandau, Boling and Young, 1941), guinea pig (Collins et al., 1938; Boling, Young and Dempsey, 1938; Bullock, 1970), mouse (Ring, 1944) and hamster (Frank and Fraps, 1945).

Estradiol implantation studies in rat brain (Lisk, 1962; Ross et al., 1971) indicated that the preoptic area and the hypothalamus were involved in the control of female sexual behavior. Since then detailed studies have been completed, using radioactively labeled estradiol to localize and characterize putative binding sites for estradiol in the brain and pituitary of rats and other mammals.

Utilizing autoradiographic techniques in rats, (^3H)-estradiol concentrating cells were located in the anterior pituitary, preoptic area, hypothalamus, amygdala and, to a lesser extent, other regions of the limbic system. Most other regions of the brain had a very low density of labeled cells (Pfaff, 1968; Stumpf, 1968; Anderson and Greenwald, 1969; Sar and Stumpf, 1971; Pfaff and Keiner, 1973). Similar patterns of estradiol concentrating cells have been

found in other mammals including the squirrel monkey (Keefer and Stumpf, 1975) and the Rhesus monkey (Pfaff et al., 1976).

Using scintillation counting techniques, selective retention of ^3H -estradiol was again demonstrated in the rat hypothalamus, preoptic area, septum, amygdala and anterior pituitary (Eisenfeld and Axelrod, 1965; Kato and Villedy, 1967). Subcellular localization of ^3H - 17β -estradiol in the rat brain has shown that the highest level of the steroid retention occurs in the nuclei of the cells of these areas (Zigmond and McEwen, 1970). This cell nuclear uptake can be blocked by relatively large doses of nonradioactive 17β -estradiol, indicating saturability of the binding sites, but this hormone uptake is not blocked by pretreatment with the stereoisomer, 17α -estradiol, or nonestrogenic steroids, indicating specificity of the estradiol-binding site interaction (Kato and Villedy, 1967; Zigmond and McEwen, 1970; Eisenfeld, 1970).

The mechanism of action of estradiol in the brain and the pituitary remains to be elucidated. However, dosages of synthetic antiestrogens which are sufficient to block behavioral responses to estradiol also inhibit cell nuclear uptake of estradiol in the hypothalamus and preoptic area (Roy and Wade, 1976). This indicates that nuclear translocation of estradiol or the estradiol-receptor complex may be necessary for the initiation of the biochemical events which

lead to estrogenic sexual responses. This corresponds well with the current model of steroid mechanism of action in which it is proposed that the hormone-receptor complex enters the nucleus and initiates a chain of responses which lead to the induction of protein synthesis (Gorski and Gannon, 1976; Yamamoto and Alberts, 1976).

When in vitro binding techniques are used, the areas of highest uptake of ^3H -estradiol within the brain and pituitary are also found to be the anterior hypothalamus and the anterior pituitary (Kato, 1969; Eisenfeld, 1970; Kahwango, Heinrichs and Herrmann, 1970). This binding is estrogen specific as it can be blocked by excess unlabeled estradiol (Kato, 1969; Eisenfeld, 1970), but (^3H)estradiol binding is not inhibited by high doses of nonestrogenic competing steroids (Eisenfeld, 1970; Korach and Muldoon, 1975).

An estrogen specific receptor component of the cytosol of the pituitary and hypothalamic tissues of rat (Kato, Atsami and Inaba, 1970; Korach and Muldoon, 1974) and cow (Kahwanago, Heinrichs and Herrmann, 1970) has been isolated. From binding studies completed with the addition of proteases, sulfhydryl blocking compounds, DNase and RNase, it has been determined that the cytosol estrogen receptor molecule is protein in nature and contains a sulfhydryl group in the active binding site for estradiol (Eisenfeld, 1970; Kahwanago Heinrichs and Herrmann, 1970; Kato, Atsami and Inaba, 1970).

The sedimentation coefficient of the receptor is estimated to be approximately 8S in low-salt buffers (Kato, Atsami and Inaba, 1970; Korach and Muldoon, 1975).

Using Scatchard analysis of in vitro cytosol-estradiol binding studies, an affinity constant (Kd) of 10^{-9} to 10^{-10} M has been determined for the hypothalamic-preoptic area receptor of the rat (Korach and Muldoon, 1974; Kato, 1973, Ginsburg et al., 1974).

The goal of this thesis was to replicate some of the above cited studies, using as an experimental subject the Mongolian gerbil, Meriones unguiculatus. Gerbils have become increasingly popular laboratory research animals (Schwentker, 1976; Theissen and Yahr, 1977); various neurological, endocrine and behavioral studies have been completed with gerbils as subjects. Some data have been generated regarding the reproductive physiology and behavior of the gerbil, and preliminary data from our laboratory indicate that gerbils might be an ideal species in which to study the effects of social and environmental conditions on reproductive physiology and behavior. To date, however, no studies have been reported which examine the physiological mechanisms by which reproductive hormones act in gerbils. The rationale for this project was therefore twofold. One purpose of this series of studies was to extend the comparative literature in the field of hormone-receptor interactions. The second was to generate baseline or control data typical of the

Mongolian gerbil as maintained under specific laboratory conditions. The immediate aim of this thesis is to report the results of a series of experiments designed to localize and characterize estradiol binding sites in the brain and pituitary of female gerbils, and to compare these results with parallel data previously reported for rats.

