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PREGNENOLONE BINDING SITES IN THE RAT OLFACTORY BULB

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Summary—High concentrations of pregnenolone and its sulfate have been found in several areas of rat and human brain and seem to be controlled by local mechanisms. In the present experiments we have demonstrated pregnenolone binding sites in the cytosolic fraction of the rat olfactory bulb. The pregnenolone binding component showed a $K_d = 2.34 \pm 0.66 \times 10^{-7}$ M and $N_{max} = 7.25 \pm 1.20$ pmol/mg protein. Pregnenolone, pregnenolone sulfate and 17OH-pregnenolone competed equally for the binding sites while other steroids were less competitive. Protease and trypsin inhibited binding by 48 and 60% respectively. Sucrose density gradient analysis showed a minor peak at 4.6 *s* and a major one at 3.6 *s*. After gel filtration chromatography the pregnenolone binding component appeared as 2 peaks corresponding to molecular weights of approximately 150 and 220 kDa. Heating at 60°C increased binding by 150%. These results indicate that the olfactory bulb pregnenolone binding component is complex in nature. Rat plasma also bound pregnenolone. Plasma binding sites could be partially differentiated from those in the olfactory bulb on the basis of susceptibility to lipoprotein lipase, effect of heating and mobility during polyacrylamide gel electrophoresis.

INTRODUCTION

Steroids are found in various areas of the brain and by interaction with the central nervous system produce diverse neuroendocrine and behavioral effects [1, 2]. Specific binding sites have been identified in the cytosol and nuclei of brain cells for estrogens, progesterone, androgens and corticosteroids [2, 3]. The physicochemical characteristics of these receptors resemble [2] those reported for steroid receptors in non-neural tissues. Recently, high concentrations of pregnenolone, dehydroepiandrosterone and their sulfates have been demonstrated in the rat [4, 5] and human [6] brain. Their concentration in the rat brain seems to be controlled by local mechanisms independent of the peripheral endocrine glands [4, 5]. Further, the concentration of these steroids in some brain structures varies under olfactory and visual stimuli. Thus, there was a significant decrease in the concentration of total pregnenolone (pregnenolone and its sulfate) in the olfactory bulbs of male rats exposed to the scent of adult female rats for a period of seven days [7]. When male rats were exposed to the scent and view of female rats the decrease in pregnenolone concentration in the olfactory bulb was accompanied by a significant increase in total dehydroepiandrosterone concentration in that tissue [8]. These changes in the concentration of pregnenolone and dehydroepiandrosterone were not reflected in their plasma concentrations [7, 8], again indicating local mechanisms regulating the concentration of these steroids in brain structures. The local regulatory mechanisms could include (1) biosynthesis of these steroids, (2) release from conjugated forms such as fatty acid esters, (3) metabolic enzyme activities and (4) local pregnenolone and dehydroepiandrosterone binding proteins. The biosynthetic capacity of rat brain tissue to form pregnenolone from cholesterol had not been demonstrated until recently [9]. The release of steroids from fatty acid conjugates is thought to be unlikely [5]. We have been studying the possible presence of binding sites for pregnenolone and dehydroepiandrosterone in the rat brain and the metabolism of these steroids in that tissue. Because of our observation [8] on the effect of olfactory and visual stimulation on the concentration of pregnenolone and dehydroepiandrosterone in the olfactory bulb, our studies have been done principally with that tissue. Here we report the presence of pregnenolone binding sites in the rat olfactory bulb and plasma and the partial characterization of these sites. We have also studied the *in vivo* uptake of [^{3}H]pregnenolone by the olfactory bulb.

†Dr Lanthier died on November 13, 1988. This paper is dedicated to his memory.

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EXPERIMENTAL

Chemicals

[7-^{3}H]Pregnenolone (SA 13.1–22.6 Ci/mmol) was obtained from New England Nuclear, Montreal, Canada, and purified before use by celite column chromatography. Non-radioactive steroids were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. and from Ramat-Gan, Israel, and crystallized before use. Sephacryl gels and Sephadex LH-20 were obtained from Pharmacia Fine Chemicals. Other chemicals were from Sigma Chemical Co.

Animals

Male Sprague–Dawley rats, 10 weeks old, were purchased from Charles River of Canada, St Constant, Québec, Canada and were kept in individual cages under light-controlled conditions (12 h light). The animals were killed by decapitation and when required the resulting blood was collected in heparinized tubes. The brains of the animals were exposed and the olfactory bulbs were removed and dissected. Olfactory bulbs from several animals (4–20, depending on the experiment) were pooled and washed several times with ice-cold normal saline.

