The Use of Protamine to Study [6,7-³H]Oestradiol-17 β Binding in Rat Uterus

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1. The binding of [6,7-³H]oestradiol-17 β to uteri has been studied by using sucrose-gradient analysis and also the property of oestradiol receptors to form insoluble complexes with protamine. 2. Protamine precipitates the 8S and part of the 4S oestradiol-binding proteins in uterine cytoplasm from mature rats. It does not precipitate the oestradiol-17 β -binding proteins present in cytoplasm from non-target tissues or serum. No tritium-labelled material was precipitated by protamine after equilibration of [6,7-³H]oestradiol-17 β with either serum albumin or phosvitin. 3. Protamine precipitated a small amount of progesterone but not testosterone or cortisol that had been equilibrated with uterine cytoplasm. It did not precipitate any tritium radioactivity from muscle cytoplasm that had been equilibrated with either [1,2-³H]testosterone sulphate or [1,2-³H]dehydroepiandrosterone. 4. A simple method has been devised for measuring binding constants of tissue extracts for [6,7-³H]oestradiol-17 β , based on precipitation with protamine. Reasonable agreement was obtained between the values obtained by this method and those obtained by sucrose-gradient analysis. 5. This method has been used to study the effect of maturity, ovariectomy, adrenalectomy and hypophysectomy on the cytoplasmic binding of [6,7-³H]oestradiol-17 β . None of these procedures affected the dissociation constant K_D or the number of binding sites/mg of cytoplasmic protein. When measured per uterus or per mg of DNA, ovariectomy and hypophysectomy decreased the number of binding sites. Adrenalectomy had no effect. 6. The properties of the 4S oestradiol-binding protein present in cytoplasm from mature uteri have been studied. It is not present in uteri from immature, ovariectomized, or hypophysectomized rats and it does not bind testosterone or cortisol. Unlabelled oestradiol-17 β , U-11,100A, *N*-ethylmaleimide and *N*-bromosuccinimide all decrease the binding of [6,7-³H]oestradiol-17 β to both 8S and 4S receptors. Binding to both 8S and 4S receptors decreases when oestradiol is transported to the nucleus. The 4S receptor is not the same as the 4S binding component formed by salt dissociation of the 8S receptor.

One of the major problems in work on the binding of tritiated steroids to tissues is to distinguish between specific and non-specific binding. To date the only completely satisfactory method of doing this is by sucrose-gradient analysis (Toft & Gorski, 1966) or Sephadex chromatography (King & Gordon, 1966) but these are not practical for monitoring large numbers of fractions. The use of charcoal to separate free and protein-bound oestradiol-17 β in biological fluids has also been used (Korenman, Perrin & McCallum, 1969). The observation that both nuclear and cytoplasmic oestradiol-17 β receptors were precipitated from a number of oestrogen-sensitive tissues by polycations (King, Gordon & Steggles, 1969) suggested

a relatively simple test system. They described the use of a simple polycation, protamine, for distinguishing between specific and non-specific binding of [6,7-³H]oestradiol-17 β to the cytoplasmic fraction of a number of oestrogen-sensitive tissues. The method has now been used to measure the binding constants of rat uterine supernatant for [6,7-³H]oestradiol-17 β under different hormonal conditions. During these experiments it was noted that cytoplasm from the uteri of mature, but not of immature or ovariectomized, rats contained a protamine-precipitable component that bound [6,7-³H]oestradiol-17 β and had a sedimentation coefficient of 4S. The present paper describes some of the properties of this component.

MATERIALS AND METHODS

Animals. Mature (2.3–5 months) or immature (21–23 days) Sprague–Dawley rats were used. Livers were also obtained from mature hamsters (3 months). They were killed by cervical dislocation. Uteri were slit open before use. All operations were carried out under ether anaesthesia. Hypophysectomy was performed by the parathyroid route, adrenalectomy and ovariectomy by dorsal incision.

