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HYPOTHALAMIC AND HYPOPHYSIAL PROGESTERONE RECEPTORS: ESTROGEN-PRIMING EFFECT, DIFFERENTIAL LOCALIZATION, 5α-DIHYDROPROGESTERONE BINDING, AND NUCLEAR RECEPTORS

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SUMMARY

Specific progesterone receptor of 7*S* which can be isolated from rat female hypothalamic and hypophysial cytosols was further investigated in terms of its induction effects by estrogen-priming, intracerebral localization, 5α-dihydroprogesterone (DHP) binding, and nuclear receptors. Estrogen-priming was necessary for the appearance of the cytosol receptors. The 7*S* hypothalamic and hypophysial receptors were increased in a dose dependent fashion by estradiol injections. The maximal effective doses of estradiol benzoate for both tissues were 1 and 10 μg, respectively.

The receptors were mostly localized in the median eminence, preoptic-anterior hypothalamus and anterior hypophysis of estrogen-primed immature and mature rats, but little or no 7*S* binding was detected in the cerebral cortex, reticular formation, amygdaloid complex and posterior hypophysis. This differential localization of progesterone receptors resembles that of estrogen receptors, suggesting possible induction of progesterone receptors in these specific brain regions by estrogen.

Progesterone receptor complexes of 5*S* were isolated from "purified" nuclei of anterior pituitaries from estrogen-primed adult female rats. These results on the cytosol and nuclear receptors suggest that progesterone can directly act on the brain through its interaction with specific progesterone receptors in the hypothalamus and hypophysis. It is noteworthy that 5α-DHP can bind the progesterone receptors in both tissues, suggesting its direct feedback action on the brain through the receptors.

INTRODUCTION

Despite the established central action of progesterone [1–3], specific progesterone binding in the hypothalamus and hypophysis has been controversial and conflicting [4–13] for three reasons; firstly the difficulty of identification of true progesterone receptors from contaminating CBG, CBG-like progesterone binding proteins or corticoid binding proteins, secondly the instability of progesterone receptors complexes and thirdly the small amount of the receptors [12, 13]. Our previous reports [15, 16] have confirmed and extended the presence of high affinity and low capacity specific receptors for progesterone of 7*S* in the hypothalamic and hypophysial cytosols from estrogen-primed rats by the use of R5020, a synthetic progestin, which binds specifically to progesterone receptors [14]. Its possible role was suggested in the mechanism of feedback action of progesterone on the brain.

Since estrogen-priming is necessary for the appearance of specific progesterone receptors in rat hypothalamus and hypophysis [15, 16], how the brain receptors are induced by the estrogen-priming was examined. The localization of the receptors in the brain was also investigated to elucidate the possible site of the feedback action of progesterone on the brain. Furthermore, little data are available on the isolation of nuclear progesterone receptors from the hypothalamus and hypophysis. Whether or not the nuclear receptors can be isolated from these tissues was examined in the present communication.

METHODS AND MATERIALS

Preparation of the cytosol and gradient centrifugation

Immature female rats of the Wistar strain were estrogen-primed with estradiol benzoate. Estradiol benzoate (5 μg) in sesame oil was injected s.c. once daily for 5 consecutive days and, 20 h after the final injection, the rats were killed at 28 days of age. Ten μg of estradiol benzoate was also injected s.c. once daily for 5 consecutive days prior to sacrifice at 71–77 days of age. In the experiments on receptor localization (Fig. 5) and nuclear receptors (Fig. 7) rats were primed with estradiol valerate (2.5 mg) and killed at 71–77 days of age as previously described [16].

The whole hypothalamus, including the preoptic nucleus, was cut as a block in a way similar to that previously described [17, 18]. After resection of the median eminence, the hypothalamus was divided into three parts; the preoptic-anterior hypothalamic region, the middle hypothalamus and the posterior hypothalamus, as described previously [17, 19]. The cortical tissue was excised from the frontal lobes of the brain. The tissues were rinsed in ice-cold 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA and 12 mM thioglycerol, and then blotted on filter paper. The hypothalamic tissues, the cortex, amygdala and reticular formation (1.4 vols, wt/vol. of buffer), and the anterior pituitaries (5.2–5.9 vols, wt/vol. of buffer), were homogenized with a Teflon pestle in a homogenizer (Takashima, Tokyo) (five strokes, 600–1,000 rev./min, 3 min) at 4°C. The homogenates were then cen-

trifuged at 105,000 *g* for 1 h in a Hitachi RP65 Model ultracentrifuge.