Preparation of cytosol

The pooled tissue was homogenized in 10 mM Tris–HCl buffer, pH 7.4, containing 1.5 mM EDTA, 15 mM sodium molybdate, 12 mM monothioglycerol and 10% glycerol (TEMG buffer). The homogenate was filtered through gauze and centrifuged at 800 ***g*** for 10 min in a Beckman refrigerated centrifuge. The supernatant was then centrifuged at 100,000 ***g*** for 1 h in a Beckman ultracentrifuge. The resulting supernatant was made to 4 ml or more as required and designated as cytosol. 4 ml of cytosol was then mixed with a charcoal pellet resulting from the centrifugation of 0.4 ml of a charcoal suspension in TEMG buffer (2.5% charcoal + 0.25% dextran) and kept at 4°C for 45 min. Charcoal was removed by centrifugation in the Beckman centrifuge. Such charcoal-treated cytosol was used in all experiments. The charcoal treatment was done to minimize the effect of endogenous pregnenolone on binding with the incubated ligand.

Binding assay

A series of tubes containing 10–500 nM [^{3}H]pregnenolone was set up and 0.2 ml of cytosol (approximately 200 μg protein) was added to each tube. A parallel series contained the tracer, 200-fold excess of non-radioactive pregnenolone and cytosol. The tubes were gently vortexed and kept at 0°C for 4 h. Separation of bound and free radioactivity was then done by use of Sephadex LH-20 minicolumns. A 5 ml disposable pipette cut at 2 ml mark, with a piece of glass wool at the bottom, was used as a column. It was packed with Sephadex LH-20, previously swollen with TEMG buffer to the height of 2.5 cm. All columns were kept at 4°C at least for 1 h before use. The contents (0.2 ml) of an incubated tube were deposited on the column and allowed to enter the bed. 1 ml of ice-cold TEMG buffer was then added to the column and passed through under nitrogen pressure. The entire procedure took less than one min. The eluate was collected in a vial and mixed with 10 ml of scintillation fluid. The radioactivity in the vials was determined by counting in a Beckman Liquid Scintillation counter. The results of a binding assay were analyzed by microcomputer assisted linear regression and plotted according to the method of Scatchard[10]. Protein was determined by the method of Bradford[11].

Effect of enzymes on binding

One ml aliquots of cytosol were incubated at 0°C for 60 min with 0.2 ml of an enzyme solution in TEMG buffer or with 0.2 ml of the buffer. A series of tubes containing 100 nM [^{3}H]pregnenolone without (3 tubes per treatment group) or with (2 tubes per treatment group) 200-fold excess pregnenolone was set up. 0.2 ml of enzyme-treated or buffer-treated cytosol was added to a tube and the tubes were kept at 0°C for 4 h. The bound activity was isolated by Sephadex LH-20 minicolumns. The enzymes used and their concentrations are shown in Table 3.

Effect of heating on binding

1.2 ml aliquots of cytosol were kept at 0, 30 or 60°C for 30 min. Binding to [^{3}H]pregnenolone was then determined as described for enzyme-treated cytosol.

Competition for binding by different steroids

0.2 ml of cytosol was incubated in triplicate at 0°C with (a) 100 nM [^{3}H]pregnenolone and (b) 100 nM [^{3}H]pregnenolone + 200-fold excess of a non-radioactive steroid. After 4 h of incubation bound and free fractions were separated as described before. Results for a competitor were expressed in comparison with the displacement of radioactivity obtained with non-radioactive pregnenolone according to the formula (dpm displaced with competitor/dpm displaced with excess pregnenolone) × 100.

Polyacrylamide tube-gel electrophoresis

These experiments used 12 cm long tube gels. 0.2 ml cytosol samples were stacked on 2.5% polyacrylamide gels (prepared in 50 mM Tris, pH 6.7) and separated with 7% gels (prepared in 50 mM Tris, pH 8.9). Both the stacking (6.3×10^5 dpm/tube) and the separating (1.2×10^6 dpm/tube) gels contained [^{3}H]pregnenolone to allow binding to take place during electrophoresis. The electrode chamber buffer ions were Tris (50 mM) and glycine (0.38 M) at pH 8.3. Electrophoresis was carried out at 1 mA/tube in a pre-chilled apparatus (4°C) and terminated when the tracking dye approached 1 mm from the bottom of the tube. The gels were cut into 1.5 mm slices and their radioactivity was determined.