Labelling of tissues. Two methods were used. In method 1, intact tissues were incubated for 2 h at 4°C in Krebs–Ringer phosphate medium II (Dawson, Elliott, Elliott & Jones, 1959) containing the ^3H -labelled steroid. The medium was changed twice during this period. The tissues were then washed twice with steroid-free medium and an approx. 30% (w/v) homogenate in 10mM-tris-HCl buffer (pH 7.4)–1.5mM-EDTA–2mM- β -mercaptoethanol was prepared with a Silverson homogenizer (Silverson Machines Ltd., London S.E.1, U.K.). This was centrifuged at 10^5g for 1 h to give a clear supernatant fraction.

In the experiments where the supernatant only was labelled (method 2), the ^3H -labelled steroid was omitted from the incubation scheme outlined above and was added directly to the supernatant and equilibrated for 1 h at 4°C.

Precipitation with protamine. Protamine [salmine sulphate from either British Drug Houses Ltd., Poole, Dorset, U.K., or Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] was used. Clupeine sulphate, calf thymus histone or poly-L-lysine will also precipitate the bound $[6,7\text{-}^3\text{H}]\text{oestradiol-17}\beta$. Freshly prepared protamine (0.5 vol., 7.5 mg/ml of water) was added to the test solution and left at 4°C for 5 min. The precipitate was sedimented at 500 g for 10 min at 4°C and the tritium in the pellet and supernatant was estimated as the bound and free steroid respectively. The binding constants were derived from a plot of the bound/free oestradiol ratio against bound oestradiol (Scatchard, 1949). The intercept on the abscissa gives the molar concentration of binding sites (n) and on the ordinate nK_A .

Sucrose gradients. Linear 5–20% (w/v) sucrose gradients in 10mM-tris-HCl buffer, pH 7.4, were used. In most experiments these were centrifuged for 16–18 h in a Beckman SW65 rotor at 125 000 g_{av} at 5°C. To assess the sedimentation coefficients accurately, some gradients were centrifuged for 16–18 h in a Beckman SW40 rotor at 199 000 g_{av} at 7°C.

Miscellaneous methods. $[6,7\text{-}^3\text{H}]\text{Oestradiol-17}\beta$ (235 $\mu\text{Ci}/\mu\text{g}$), $[1,2\text{-}^3\text{H}]\text{cortisol}$ (30 $\mu\text{Ci}/\mu\text{g}$) and $[7\alpha\text{-}^3\text{H}]\text{testosterone}$ (30 $\mu\text{Ci}/\mu\text{g}$) were given by Mr N. Deshpande (of this Institute). $[7\alpha\text{-}^3\text{H}]\text{Testosterone sulphate}$ (10 $\mu\text{Ci}/\mu\text{g}$) and $[7\alpha\text{-}^3\text{H}]\text{dehydroepiandrosterone}$ (65 $\mu\text{Ci}/\mu\text{g}$) were donated by Dr D. Y. Wang (of this Institute). All the steroids were purified by paper chromatography before use. All radioactivity counting was performed in a Packard model 3003 scintillation spectrometer. DNA and protein were determined by the diphenylamine (Burton, 1956) and Folin (Lowry, Rosebrough, Farr & Randall, 1951) methods respectively.

Treatment with *N*-ethylmaleimide was carried out by incubating the uteri in the Krebs–Ringer saline containing 1 mM-*N*-ethylmaleimide for 1 h at room temperature and

then labelling by method 1. Oxidation of tryptophan residues was carried out with 4 mM-*N*-bromosuccinimide for 1 h at 4°C as described by Spande & Witkop (1967) except that 4 mM- KH_2PO_4 –0.4 mM-EDTA buffer, pH 6, was used. This was to minimize acid dissociation of the $[6,7\text{-}^3\text{H}]\text{oestradiol-17}\beta$ binding. This treatment was performed on the supernatant before labelling by method 2. Similar results were obtained when the *N*-bromosuccinimide treatment was carried out after labelling with $[6,7\text{-}^3\text{H}]\text{oestradiol-17}\beta$.