The supernatant fraction, cytosol, (0.29 ml) was incubated with gentle shaking for an appropriate time at 0°C with [^{3}H]-R5020 (SA 51 Ci/mmol, Roussel-Uclaf, France), [^{3}H]-5α-dihydroprogesterone (S.A. 55.7 Ci/mmol), in the absence or presence of excess 100-fold unlabeled steroid hormones dissolved in 5 μl of ethanol. Incubation time was 4–8 h [16]. The mixture was layered on a sucrose density gradient (4.8 ml, 5–20% linear) in 10% glycerol–10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 12 mM thioglycerol and centrifuged for 16 or 18 h at either 45,000 or 50,000 rev./min in a Beckman Model L3-50 ultracentrifuge with a SW 50.1 rotor at 4°C. The above procedures were performed in a cold room at 4°C. The pH of Tris–HCl buffer was determined at room temperature.

After sucrose density gradient centrifugation, the tubes were kept at room temperature for 30 min before fractionation (0.2 ml fractions) with an ISCO Model Density Gradient Fractionator. Protein was measured by the method of Lowry *et al.* [20]. To determine apparent sedimentation coefficients (*S*) by the method of Martin and Ames [21], crystalline bovine albumin (4.6*S*), yeast alcohol dehydrogenase (7.6*S*), and beef liver catalase (11.3*S*) were used as standards. In order to absorb unbound radioactivity in each fraction after gradient centrifugation, 1.0 ml suspension of dextran-coated charcoal (0.25% Norit A, 0.0025% Dextran T70 in 0.01 M Tris–HCl, pH 7.4 at 4°C) was added to each fraction (0.2 ml aliquots), mixed, and incubated 15 min at 4°C. After centrifugation for 10 min at 800 *g* in a refrigerated centrifuge, a 1.0 ml aliquot of the supernatant was transferred into a scintillation vial.

Radioactivity was measured in an Aloka Model LSC-653 scintillation counter (Nihon Musen Irigaku-ken, Mitaka, Tokyo) in a Toluene–Triton–PPO-POPOP system. The counting efficiency was 38%.

Preparation of the nuclei and extraction

Anterior pituitaries and whole hypothalami were collected from 49 female rats of 77 days old treated with estradiol valerate (2.5 mg) as previously described [16]. Quartered anterior pituitaries and divided hypothalami (3–4 portions) were incubated in 15 ml of Krebs–Ringer bicarbonate–glucose buffer; pH 7.4, containing 2% BSA and [^{3}H]-R5020 (5.1 nM). Incubation was performed at 37°C with gentle shaking for 30 min. The tissues removed from the medium were rinsed with ice-cold Krebs–Ringer solution.

"Purified" nuclei were prepared from the homogenate according to the method of Shaw and Huang [22], followed by the extraction of R5020–receptor complexes with 0.4 M KCl from the nuclei as previously described [23, 24]. The extracts of anterior pituitary (0.41 ml, 1.45 mg protein) and hypothalamus (0.41 ml, 0.67 mg protein) were layered on a 5–20% sucrose density gradient containing 10% glycerol, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 12 mM thioglycerol and 0.4 M KCl, and centrifuged at 50,000 rev./min in a SW 50.1 rotor in L3-50 Beckman ultracentrifuge for 22 h at 4°C. For determination of the sedimentation coefficient (*S*) cytochrome C (1.9*S*), bovine serum albumin and alcohol dehydrogenase were used as standard enzymes [21].

Chemicals

Redistilled and purified solvents and reagent grade chemicals were used in all experiments. [1,2-^{3}H]-5α-Dihydroprogesterone (S.A. 55.7 Ci/mmol) as obtained from New England Nuclear Corp., Boston, MA, and purified by thin layer chromatography as previously described [17]. [6,7^{3}H]-17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione ([^{3}H]-R5020, S.A. 51 Ci/mmol) and radioinert R5020 were supplied by Dr. J. P. Raynaud, The Roussel-Uclaf, France. Steroids were supplied by Steraloids, Inc., Pawling, NY, Sigma Chemical Co., St. Louis, MO, and Ikapharm, Israel. Estradiol benzoate and progestins were obtained from Teikoku Zoki Co., Tokyo.

Crystalline bovine serum albumin (Nutritional Biochemical Corp., Cleveland, Ohio), yeast alcohol dehydrogenase (Sigma Chemical Co., St. Louis, MO), beef liver catalase, 2× crystallized (Sigma Chemical Co., St. Louis, MO), cytochrome C (Miles Lab., Slough, England) and pronase (Kaken Kagaku, Tokyo) were used. Sucrose, glycerol and thioglycerol were purchased from Merck, Japan and EDTA from Dotite Co., Kumamoto, Japan. Dextran T70 (Pharmacia Fine Chemicals) and Norit A (American Norit Co.) were used.

Identification of R5020

Radioactivity bound in the peak at 7*S* (fractions 11–15) in the gradients of the hypothalamic and anterior hypophysial cytosols extracted with chloroform methylene chloride (1:1, v/v) dried at 50°C under nitrogen, and subjected to t.l.c. in cyclohexane–ethylacetate (1:1, v/v) system as previously described [17]. The radioactivity corresponding to standard R5020 was counted.