Sucrose density gradient analysis

In these experiments and in those described for gel filtration analysis a protease inhibitor, phenylmethylsulfonyl fluoride (1 mM), was added to the TEMG buffer. Linear sucrose density gradients (10–30%) were prepared in the buffer. 0.2 ml of cytosol was put on a centrifuge tube containing 3.8 ml of the gradient. The tubes were centrifuged at 330,000 *g* for 18 h at 2°C in a swinging bucket rotor (SW 60 Ti) in Beckman ultracentrifuge model L2-65B. After centrifugation the gradient was fractionated into 24 fractions with a Buchle device. Each fraction was collected in a tube containing 100 nM [^{3}H]pregnenolone, then incubated at 0°C for 4 h and the bound activity was isolated. Ovalbumin-(methyl-$^{-14}$C), 3.6 *s*, and human globulin-(methyl-$^{-14}$C), 7.0 *s*, were used as markers.

Gel-filtration chromatography

Cytosol (approximately 20 mg protein) prepared from the olfactory bulbs of 20 animals was used in each experiment. Sephacryl gels S-400 and S-500 were used and the gels were packed and equilibrated with TEMG buffer in a column (2.6 cm in diameter, up to a height of 60 cm). All chromatography procedures were conducted in a cold room at 3°C. Four protein standards were used to calibrate the columns (Figs 5 and 6). The [^{3}H]pregnenolone–cytosol complex was found to be unstable on the columns during the chromatography. Therefore, the cytosol (4 ml) was put on the column without pre-incubation with [^{3}H]pregnenolone. The column was eluted with TEMG buffer at 15 ml/h and 110 or 125 (3 ml) fractions were collected. 0.2 ml of a fraction was then added to a tube containing 200 nM [^{3}H]pregnenolone and incubated at 0°C for 4 h. The bound radioactivity was isolated by LH-20 minicolumns and counted.

Studies with plasma

Plasma was prepared from the blood collected at the time of decapitation of an animal. All the experiments described above for the olfactory bulb cytosol were also done with plasma. The plasma was diluted with TEMG buffer to obtain appropriate protein concentration for an experiment and treated with a pellet of dextran-coated charcoal before use in that experiment.

Uptake of [^{3}H]pregnenolone by various regions of the rat brain

Adrenalectomized and castrated male rats were maintained on normal saline and their usual diet for 5 days. The animals were divided in two groups, control and experimental. Control animals were injected i.p. with 0.2 ml of 50% ethanol and those in the experimental group with 200 μg pregnenolone in 0.2 ml of 50% ethanol. 30 min after this treatment each animal in the 2 groups was injected i.p. 40 μCi [^{3}H]pregnenolone in 0.2 ml of 20% ethanol and was killed by decapitation 1 h after this injection. Blood was collected at the time of decapitation. The brain was isolated and amygdala, hippocampus, hypothalamus, olfactory bulbs, pituitary and different regions of the cortex were dissected out. Each tissue was weighed and homogenized in 2 ml distilled water. The homogenate was processed for isolation of radioactivity associated with pregnenolone and its sulfate by our previously described technique [8]. Isolated radioactivity was expressed as dpm/10 mg tissue.

RESULTS

In preliminary experiments it was observed that olfactory bulb cytosol and diluted plasma of the rat bound [^{3}H]pregnenolone. The specific binding to cytosol was maximal at 4–6 h and declined thereafter. The binding to plasma continued to increase after the 4 h period and was maximal at 16 h and remained at that level up to 24 h. In further experiments incubations of [^{3}H]pregnenolone with the cytosol were kept for 4 h and those with the plasma for 18–20 h.

Binding to different regions of the brain

Different regions of the brain of an animal were dissected out and cytosolic fractions were prepared as described for the olfactory bulb. The cytosols were then used at approximately equal protein concentration for binding with [^{3}H]pregnenolone. Cytosols of all the regions that were tested bound pregnenolone (see Table 1). The binding capacity was roughly equal in the regions although the highest capacity was seen with the bulb cytosol.

Identity of the bound radioactivity

In one experiment, olfactory bulb cytosol (3.0 ml) was incubated with [^{3}H]pregnenolone (1.28×10^6 dpm) and the bound activity was isolated by a series of Sephadex LH-20 columns. The bound activity was extracted with ethyl acetate and after addition of non-radioactive pregnenolone (25 μg), was chromatographed on silica gel thin-layer plates in the system benzene:ethanol (95:5). 85% of the extracted radioactivity co-migrated with authentic pregnenolone standard. The remainder was attributable to some transformations during the extraction procedure and chromatography.

Table 1. Specific [^{3}H]pregnenolone binding by cytosolic fractions of rat brain structures and adrenals

Tissue	[^{3}H]pregnenolone bound pmol/mg protein
Olfactory bulb	2.83
Amygdala	1.82
Hippocampus	1.51
Hypothalamus	1.81
Frontal cortex	1.65
Parietal cortex	1.32
Occipital cortex	1.05
Temporal cortex	1.22
Adrenals	3.75

Cytosols were incubated with 100 nM of the ligand. Values are means of two experiments.