GENERAL METHODS

Subjects. Female gerbils, 14 weeks old, (Tumblebrook Farms, West Brookfield, MA) were housed individually in 17.5 x 17.5 x 25 cm. wire-bottom cages and maintained on a 14 h:10 h light-dark cycle. Gerbils were given ad libitum access to Purina Laboratory Chow and tap water. One week prior to sacrifice, gerbils were ovariectomized via a single midventral incision under methoxyflurane (Metofane) anesthesia.

In vivo uptake of (³H)estradiol. Forty μ Ci 17β -(2,4,6,7-³H)-estradiol (specific activity 115 Ci/mmol.; New England Nuclear) were injected interperitoneally in the first experiment and into the femoral vein under light methoxyflurane anesthesia for the other experiments in 0.1 ml 20% ethanol. After one hour (except as noted in the time-course study), gerbils were anesthetized with sodium pentobarbital (Nembutal), a blood sample was drawn, and the animals were perfused with cold saline. Tissues were rapidly dissected and homogenized. Cell nuclei were isolated and tissue whole homogenate and isolated cell nuclear (³H)estradiol content

were determined according to the method of Zigmond and McEwen (1970) as modified by Gentry, Roy and Wade (1976). Protein in whole homogenate and cell nuclear fractions were precipitated with ethanol and estimated by the method of Lowry et al. (1950).

Regional differences in whole homogenate and cell nuclear (^3H)estradiol uptake were determined in 6 brain areas and whole pituitary. Brain dissections were as follows: The olfactory bulbs were removed by transection and the brain was placed with the ventral side up on a chilled glass plate. The brain was transected as described by McEwen and Pfaff (1970). An additional coronal slice was made posterior to the mammillary bodies, dividing the brain into hypothalamic and midbrain slices. The preoptic section was cut as a cube, the depth and width of the fornix. The amygdala and overlying cortex were removed from the hypothalamic slice by sectioning the area bordered coronally by the rhinal fissure and sagittally at the optic tract. The hypothalamic section was then dissected from the remainder of this slice. This section consisted of the area the width of the median eminence cut horizontally through and ventral to the third ventricle. The pons was removed from the midbrain slice. A horizontal cut was made just below the posterior commissure, saving the aqueduct and the surrounding tissue. Sagittal cuts were made lateral to the medial lemnisci, and the remaining tissue was considered the midbrain section. Cortical

tissues were removed from both the hypothalamic and preoptic area slices, with care being taken not to cut below the level of the corpus collosum.

To test the saturability of (^3H)estradiol binding in these areas, a second group of animals was injected with 200 ug. unlabeled estradiol 15 minutes prior to the administration of (^3H)estradiol and uptake of radioactivity was measured as described above.

To test the specificity of (^3H)estradiol binding, animals were injected with 100 ug. unlabeled estradiol, progesterone, 5 α -dihydrotestosterone or cortisol or 1 mg. of the synthetic antiestrogen, nafoxidine, 15 minutes prior to an intravenous injection of (^3H)estradiol. Whole tissue homogenate and cell nuclear uptake of radioactivity were determined for cortex, pooled hypothalamus-preoptic area, amygdala and pituitary.

The time-course of estradiol binding in whole homogenate and cell nuclear fractions of cortex, hypothalamus-preoptic area, amygdala and pituitary was determined by sacrificing animals .25, 1, 3, 6 and 12 hours after an intravenous injection of (^3H)estradiol.

Data for the specificity and localization studies were calculated as disintegrations per minute per mg. protein: disintegrations per minute per microliter plasma. Data for the time-course experiment are presented as disintegrations per minute per mg. protein. Differences to be reported

were significant at the $p < .05$ level using a Student's t-test.

In vitro cytosol (^3H)estradiol binding. Animals were sacrificed, and the hypothalamus, preoptic area and amygdala were rapidly dissected. Tissues were pooled and homogenized at a concentration of 100 mg. tissue per ml. buffer containing 10 mM Tris-HCl, 1.5 mM EDTA, and 0.1 M mercaptoethanol, pH 7.4. Homogenates were centrifuged for 2 hours at $48,000 \times g$, and cytoplasmic estrogen-receptor binding was determined according to the method of Ginsburg et al (1974) as modified by Wade and Blaustein (in press).