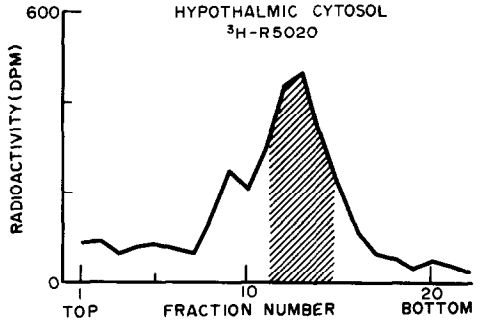
RESULTS

Steroid analysis

When radioactivity collected from the 7*S* peak area on gradients of hypothalamic and hypophysial cytosols labeled with [^{3}H]-R5020 was subjected to steroid analysis, most of the radioactivity was found to be unaltered R5020 in the hypothalamus (Fig. 1) and hypophysis (not illustrated).

Effects of 5α-dihydroprogesterone (5α-DHP)

There have been reports that the metabolism of progesterone into 5α-reduced metabolites may play an important role as the intracellular mediators of the central action of progesterone [25–27]. In an analogy to receptors for 5α-dihydrotestosterone isolated from the brain and hypophysis [28–30], it is of special



Radioactivity was collected from the peak of the gradients of the hypothalamic cytosol and extracted. The extract was subjected to thin layer chromatography, cyclohexane and ethyl acetate (1:1, v/v).

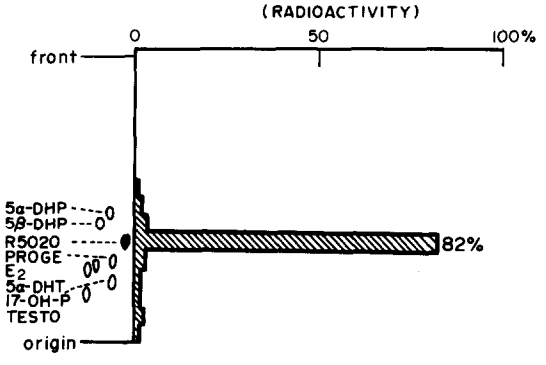


Fig. 1. Steroid analysis of the radioactivity associated with 7*S* [^{3}H]-R5020 binding in hypothalamic cytosols from intact estrogen-primed 28-day-old female rats. Cytosols from the hypothalamus (0.29 ml 3.0 mg protein) were incubated with [^{3}H]-R5020 (7.7 nM), and the mixture was layered on 5–20% sucrose density gradients containing 10% glycerol, followed by centrifugation at 50,000 rev./min in a SW 50.1 rotor in a Beckman ultracentrifuge for 18 h. The radioactivity collected from the 7*S* region was subjected to thin layer chromatography in silica gel in a cyclohexane–ethyl acetate (1:1, v/v) system. 5α-DHP: 5α-dihydroprogesterone; 5β-DHP: 5β-dihydroprogesterone; R5020: 17,21 Dimethyl-19-nor-4,9-pregnadiene 3,20-dione; Proge: progesterone; E_2: estradiol; 5α DHT: 5α-dihydrotestosterone; 17OH-P: 17-Hydroxyprogesterone; Testo: testosterone.

interest to examine the possibility of binding of the progesterone metabolites in neural structure.

As shown in Fig. 2, unlabeled 5α-DHP inhibited the R5020 binding to hypothalamic progesterone receptors, but its 5β-isomer did not. Other progestational compounds such as chlormadinone acetate, norethindrone and provera are found to compete for binding sites on the receptor.

Effects of 5α-DHP and its 5β-isomer, and progestins on the hypophysial R5020 binding were similar to that on the hypothalamus (Fig. 2). Thus, it is suggested that 5α-DHP can act on the brain through the progesterone receptors.

Inductive effects of estrogen

Immature female rats were treated with 5 consecutive injections of estradiol benzoate in dosage of 0, 0.1, 1, 10 and 100 μg, and brain progesterone receptors were isolated by density gradient centrifugation.

As clearly shown in Fig. 3, the 7*S* hypothalamic progesterone receptors increased in a dose dependent fashion with increasing dosage of estradiol benzoate. It was noticeable that the maximum induction of the receptor proteins was obtained with 1 μg or more of estradiol benzoate.

Similarly, hypophysial progesterone receptor proteins were induced dose-dependently with the maximal effect with a dose of 10 μg estradiol benzoate. In this context it is noteworthy to mention our previous data [19] that hypothalamic and hypophysial uptake of [^{3}H]-estradiol reached a maximum with 1.8 μg and 10 μg of estradiol *in vivo*, respectively.

Specific localization

To localize the progesterone receptors induced by the estrogen priming, the hypothalamus was divided into the median eminence, the preoptic-anterior hypothalamus, the middle hypothalamus and the posterior hypothalamus. Representative sedimentation profiles of the cytosols containing the same amount of protein from these hypothalamic tissues, cerebral cortex, cerebellum, amygdaloid complex and reticular formation from 28-day-old female rats are shown in Fig. 4. The pattern of hypophysial cytosol containing half the amount of protein as brain tissues is also shown in Fig. 4.