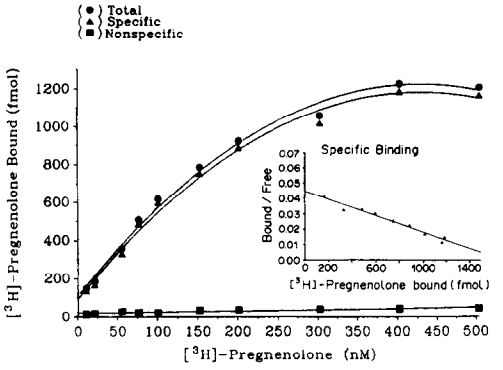

Fig. 1. Saturation curve and Scatchard analysis (Insert) of [^{3}H]pregnenolone binding to rat olfactory bulb cytosol. Increasing amounts (10–500 nM) of [^{3}H]pregnenolone ± 200-fold excess of non-radioactive pregnenolone were incubated with 0.2 ml of bulb cytosol (190 μg protein). The data for total and specific binding were analyzed by non-linear regression and the data for non-specific binding by linear regression. For Scatchard analysis specific binding data were analyzed by linear regression. The Scatchard plot ($r^2 = -0.98$) for this experiment gave a $K_d = 1.88 \times 10^{-7}$ M and $N_{max} = 8.56$ pmol/mg protein.

Binding parameters

Specific binding represented by far the major portion of the total [^{3}H]pregnenolone bound to the olfactory bulb cytosol (Fig. 1). Specific binding was saturable at 400–500 nM concentration of the ligand. Scatchard transformations of the specific binding data revealed a $K_d = 2.34 \pm 0.66 \times 10^{-7}$ M and $N_{max} = 7.25 \pm 1.20$ pmol/mg protein ($n = 3$). Representative saturation and Scatchard plots are shown in Fig. 1. The binding characteristics for plasma are illustrated in Fig. 2. Non-specific binding was again minor and the specific binding was saturable. The K_d for binding, 3.15×10^{-7} M was similar to that of the bulb cytosol but plasma showed a much higher binding capacity, 31.17 pmol/mg protein.

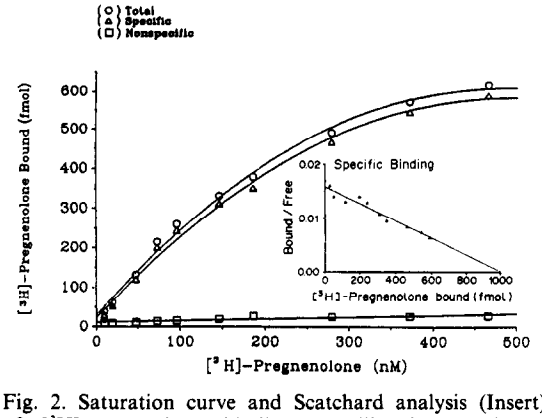

Fig. 2. Saturation curve and Scatchard analysis (Insert) of [^{3}H]pregnenolone binding to diluted rat plasma (32 μg protein) as described in the legend to Fig. 1. The Scatchard plot ($r^2 = -0.97$) gave a $K_d = 3.15 \times 10^{-7}$ M and $N_{max} = 31.17$ pmol/mg protein.

Table 2. Displacement of [^{3}H]pregnenolone bound to rat olfactory bulb cytosol or plasma by various steroids (200-fold excess)

Steroid added	Displacement (%) Cytosol	Plasma
Pregnenolone	100	100
17OH-pregnenolone	99.5	96.2
Pregnenolone sulfate	97.2	90.3
Progesterone	77.6	74.1
Dehydroepiandrosterone	65.1	32.9
Dehydroepiandrosterone Sulfate	33.5	00.0
17OH-progesterone	46.6	49.2
Androstenedione	49.3	24.9
Testosterone	47.0	30.2
Estrone	62.5	38.7
Estradiol	63.0	37.4
Corticosterone	23.8	28.8
Cholesterol	25.0	29.2

Values (means of 2 experiments) are expressed according to the formula:

$$\frac{\text{dpm displaced with competitor}}{\text{dpm displaced with excess pregnenolone}} \times 100.$$

Displacment of binding by different steroids

Pregnenolone, 17-hydroxy-pregnenolone and pregnenolone sulfate displaced to the same extent the [^{3}H]pregnenolone bound to olfactory bulb cytosol. Other steroids showed less competition for the bound ligand. All the steroids in this latter group displaced [^{3}H]pregnenolone to some extent, progesterone being the most effective. Similar results were obtained with the plasma (Table 2) although dehydroepiandrosterone sulfate did not displace the ligand at all.