RESULTS

Effect of protamine on $[6,7\text{-}^3\text{H}]\text{oestradiol-17}\beta$ binding in rat uterus. Fig. 1(a) shows the sucrose-gradient profiles of cytoplasm from mature rat uteri after labelling with different amounts of $[6,7\text{-}^3\text{H}]\text{oestradiol-17}\beta$ (method 2). The uteri were washed before homogenization to remove non-specifically bound material (see the Materials and Methods section). Protamine precipitated all of the 8S material and a large part of the 4S region. Good agreement was obtained between the c.p.m. precipitated by protamine and the c.p.m. in the 8S+4S region of the gradient (Table 1) except at very high concentrations of $[6,7\text{-}^3\text{H}]\text{oestradiol-17}\beta$. This may be due to the large overlap of free $[6,7\text{-}^3\text{H}]\text{oestradiol-17}\beta$ into the 4S region of the gradient. When this experiment was repeated with uteri from immature rats, no ^3H appeared in the 4S region of the gradient until the $[6,7\text{-}^3\text{H}]\text{oestradiol-17}\beta$ concentration reached 2 nM (Fig. 1b). At this concentration, about 10% of the ^3H sedimented in the 4S region as compared with 40% in the comparable experiment with cytoplasm from mature rats.

Steroid specificity. Only trace amounts of $[1,2\text{-}^3\text{H}]\text{cortisol}$ and $[7\alpha\text{-}^3\text{H}]\text{testosterone}$ were co-precipitated from uterine supernatant from mature rats by protamine (Table 1). Sucrose-gradient analysis (Fig. 1c) indicated that neither of these steroids was attached to macromolecules in the 8S regions of the gradient. Some $[7\alpha\text{-}^3\text{H}]\text{progesterone}$ was precipitated by protamine and this coincided with the appearance of bound $[7\alpha\text{-}^3\text{H}]\text{progesterone}$ in the 4S but not the 8S region of the sucrose gradient. Pretreatment of the uteri with 12 nM-*oestradiol-17}\beta depressed the subsequent binding of 1 nM- $[6,7\text{-}^3\text{H}]\text{oestradiol-17}\beta$ by 67% as judged by protamine precipitation, but had no effect on the results with the other tritiated steroids. To check the possibility of non-specific interaction between the polycation and negatively charged steroid metabolites, a soluble fraction from muscle was incubated with 1 nM- $[1,2\text{-}^3\text{H}]\text{testosterone sulphate}$ or $[1,2\text{-}^3\text{H}]\text{dehydroepiandrosterone sulphate}$. Less than 5% of either steroid was precipitated by protamine.*

Fig. 1. Sedimentation profiles of uterine supernatant labelled with [³H]steroids. The gradients [5–20% (w/v) sucrose in 10 mM-tris-HCl buffer, pH 7.4] were centrifuged for 16–18 h at 5°C at 125 000 *g*_{av} in the Spinco SW65 rotor. (a) Effect of adding increasing amounts of [6,7-³H]oestradiol-17 β to supernatant from uteri of mature animals: ----, 0.5 nM; ••••, 1 nM; —, 2 nM; —, 4 nM; - - - - , 4 nM-oestradiol, followed by treatment with protamine and removal of insoluble material. (b) Binding of 1 nM-[6,7-³H]oestradiol-17 β to supernatant from uteri of immature (—), mature (----) and ovariectomized (••••) animals. (c) Binding of 1 nM-[1,2-³H]-cortisol (—), -[7 α -³H]testosterone (••••) and -[7 α -³H]progesterone (----) to supernatant of uteri from mature animals. In no case was there a significant amount of radioactivity in fractions 1–10. (d) Effect of 100 nM-oestradiol (••••) and 10 μ M-U-11,100A (▼▼▼▼) on the binding of 1 nM-[6,7-³H]oestradiol-17 β (—) to supernatant of uteri from mature animals.

Tissue specificity. Table 2 shows the results obtained after labelling various tissues with 1 nM-[6,7-³H]oestradiol-17 β (method 1). Negligible amounts of steroid were precipitated with rat serum or phosvitin, both of which formed bulky precipitates with protamine. Bovine serum albumin did not complex with protamine and no steroid was precipitated. Less than 20% of the

steroid was precipitated from the soluble fraction of rat muscle and hamster liver. A similar value has been reported for rat brain cortex (Vertes & King, 1969). Somewhat higher values were obtained with rat liver and spleen. The reason for this is not known but with liver there is a species difference, as hamster liver contained negligible amounts of precipitable material.