The peak of 7*S* [^{3}H]-R5020 binding in the preoptic-anterior hypothalamus, the median eminence and the anterior hypophysis was found to be much greater than those of the other two parts. The values of sedimentation coefficients for the hypothalamic tissues were almost the same as that for the anterior hypophysis.

The radioactivity associated with 7*S* progesterone receptors in the brain was in the following order; the median eminence > the preoptic-anterior hypothalamus > the middle hypothalamus ≫ the posterior hypothalamus (Fig. 4 and Table 1). Much less or no binding of R5020 were found in the cerebral cortex, amygdaloid complexes, reticular formation and posterior hypophysis (Fig. 4).

As shown in Fig. 5, distribution of progesterone receptors in the brain estrogen-primed adult female rats was essentially the same as that in immature rat brain.

These data show that the progesterone receptors are predominently localized in the preoptic-anterior hypothalamic region and the median eminence, and the anterior hypophysis of the estrogen-primed immature and mature female rat. It is very interesting that this differential localization of specific brain progesterone receptors induced by the estrogen priming is similar to that of estrogen receptors [31–34].

Figure 6 shows a schematic representation on the distribution of progesterone receptors along with receptors for estrogen and androgen [28–30, 35–38] in the brain of the rat. Progesterone receptors in the preoptic-anterior hypothalamic region, median