High speed supernatant from gerbil brain was incubated in duplicate with eight different concentrations of (^3H)-estradiol, ranging from 0.1 to 5 nM for 2 hours at 0°C . Corresponding duplicates at four concentrations were run, each containing a 100-fold excess of unlabeled estradiol to determine nonspecific binding. Specific binding was determined by subtracting nonspecific binding (samples containing unlabeled estradiol) from total binding (samples containing only labeled estradiol). Scatchard analysis of these data yielded an approximation of the dissociation constant (Kd) of the brain cytoplasmic receptor-estradiol binding.

Specificity of the cytosol receptor binding was determined by incubating high speed supernatant from gerbil brain with 1 nM (^3H)estradiol plus a 100-fold excess of unlabeled estradiol, the synthetic estrogen, R 2858 (Rousseau Uclaf),

5 α -dihydrotestosterone, progesterone or cortisol. Data are presented as femtomoles (³H)estradiol bound per mg. protein.

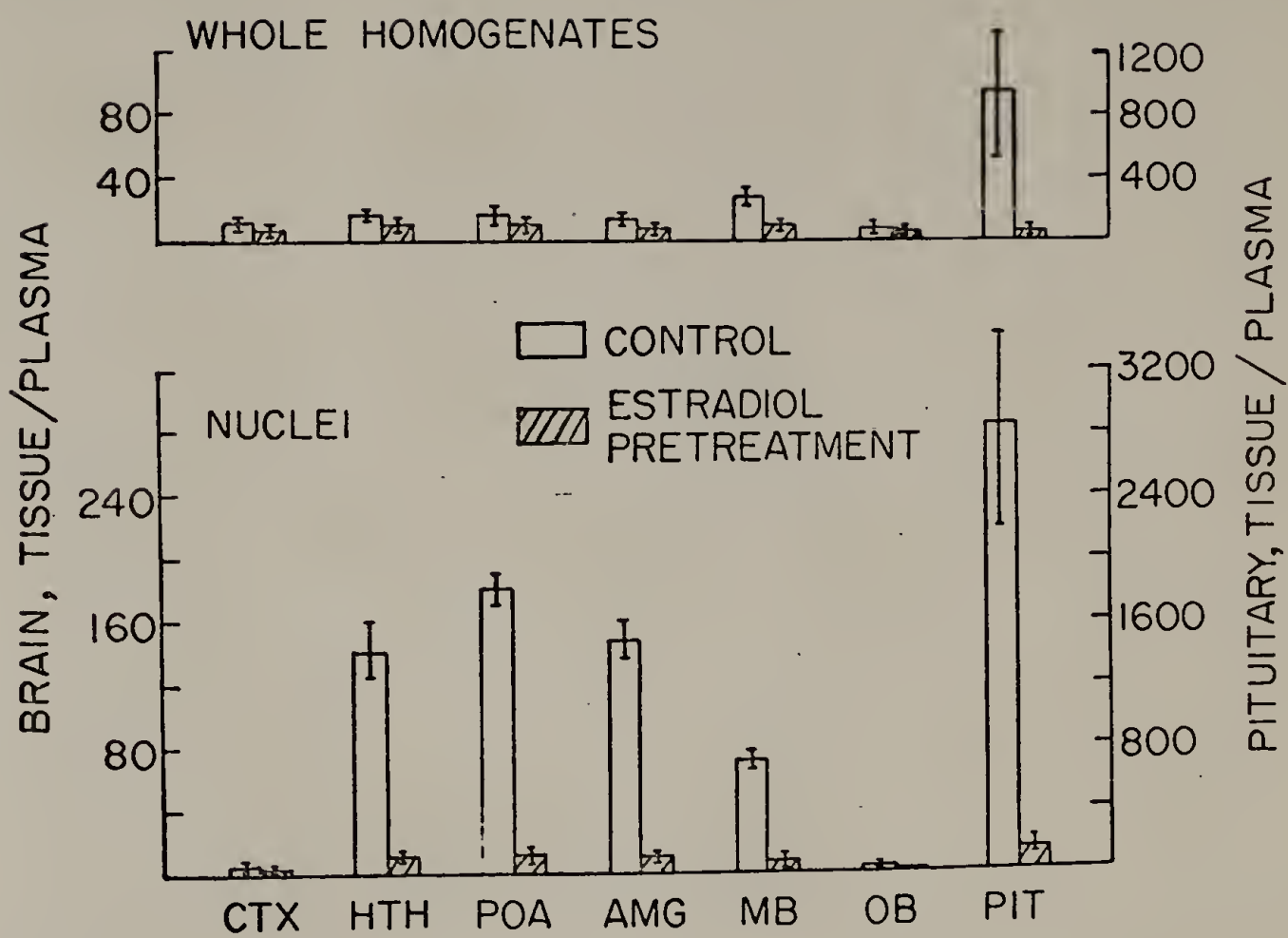
To determine the chemical nature of the receptor, high speed supernatant was incubated for 1 hour at 24°C with 1 nM (³H)estradiol plus DNase (Worthington, bovine pancreas; .5 mg./ml.), RNase (Worthington, bovine pancreas; .5 mg./ml.) or pronase (Sigma; .5 mg./ml.).