Effect of enzymes

Protease and trypsin inhibited binding of [^{3}H]pregnenolone to olfactory bulb cytosol by approximately 48 and 60% respectively, while DNase, RNase and lipoprotein lipase had no effect. Binding to plasma was inhibited to a greater extent by protease and the plasma binder showed marked sensitivity to lipoprotein lipase (Table 3).

Table 3. Effect of enzymes on [^{3}H]pregnenolone binding by rat olfactory bulb and plasma

Enzyme added	Cytosol (% control)	Plasma (% control)
Protease		
1 mg/ml	55.2	36.0
2 mg/ml	52.4	10.3
Trypsin		
1 mg/ml	45.8	—
2 mg/ml	40.0	44.0
Lipoprotein lipase		
1 unit/ml	103.2	19.3
DNase		
1 mg/ml	102.0	97.7
RNase		
1 mg/ml	97.0	102.0

Cytosol or plasma was pre-incubated with the enzyme for 60 min at 0°C before measuring specific binding to [^{3}H]pregnenolone. Values are means of 2 experiments and are expressed as % of specific binding in control samples. Protease was type XIV (Sigma Chemical Co.), derived from streptomyces griseus.

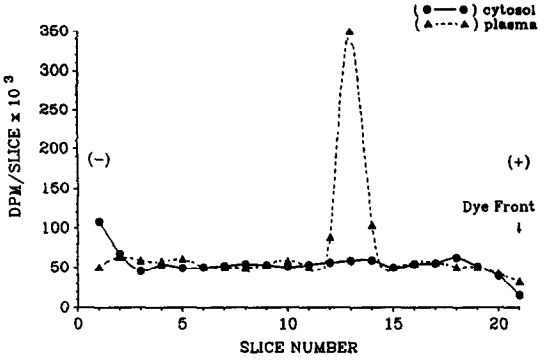

Fig. 3. Binding of [^{3}H]pregnenolone to rat olfactory bulb cytosol and plasma as determined by tube gel electrophoresis. [^{3}H]pregnenolone was mixed with the stacking and separating gels and aliquots (350 μg protein) of cytosol or diluted plasma were put on the gel. Following electrophoresis gels were cut into 1.5 mm slices and their radioactivity determined.

Effect of heat

Heating the cytosol to 30°C had no effect on its capacity to bind [^{3}H]pregnenolone while heating to 60°C increased the capacity by 150%. Similar treatment of plasma resulted in decreases in binding capacity of 20 and 75% respectively.

Polyacrylamide gel electrophoresis

When electrophoresis was carried out after putting cytosol on polyacrylamide gel containing [^{3}H]pregnenolone, the binding component did not migrate from the stacking gel (Fig. 3). On the other hand, the binding component in plasma migrated during electrophoresis (Fig. 3).

Sucrose density gradient analysis

In experiments where cytosol containing bound [^{3}H]pregnenolone was subjected to sucrose gradient centrifugation for 18 h the binding did not remain stable. The analysis was therefore done by incubating post-centrifugation fractions with [^{3}H]pregnenolone. Binding component in the cytosol sedimented as a minor peak at 4.6 *s* and a major one at 3.6 *s* (Fig. 4). Pregnenolone binding macromolecules in the plasma migrated as a major peak at 3.6 *s* and a minor one at 1.3 *s* (Fig. 4).

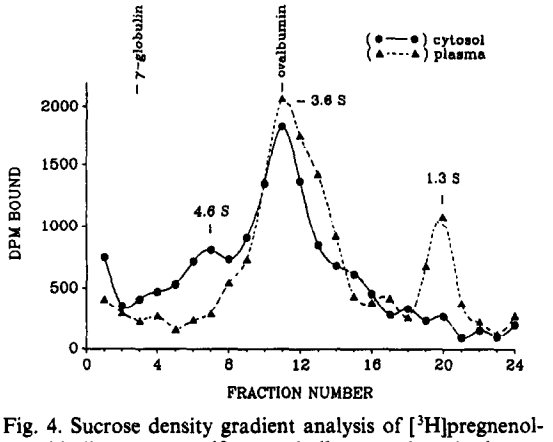

Fig. 4. Sucrose density gradient analysis of [^{3}H]pregnenolone binding to rat olfactory bulb cytosol and plasma. Cytosol or diluted plasma was put on a 10–30% sucrose gradient. After centrifugation at 330,000 g for 18 h, the gradient was fractionated into 24 fractions. Each fraction was incubated with 100 nM [^{3}H]pregnenolone and the bound radioactivity (values shown in the figure) was isolated as described in the text.