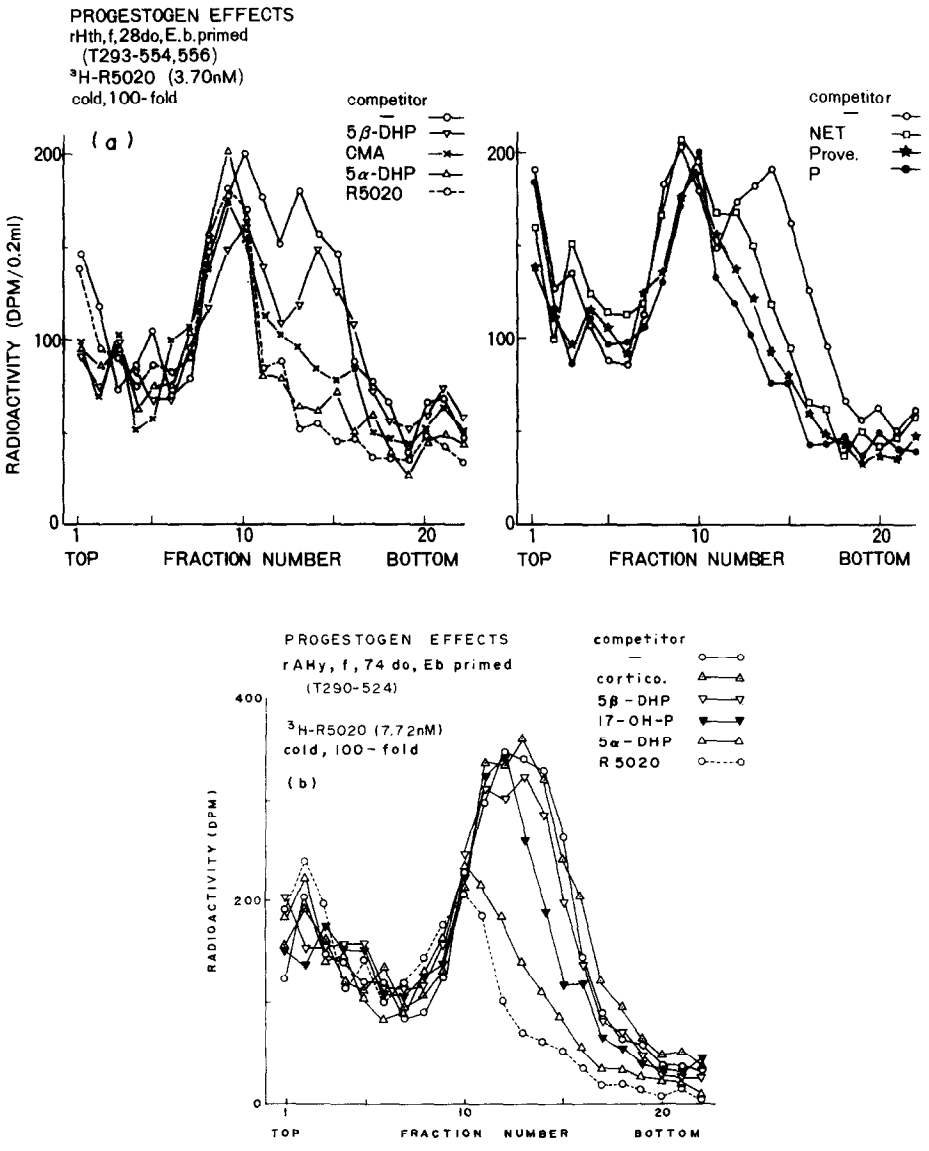


Fig. 2. Effect of progesterone metabolites and progestins on female rat hypothalamic and hypophysial [^{3}H]-R5020 binding. Representative sucrose density gradient patterns of cytosols from immature rat hypothalamus and adult anterior hypophysis incubated *in vitro* with [^{3}H]-R5020 in the presence or absence of 100-fold excess 5α- and 5β-dihydroprogesterone and various progestins. Cytosols (0.29 ml, 3.0 mg protein) labeled with [^{3}H]-R5020 for 4 h at 0°C were layered on sucrose density gradients (5–20%) containing 10% glycerol, which were centrifuged at 50,000 rev./min in a SW 50.1 rotor in a Beckman ultracentrifuge for 18 h.

eminence and anterior hypophysis are presumed to be induced by estrogen through its receptors concentrated in the specific regions.

Nuclear receptors

Purified nuclei from anterior pituitaries of adult rats incubated *in vitro* with [^{3}H]-R5020 were treated with 0.4 M KCl to extract nuclear receptor complexes. Figure 7 shows representative sucrose density pattern of the nuclear extract, on which a single definite peak of [^{3}H]-R5020 is recognized in the 5*S* region. Nuclear receptor complexes from the hypothalamic extract tended to appear in the region corresponding to that from the anterior pituitary.

Thus, nuclear temperature-dependent translocation of progesterone receptors is suggested in the pituitary cell and, possibly, the hypothalamus.

DISCUSSION

There is evidence for the role of 5α-reduced metabolites of progesterone as the intracellular mediators of the central action of progesterone [25–27]. It is interesting to learn whether or not specific receptors

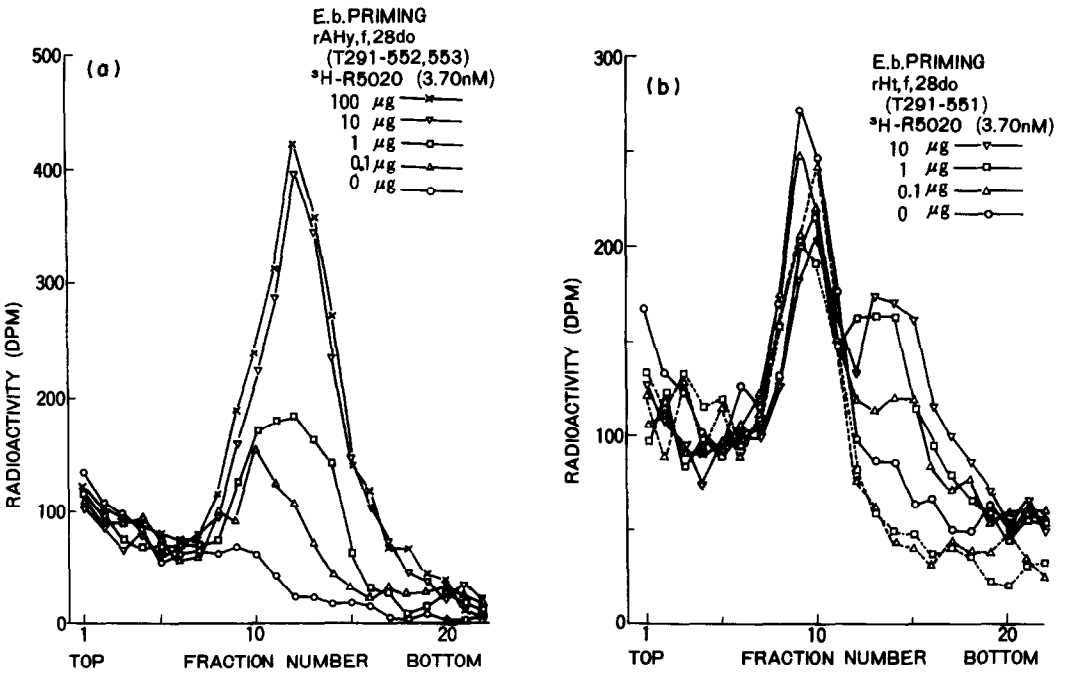


Fig. 3. Inductive effects of the estrogen-priming on hypothalamic and hypophysial progesterone receptors. Hypothalamic and anterior hypophysial cytosols were obtained from 27-day-old female rats treated with varying doses of estradiol benzoate. The cytosols (0.29 ml, 3.0 mg protein for the hypothalamus; 1.0 mg protein for the hypophysis) labeled with [^{3}H]-R5020 (3.7 nM) for 4 h at 0°C were layered on 5–20% sucrose density gradient, containing 10% glycerol, 10 mM Tris–HCl, 1 mM EDTA and 12 mM thioglycerol, and were centrifuged at 50,000 rev./min in a SW 50.1 rotor in a Beckman ultracentrifuge for 18 h. AHy, Anterior hypophysis; Ht, Hpothalamus.

for 5α-DHP are present in the hypothalamus. In our previous experiments [15, 16], no appreciable amount of radioactivity was associated with the 7*S* region on gradients of rat hypothalamic and hypophysis cytosols incubated with [^{3}H]-DHP. As shown in Fig. 2, however, 5α-DHP strongly competed for binding sites on the specific progesterone receptors isolated from hypothalamic and hypophysial rats (Fig. 2). No effects of its 5β-isomer were found. It is presumed that 5α-DHP acts upon the brain through its binding to the progesterone receptors.