RESULTS

In vivo (³H)estradiol binding. One hour after an intraperitoneal injection of (³H)estradiol, the highest concentration of radioactivity was found in cell nuclei from the pituitary (Figure 1), followed by preoptic area, hypothalamus, amygdala, and midbrain, with very low levels in the olfactory bulbs and the cerebral cortex. That the regional specificity is more pronounced in the cell nuclear fractions than in whole homogenates can be seen by comparing ratios of radioactivity in different regions to that found in cortex. While whole homogenate tissue/cortex ratios for hypothalamus, preoptic area and amygdala range from 1.3 to 1.5, cell nuclear tissue/cortex ratios for these same areas range from 25.7 to 32.9. The midbrain/cortex whole homogenate ratio was 2.4 while the nuclear ratio was 13.0. Injection of unlabeled estradiol 2 hours prior to sacrifice significantly lowered (³H)estradiol uptake in whole homogenate of amygdala and midbrain and in the nuclear fractions of all tissues

Cover page for Figure 1 (page 10).

Anatomical distribution of (³H)estradiol uptake in whole homogenate and cell nuclear fractions of 6 brain regions and whole pituitary. Ovariectomized gerbils received no pretreatment (n=7) or were given an ip. injection of 200 ug. unlabeled estradiol (n=5) 15 minutes prior to an injection of 40 uCi (³H)estradiol. Whole homogenate and nuclear uptake of (³H)estradiol were determined for cortex (CTX), hypothalamus (HTH), preoptic area (POA), amygdala (AMG), midbrain (MB), olfactory bulbs (OB) and whole pituitary (PIT). Data are presented as tissue/plasma ratios (Mean \pm SEM).



studied except olfactory bulbs, indicating saturability of the estradiol binding sites in brain and pituitary.

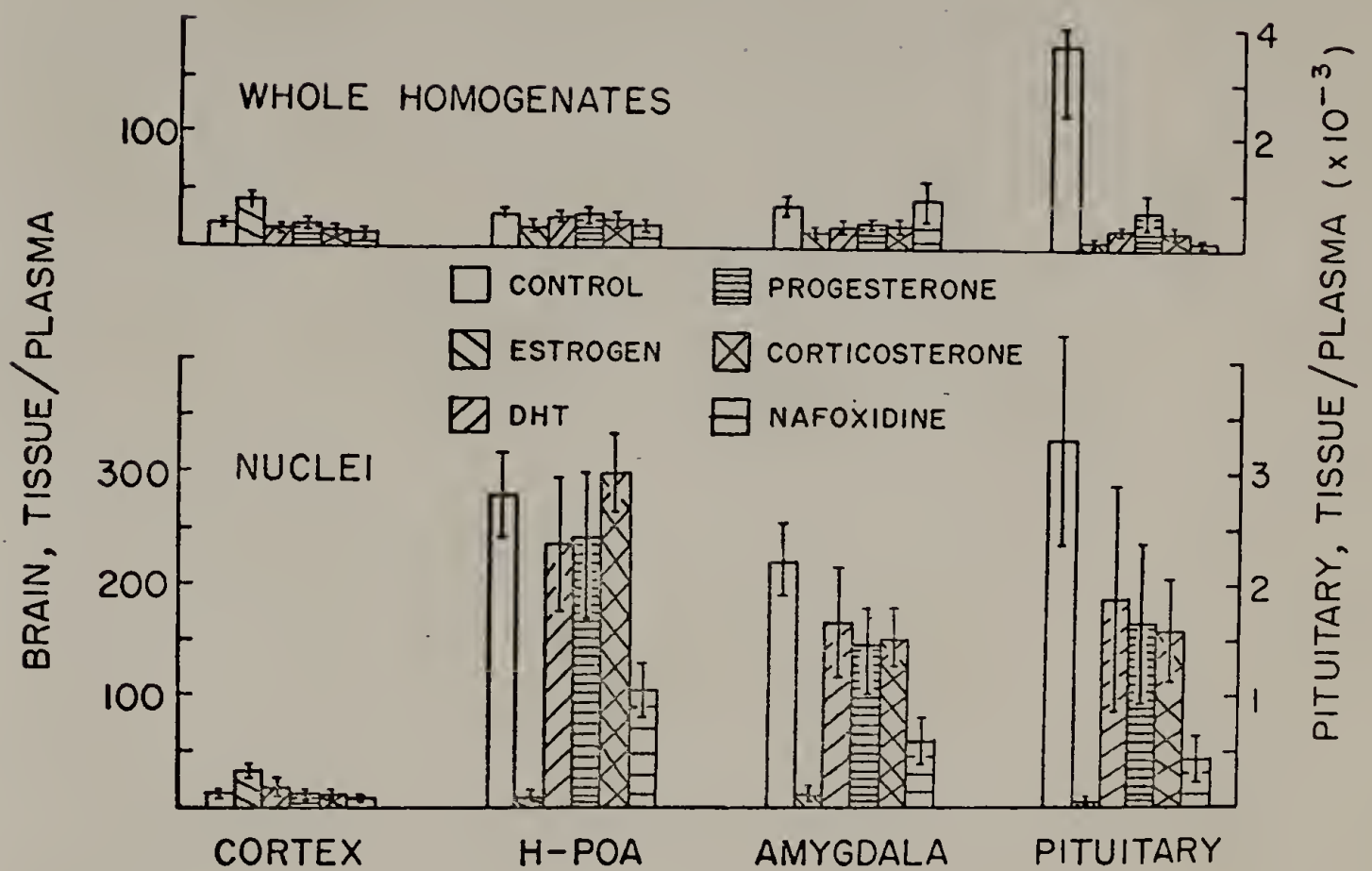
Estradiol binding in pooled hypothalamus-preoptic area and amygdala was shown to be estrogen-specific, because cell nuclear binding was significantly reduced by pretreatment with unlabeled estradiol and nafoxidine, but not by pretreatment with progesterone, 5 α -dihydrotestosterone or cortisol (Figure 2). Some competition by nonestrogenic steroids was observed in the pituitary.