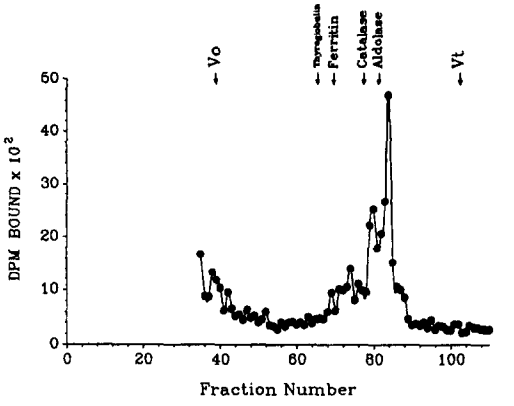

Fig. 5. [^{3}H]Pregnenolone binding by rat olfactory bulb cytosol fractions after chromatography on Sephacryl S-400 columns. Cytosol (20 mg protein) was put on the column; was eluted with TEMG buffer and 110 (3 ml) fractions were collected. An aliquot (0.2 ml) from fractions 35–110 was incubated with 200 nM [^{3}H]pregnenolone. Bound radioactivity (values shown in the figure) was isolated as described in the text. The column was calibrated with aldolase (M_r 158 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (660 kDa).

Gel filtration chromatography

The columns were first calibrated with protein standards to give a plot of K_{av} vs log molecular weight which was used to estimate the apparent molecular weight of the pregnenolone binding component. Pregnenolone binding was determined on cytosolic fractions collected during the gel chromatography. The results are shown in Figs 5 and 6. On the S-400 column the binding component was resolved in two

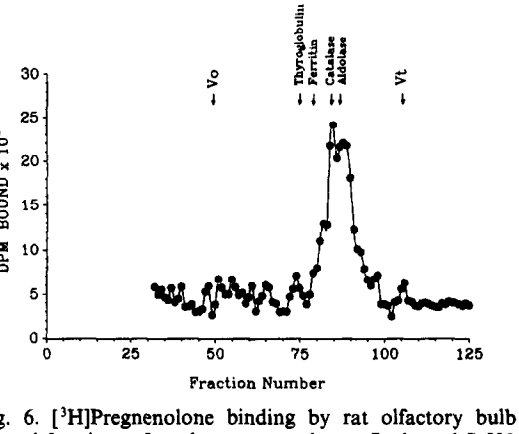

Fig. 6. [^{3}H]Pregnenolone binding by rat olfactory bulb cytosol fractions after chromatography on Sephacryl S-500 column. Details were as described in the legend to Fig. 5; 125 (3 ml) fractions were collected.

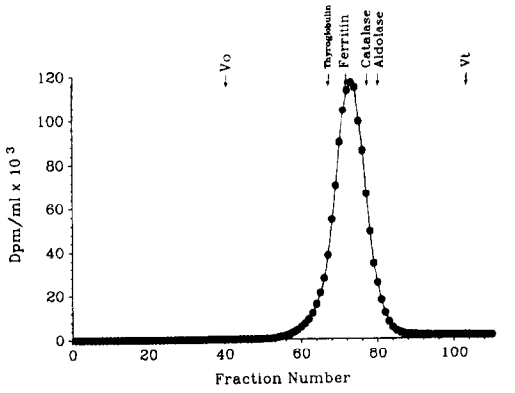

Fig. 7. Gel filtration chromatography of [^{3}H]pregnenolone-rat plasma complex on Sephacryl S-400 column. 4 ml diluted plasma (equivalent to 30 mg protein) was incubated with 200 nM [^{3}H]pregnenolone. The bound fraction was isolated by treatment with a pellet of dextran-coated charcoal and put on the column. Elution was done with TEMG buffer; 110 (3 ml) fractions were collected and their radioactivity was determined.

peaks corresponding to M_r 150 and 220 kDa. Similarly two peaks in binding were evident after S-500 column chromatography and corresponded to M_r 145 and 232 kDa. The [^{3}H]Pregnenolone–plasma complex was stable on the column and showed a single peak corresponding to M_r 340 kDa (Fig. 7).

In vivo uptake of [^{3}H]pregnenolone by different regions of the brain

Preliminary experiments had shown that the uptake of [^{3}H]pregnenolone by brain areas was maximal 1 h after its administration. This period was chosen for further experiments. After administration of the tracer its concentration in brain regions was much higher than in the plasma (Table 4). Uptake in the pituitary was the lowest while differences in uptake by other regions were not statistically significant. The uptake of [^{3}H]pregnenolone by the brain structures was quantitatively similar in animals pre-injected with non-radioactive pregnenolone and in those not receiving such pre-treatment (Table 4).