As shown in Figs 3 and 4, the progesterone receptor proteins in the hypothalamus and hypophysis, like the uterus [39], are induced in a dose dependent fashion by the estrogen priming with increasing dosage in a range of 0.1 to 1 μg and 0.1 to 10 μg, respectively. The maximal effective doses of estradiol benzoate in both tissues are in good agreement with the uptake data [19] that *in vivo* uptake of [^{3}H]-estradiol by the hypothalamus and hypophysis can reach a plateau with 1.8 μg and 10 μg of estradiol, respectively. These data clearly demonstrate that the progesterone receptor proteins depend upon the binding of estrogen to the brain and hypophysis.

Table 1. Total specific progesterone binding in various parts of the estrogen-primed immature female rats*

Tissue	Relative total specific progesterone bound† (fmol/mg cytosol protein)
Preoptic-anterior hypothalamus	5.5‡ ± 1.66 (3)
Middle hypothalamus	3.2 ± 1.21 (3)
Posterior hypothalamus	0.71 ± 0.13 (3)
Median eminence	6.3 ± 1.15 (3)
Anterior hypophysis	9.6§ (10.6; 8.6)
Posterior hypophysis	0.21§ (0.11; 0.31)

* Estradiol benzoate (5 μg) in sesame oil was injected s.c. once daily for 5 consecutive days and, 20 h after the final injection, the rats were killed at 28 days of age. † Total specific progesterone binding was calculated as difference in the radioactivity of [^{3}H]-R5020 on gradients in the presence and absence of excess 100-fold unlabeled R5020. ‡ Mean and standard deviation (number of determinations). § Mean value of two determinations.