Animals were sacrificed at .25, 1, 3, 6 or 12 hours following an intravenous injection of (^3H)estradiol. While whole homogenate levels of radioactivity were highest at .25 hours, cell nuclear uptake in all tissues peaked at one hour (Figure 3). Radioactivity was still present in cell nuclei from pituitary, hypothalamus-preoptic area and amygdala 12 hours after an injection of (^3H)estradiol, although at levels only 2 per cent of one hour peak levels. Nuclear to whole homogenate ratios increased as a function of time through six hours for these areas. At 12 hours the nuclei/whole homogenate ratio for pituitary, amygdala and hypothalamus-preoptic area were 7.7, 9.6 and 2.8 respectively.

Cytoplasmic estrogen receptors. There was a substantial amount of macromolecular binding of (^3H)estradiol in the cytoplasmic fraction of pooled hypothalamus-preoptic area-amygdala (Figure 4). Scatchard analysis indicated a K_d of $5.41 \times 10^{-10}\text{M}$ for the estradiol-cytoplasm receptor binding.

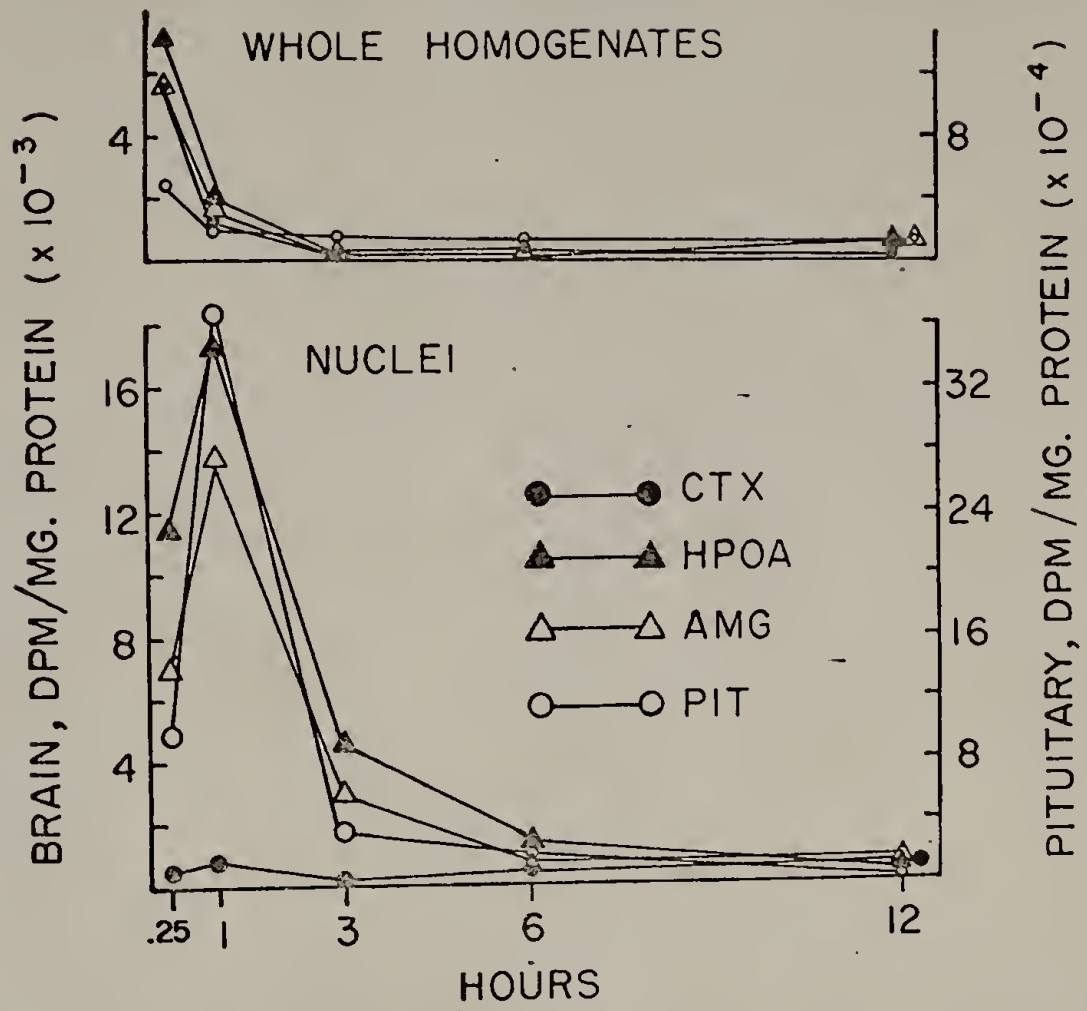
Cover page for Figure 2 (page 12).