Table 4. Uptake of [^{3}H]pregnenolone by regions of rat brain

Tissue	Uptake (dpm/10 mg tissue) Group C	Group E
Olfactory bulb	285 ± 75	258 ± 45
Amygdala	346 ± 76	322 ± 50
Hippocampus	499 ± 101	462 ± 68
Hypothalamus	438 ± 94	400 ± 61
Frontal cortex	386 ± 72	325 ± 54
Pituitary	124 ± 35	93 ± 19
Plasma (dpm/10 μl)	36 ± 11	30 ± 8

Radioactivity (dpm) isolated as pregnenolone + pregnenolone sulfate; group C: animals were injected (i.p.) 40 μCi [^{3}H]pregnenolone; group E: animals pre-injected 200 μg non-radioactive pregnenolone before injection of 40 μCi [^{3}H]pregnenolone; other areas of the cortex had values similar to those shown for frontal cortex. Values are means ± SEM (n = 6).

DISCUSSION

The results reported above show the presence of pregnenolone binding sites in the olfactory bulb of the rat. Similar binding was also detected in other areas of the brain (Table 1). Because of our interest in pregnenolone metabolism in the olfactory bulb the binding was studied in more detail in that tissue.

In competition studies (Table 2) pregnenolone and pregnenolone sulfate competed equally for the binding sites. This is of some significance since pregnenolone sulfate forms more than 50% of total pregnenolone in the rat olfactory bulb [8]. Progesterone and other steroids also displaced bound [^{3}H]pregnenolone but to a lesser extent than pregnenolone. Total pregnenolone concentrations (30–50 ng/g) in the bulb [8] are much higher than those of progesterone and most of the other steroids [8, 12]. Therefore, the binding sites may have a physiological role in pregnenolone metabolism in that tissue.

The binding parameters for pregnenolone binding in the bulb ($K_d = 2.34 \times 10^{-7}$ M and $N_{max} = 7.25$ pmol/mg protein) indicate that the binding component is not similar to the high affinity ($K_d = 0.4$–1.0×10^{-9} M) and low capacity (23–43 fmol/mg protein) binding sites in the rat brain that have been reported for estradiol, progesterone, androgens and corticosteroids [13–16]. Further, after sucrose density gradient centrifugation in low salt buffer the latter binding components sediment at 7–8 s [2]. Pregnenolone binding component sedimented at 3.6 and 4.6 s (Fig. 4). Steroid binding sites with characteristics similar to those mentioned above for estradiol, progesterone, androgens and corticosteroids function as steroid receptors in the brain and other tissues [2, 17]. The pregnenolone binding component in the bulb will thus not act as a receptor. It may be similar in function to some other tissue binding proteins such as the fatty acid binding protein which binds fatty acids and regulates their tissue metabolism [18] and the sterol carrier protein which binds cholesterol and is involved in tissue cholesterol transport and metabolism [19].

The considerable reduction in pregnenolone binding by the bulb cytosol after treatment with protease and trypsin (Table 3) indicates that a significant part of the binding component is protein in nature. The binding sites do not seem to be lipoproteins since lipoprotein lipase had no effect on pregnenolone binding (Table 3). As mentioned before, the bound ligand was identified as pregnenolone. Therefore, the binding component does not seem to be associated with steroid metabolizing enzymes. The increase in binding capacity observed after heating the cytosol to 60°C is rather surprising and difficult to explain on the basis of the present experiments.

The gel chromatographic behavior of the cytosolic pregnenolone binder was unexpected in the sense that the kinetic data (Fig. 1) indicated the presence of only one binding site whereas we obtained two binding peaks upon gel filtration (Figs 5 and 6). Sucrose

density gradient centrifugation also resulted in two peaks (Fig. 4). It is possible that the smaller binding species is a proteolytic fragment of the larger one. Although a protease inhibitor was added to the buffer for these two studies such enzyme inhibitors are not totally effective and some proteolytic activity could have been resistant. Alternatively, the larger molecule may be an aggregate of two pregnenolone binding polypeptide chains of approximately 140 kDa. Neither of these two possibilities corresponds to sucrose density gradient analysis of the binding component where it sedimented mainly at 3.6 *s* (Fig. 4). This discrepancy could be due to the complex nature of the pregnenolone binding component. Non-globular macromolecules and complex proteins (e.g. lipoproteins) do not behave hydrodynamically in the ideal manner that spherical proteins do [20, 21]. Discrepancy in behavior in different systems has been noted in the case of other steroid binding molecules. Thus, the transformed glucocorticoid receptor sediments at 4.0–4.6 *s* while its molecular weight as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis is around 95–102 kDa [22].