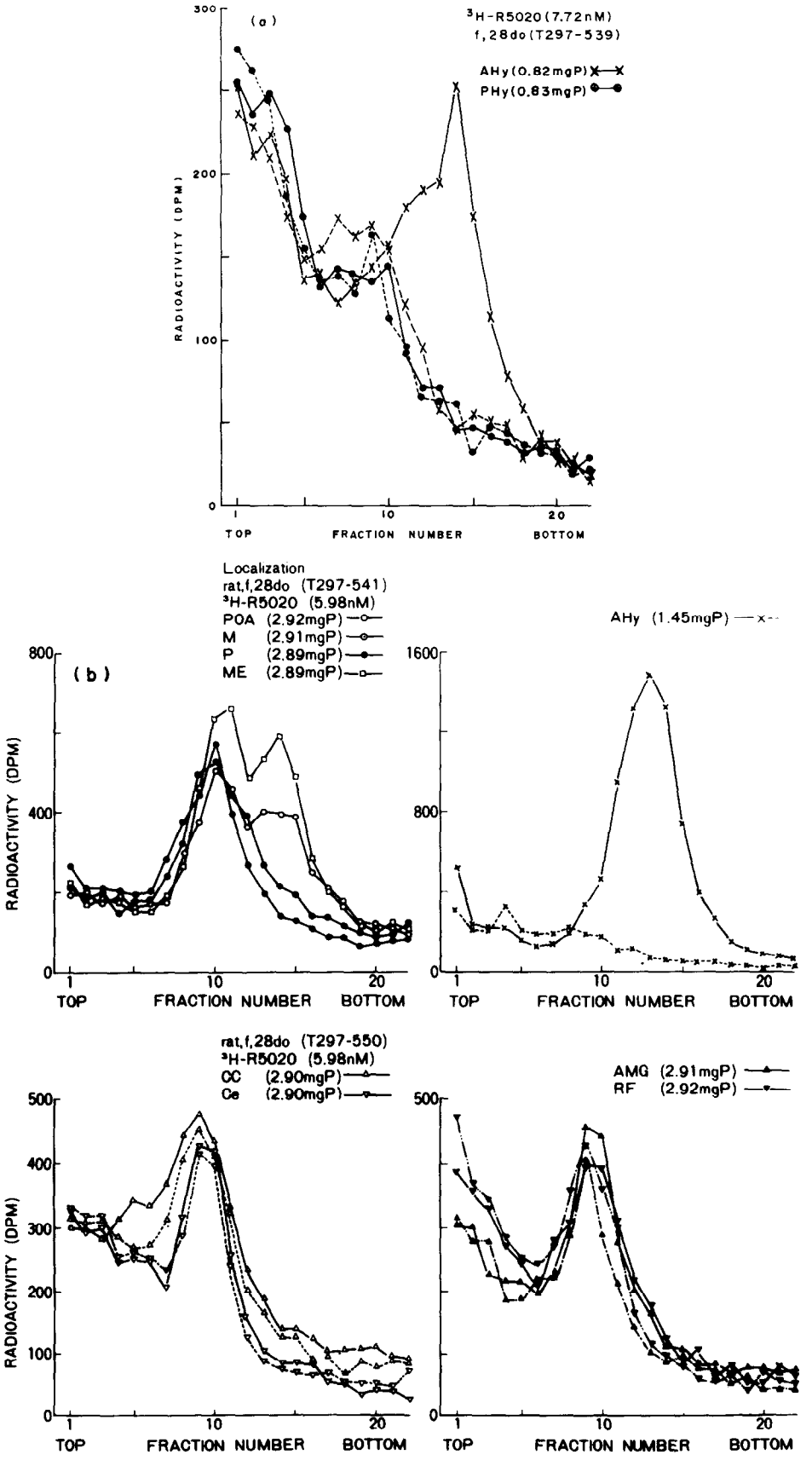


Fig. 4. Density gradient sedimentation patterns of the cytosols from the preoptic-anterior hypothalamus, the middle- and posterior hypothalamus, the median eminence, various brain tissues and the hypophysis of the estrogen-primed 28-day-old rats. The cytosols were incubated with [^{3}H]-R5020 at 0°C for 4–8 h. Details are given in the text. Radioactivity was expressed as disintegrations/0.2 ml fraction. POAH, preoptic-anterior hypothalamus; MH, middle hypothalamus; PH, posterior hypothalamus; ME, median eminence; CC, cerebral cortex; Ce, cerebellum; AMG, amygdala; RF, reticular formation;