Effect of competing steroids on whole homogenate and cell nuclear uptake of (³H)estradiol in brain and pituitary of ovariectomized gerbils. Fifteen minutes prior to an iv. injection of (³H)estradiol, gerbils were injected with nothing (controls, n=11), 100 ug. unlabeled estradiol (n=4), 5 α -dihydrotestosterone (DHT, n=5), progesterone (n=5), or cortisol (n=5) or 1 mg. nafoxidine (n=5). Data are presented as tissue/plasma ratios (Mean \pm SEM).



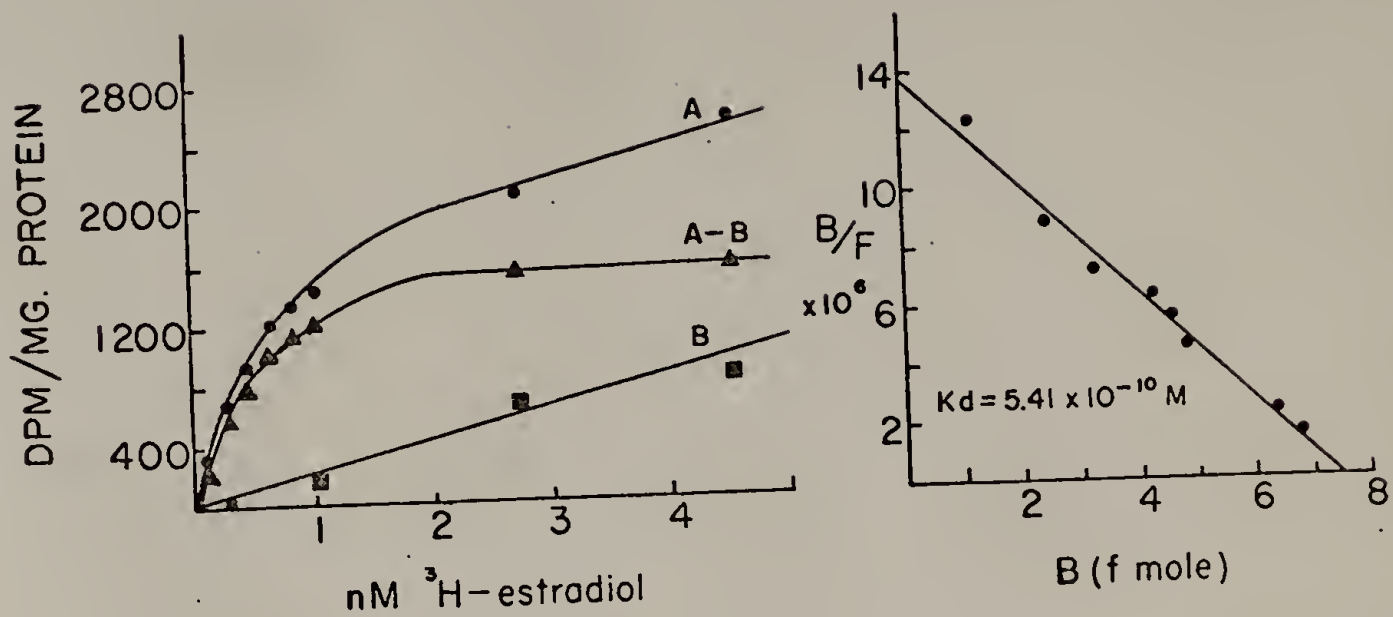
Cover sheet for Figure 3 (page 13).

Time course of uptake of (³H)estradiol in gerbil brain and pituitary. Gerbils were sacrificed .25 (n=5), 1 (n=11), 3 (n=3), 6 (n=4) or 12 (n=10) hours after iv. injection of (³H)estradiol and whole homogenate and cell nuclear uptake of radioactivity were measured. Data are presented as mean disintegrations per minute per mg. protein.