As mentioned earlier, attempts to chromatograph pre-labeled cytosolic samples failed. This was not totally unexpected since it is likely that tissular complexes with affinities of 10^{-7} M will not survive such manipulation. It should also be stated that even our post-labelling experiments (following chromatography) proved to be problematic to the extent that on some occasions the binding patterns demonstrated in Figs 5 and 6 were not evident. The olfactory bulb cytosolic pregnenolone binding component seems to be very labile and further work will be needed to determine its native structure.

In the course of these studies we have also tried to see if the olfactory bulb will extract administered pregnenolone from the circulation and whether such uptake will be selective in nature. Corpéchot *et al.*[5] had previously reported that the concentration of administered [^{3}H]pregnenolone was several-fold higher in the whole rat brain than in the plasma. This uptake of [^{3}H]pregnenolone by the brain was not selective because when it was administered with increasing quantities of non-radioactive pregnenolone the concentration of radioactivity in the brain was not diminished. This did not exclude selective uptake by specific regions of the brain. We have studied several areas of the brain individually, including the olfactory bulb, and arrived at essentially the same conclusions (Table 4) as Corpéchot *et al.* It should be noted that the high endogenous concentrations of pregnenolone, which persist even after removal of endocrine glands [5], would make it difficult to demonstrate a selective uptake of administered [^{3}H]pregnenolone by brain tissue. Nevertheless our study does show uptake of that steroid by the olfactory bulb.

In our experiments rat plasma also bound pregnenolone. This is in contrast to the observation of Kream and Sauer[23] that rat serum did not bind pregnenolone. Such binding, however, has been reported for guinea-pig serum [24]. The affinity for pregnenolone binding in rat plasma (Fig. 2) was similar to that of the bulb cytosol. The apparent molecular weight for the binding component in the plasma as determined by gel filtration chromatography was 340 kDa (Fig. 7). The results of some of our other experiments show that there are differences in the pregnenolone binding components in the olfactory bulb and the plasma. Heating to 60°C reduced binding in the plasma by 75% while that in the bulb cytosol increased by 150%. Plasma pregnenolone binding was inhibited by lipoprotein lipase but the enzyme had no effect on binding in the cytosol (Table 3). The migration pattern during electrophoresis (Fig. 3) also shows the difference between the two components. These differences make it unlikely that the bulb cytosolic pregnenolone binding may be due to contamination from the plasma. Further, the cytosolic binding capacity (N_{max} = 7.25 pmol/mg protein) although lower than that of the plasma (N_{max} = 31.17 pmol/mg protein) is of a significant order and cannot be explained on the basis of such contamination. We may add that the differences in the characteristics of the binding components of the olfactory bulb and plasma that were mentioned above are only indicative of their different nature. Because of similarities in certain other characteristics (affinity, specificity and principal sedimentation peak) and because the binding components were studied in crude preparations (cytosol and plasma) some of the observed differences could possibly also be explained as being due to the different "millieu" in which the two components were studied.

Pregnenolone binding sites are also known to be present in the soluble fraction of the rat [23], guinea-pig [24] and porcine [25] adrenals. In one experiment we also found that rat adrenal cytosol bound [^{2}H]pregnenolone (Table 1). The characteristics of the adrenal binding component have been studied in detail in the guinea-pig by Strott and co-workers [24, 26, 27]. Although the adrenal binding sites have an affinity ($K_d = 1.2 \times 10^{-7}$ M) similar to that we observed in the case of the olfactory bulb, some other characteristics of the adrenal component differ from those of the bulb component. Adrenal binding was susceptible to heating and migrated during electrophoresis [24]. Pregnenolone sulfate displaced [^{3}H]pregnenolone bound to adrenal cytosol to a much lesser extent [28] than observed in the present experiments. The molecular weight of adrenal pregnenolone binding protein has been determined to be 34 kDa [27]. The function of this protein in the adrenals is not known although a role in storage and transport of pregnenolone has been suggested [24]. It is tempting to speculate that the pregnenolone binding component in the olfactory bulb could have a role in the storage of pregnenolone in that tissue. Our study demonstrates an uptake of pregnenolone

by the olfactory bulb; the binding component may have a role in this uptake. Further, it may also be a factor in the change in total pregnenolone concentration in the bulb that occurs after olfactory stimulation [7, 8]. The physiological significance of the pregnenolone binding sites may be more evident when the significance of the high concentrations of pregnenolone and its sulfate in brain structures is clarified. Such studies are in progress and Majewska and co-workers [1] have reported that pregnenolone sulfate may function as an endogenous antagonist of the γ-aminobutyric acid–receptor complex.

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