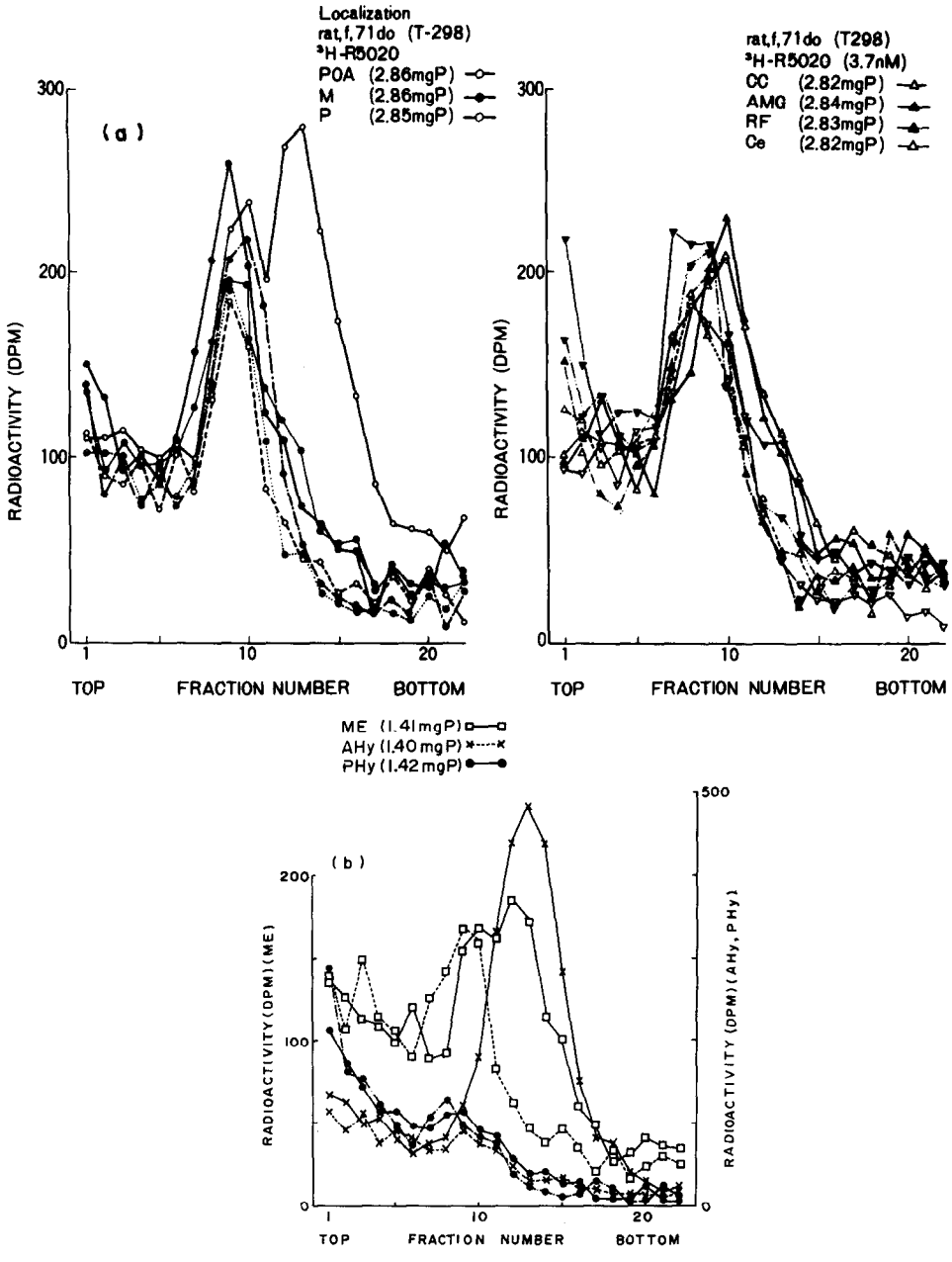


Fig. 5. Density gradient sedimentation patterns of the cytosols from various parts of the brain, anterior- and posterior hypophysis of the estrogen-primed adult female rats. Other experimental conditions were the same as the legend in Fig. 5.

The progesterone receptors are predominantly localized in the preoptic-anterior hypothalamus, the median eminence and the anterior hypophysis of the estrogen-primed immature (Fig. 4, Table 1) and mature rats (Fig. 5). Intrahypothalamic localization of the progesterone receptors, along with those of estrogen- and androgen receptors, is illustrated schematically in Fig. 6. Differential localization of the progesterone receptors in the brain is found to be almost identical to that of estrogen receptors [31–34]. It is suggested that progesterone receptors are induced in some specific brain regions containing a high amount of estrogen receptors through which estrogen affects the brain function.

It is noteworthy that in the amygdaloid complexes containing estrogen receptors [40–42] no appreciable amount of progesterone receptor proteins can be induced by estrogen priming. Since progesterone is reported to act on the amygdala [43], this interesting point remains open to further investigation.

Although as yet, there is little data available on nuclear receptors in the brain, nuclear receptors for progesterone have been demonstrated in the anterior hypophysis and, possibly, the hypothalamus of the

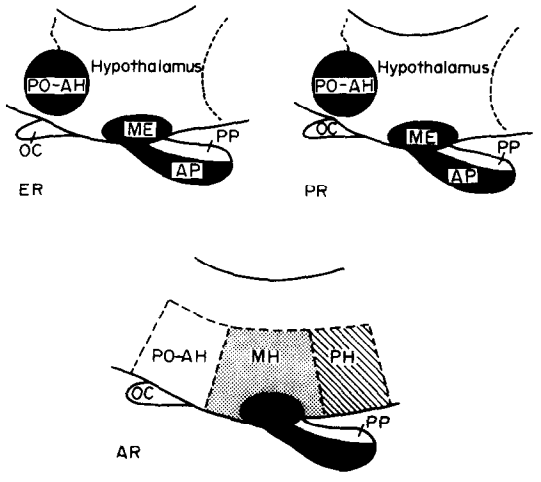


Fig. 6. Schematic representation of the localization of the receptors for estrogen, progesterone and androgen in the hypothalamus and the hypophysis of female rats. The receptors for former two steroid hormones are localized mostly in the preoptic-anterior hypothalamic region, the median eminence and the anterior hypophysis.

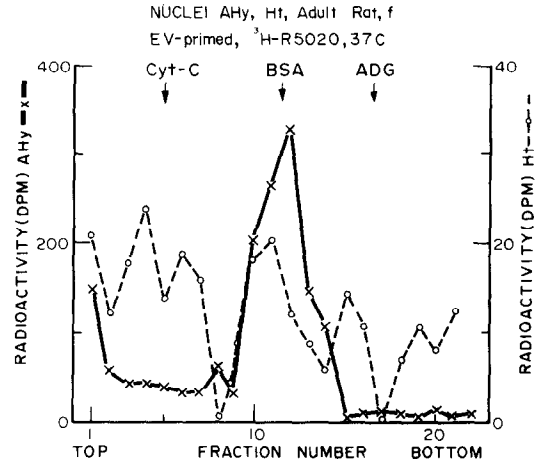


Fig. 7. A sucrose density gradient sedimentation pattern of KCl extract of purified nuclei from estrogen-primed adult rat anterior pituitaries and hypothalami. Quartered anterior pituitaries and hypothalami collected from 49 rats were incubated *in vitro* with [^{3}H]-R5020 (5.1 nM) for 30 min at 37°C. Purified nuclei were then prepared from the homogenate, followed by the extraction of R5020 receptor complexes with 0.4 M KCl from the nuclei. The extract was layered on a 5–20% sucrose density gradient containing 10% glycerol and 0.4 M KCl, and centrifuged at 50,000 rev./min in a SW 50.1 rotor in a Beckman centrifuge for 22 h. Cyto-C, cytochrome C; BSA, bovine serum albumin; ADG, alcohol dehydrogenase.

estrogen-primed adult rats (Fig. 7). These results, together with the cytosol receptors, suggest that progesterone can act upon the brain through its interaction with the receptors in the hypothalamus and hypophysis.

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