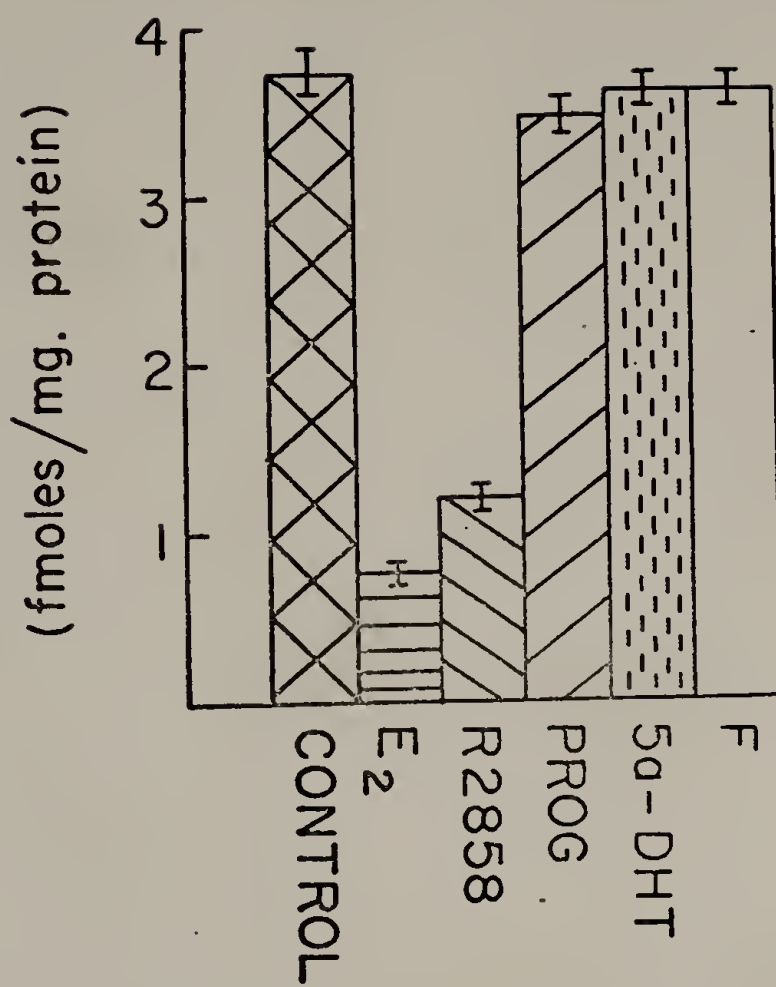
Cover page for Figure 4 (page 14).

In vitro binding of (³H)estradiol to cytoplasmic macromolecules in pooled hypothalamic-preoptic area-amygdala from ovariectomized gerbils. Concentration binding curves are shown on the left (see text for explanation of procedure). Specific binding (A-B) was determined by subtracting nonspecific binding (B) from total binding (A). Scatchard analysis of A-B is shown on the right of the figure.



Cover page for Figure 5 (page 16).

Effect of competing steroids on macromolecular binding of (³H)estradiol in cytoplasm from pooled hypothalamus-preoptic area-amygdala from ovariectomized gerbils. Estradiol, R2858, progesterone, 5 α -dihydrotestosterone and cortisol were incubated with the high speed supernatant containing 1nM (³H)estradiol. Data are presented as fmoles (³H)-estradiol bound per mg. protein (Mean \pm SEM).

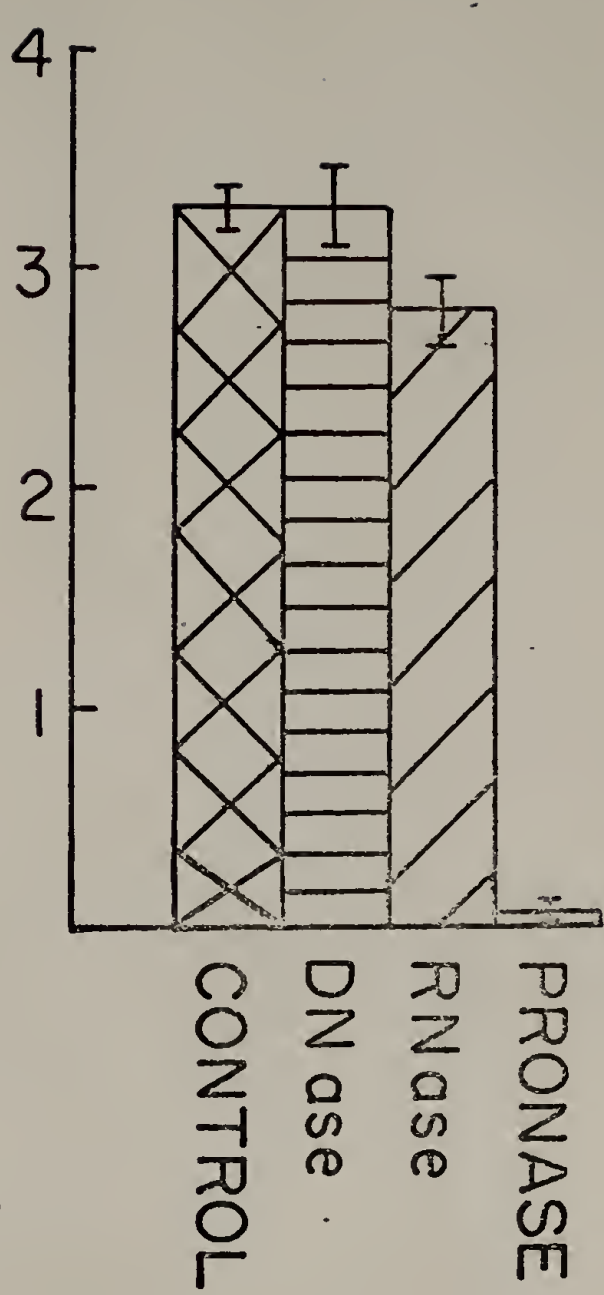
(^3H) ESTRADIOL BOUND

Cover page for Figure 6 (page 17).

Effect of enzymes on macromolecular binding of (³H)estradiol in cytoplasm from pooled hypothalamus-preoptic area-amygdala from ovariectomized gerbils. Pronase, RNase and DNase (.5 mg./ml.) were incubated with high speed supernatant containing 1 nM (³H)estradiol. Data are presented as fmoles (³H)estradiol bound (Mean \pm SEM).

(³H) ESTRADIOL BOUND

(fmoles / mg. protein)



genate ratios, however, were found to be twice as high in gerbils as in rats. The physiological significance of this species difference is not known at this time.

The binding of (³H)estradiol was estrogen specific in brain and pituitary cell nuclei. Several previous reports indicated that estradiol binding in rat pituitary and brain cell nuclei is also estrogen specific; it can be blocked by an excess of unlabeled estradiol (Eisenfeld and Axelrod, 1966; Zigmond and McEwen, 1970) but not by high doses of nonestrogenic competing hormones (Zigmond and McEwen, 1970; Korach and Muldoon, 1975).

Whole homogenate levels of radioactivity were highest at .25 hours after intravenous injections, but nuclear cell uptake in all tissues peaked at one hour. These data are comparable to those of Zigmond and McEwen (1970) who found peak levels of radioactivity in brain cell nuclei between one and two hours after administration of (³H)estradiol in rats. These results are consistent with the current model of mechanism of action of steroid hormones which suggests that after the hormone enters the cell, it first binds to a cytoplasmic receptor before being translocated to the nucleus.

Measureable, although relatively low, amounts of radioactivity were detected in cell nuclei from gerbil pituitary, hypothalamus-preoptic area and amygdala at 12 hours. Again these data are similar to those found in rats (McEwen et al., 1975; Roy and Wade, 1977), although the physiological

significance of this long term nuclear retention of estradiol is not clear at this time.

Using an in vitro receptor assay, the binding of estradiol in gerbil brain cytosol has been characterized. The calculated dissociation constant for this binding, $5.41 \times 10^{-10} \text{M}$, is comparable to the values previously reported for brain cytosol-estradiol binding in rats (Ginsburg et al., 1974; Korach and Muldoon, 1974; Kato, 1973). The protein nature of the receptor in brain cytoplasm was demonstrated by the almost total abolition of binding following the addition of pronase and the relative ineffectiveness of DNase and RNase. Similar data have been reported describing the protein nature of the brain cytosol-estradiol binding in rat (Eisenfeld, 1970).

Estrogen specificity was also demonstrated for the in vitro binding of (^3H)estradiol to brain cytoplasmic estradiol receptor molecules. While estradiol binding was unaffected by the addition of a 200-fold molar excess of progesterone, 5 α -dihydrotestosterone or cortisol, it was greatly reduced by the same quantities of unlabeled estradiol or R 2858. R 2858 has previously been shown to bind tightly to uterine estradiol receptors but not to plasma proteins (Raynaud, 1977). We have good indication in this study, therefore, that the specific binding of (^3H)estradiol being reported from these in vitro preparations is actually binding to brain cytoplasmic components, not to contaminating plasma

proteins.

In summary, these results indicate that there are binding sites for estradiol in the hypothalamus, preoptic area, amygdala and pituitary of the female gerbil. The brain binding sites are estrogen specific as determined by in vivo competition studies. Time course analysis shows a peak of radioactivity at one hour for cell nuclear components and measureable quantities of radioactivity still remaining as late as 12 hours. High affinity, estrogen specific binding of (³H)estradiol to brain cytoplasmic receptors was demonstrated.

These data, demonstrating high levels of (³H)estradiol binding in hypothalamus, preoptic area and amygdala in ovariectomized Mongolian gerbils, indicate that these brain regions might be neural sites of action of estradiol on a variety of behavioral and neuroendocrine responses (Kuehn and Zucker, 1968; Theissen and Yahr, 1977). With these data as baseline indicators, an extension of this work might include an analysis of changes in in vivo and in vitro estradiol binding as affected by environmental and social changes.

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