Table 1. Comparison of sucrose gradient profiles of labelled uterine supernatant with protamine precipitation results

Uterine supernatant from mature rats was labelled with the given concentration of ^3H -labelled steroids and then either sedimented through linear 5–20% (w/v) sucrose gradients or treated with protamine.

Steroid	Concn. of steroid (nM)	% of total supernatant ^3H	
		Precipitated by protamine	8S+4S region of the gradient
Oestradiol-17 β	1	85	91
	2	83	86
	4	76	72
	8	55	38
Cortisol	1	6	55
Testosterone	1	12	Not measurable
Progesterone	1	20	90

Table 2. Protamine precipitation of [6,7- ^3H]-oestradiol-17 β in the supernatant fraction from different tissues

Tissues from female animals were incubated with 1 nM-[6,7- ^3H]oestradiol-17 β , a 10 5 g supernatant fraction was prepared and treated with protamine. Serum, bovine plasma albumin (10mg/ml) or phosvitin (5mg/ml) were equilibrated with 1 nM-[6,7- ^3H]oestradiol-17 β and then treated with protamine. The results are expressed as mean $\pm$ S.D. of at least four results.

Tissue	Radioactivity (d.p.m./mg of supernatant protein)	% of supernatant ^3H precipitated by protamine
Rat		
Uterus	3660 $\pm$ 2290	86 $\pm$ 3
Liver	276 $\pm$ 37	38 $\pm$ 4
Spleen	695 $\pm$ 197	37 $\pm$ 9
Muscle	681 $\pm$ 188	16 $\pm$ 1
Serum	—	5 $\pm$ 1
Hamster		
Uterus	4157 $\pm$ 2037	83 $\pm$ 8
Liver	375 $\pm$ 92	19 $\pm$ 7
Bovine plasma albumin	—	1 $\pm$ 1
Phosvitin	—	6 $\pm$ 1

Use of protamine to determine binding constants of uterine supernatant for [6,7- ^3H]oestradiol-17 β . Fig. 2 compares Scatchard binding plots derived by the protamine method and by sucrose-gradient analysis. In the latter method, the amount of tritium radioactivity sedimenting in the 8S+4S region of the gradient was taken as the bound steroid. Regression lines were calculated for both methods and both gave statistically significant straight lines ($P < 0.01$ and 0.001 for the gradient and protamine methods respectively). The two lines were, however, different. By using the protamine method, the

Fig. 2. Scatchard binding plot for [6,7- ^3H]oestradiol-17 β

equilibrated with uterine supernatant from mature animals. The amounts of bound and free steroid were determined either as the ^3H in the protamine precipitate and supernatant respectively (O—O) or as the ^3H in the 8S+4S regions of the sucrose gradient and in fractions 17–20 respectively (●—●).

number of binding sites (n) was calculated as 3.1×10^{-13} mol/mg of supernatant protein and the dissociation constant, K_D , was 13.7×10^{-11} mol/l. The sucrose-gradient method gave values of $n = 1.5 \times 10^{-13}$ mol/mg of supernatant protein and $K_D = 6.0 \times 10^{-11}$ mol/l. Toft, Shyamala & Gorski (1967) quoted a value for $K_D = 70 \times 10^{-11}$ mol/l. From their data, their value for n is 1×10^{-13} mol/mg of supernatant protein. As they used immature rats, the agreement is satisfactory. The reason for the higher value of n obtained by the protamine method in our experiments is not certain. It may be due to the large errors in assessing the amount of bound oestradiol-17 β in the gradient experiments or to a real difference in the two methods. It cannot be due to the inclusion of the 4S region as bound steroid in the gradient method, as omission of it would further decrease n .

Assessment of the number of binding sites in uterine supernatant under different hormonal environments. The binding constants of uterine super-

Table 3. *Hormonal influence on the number of [6,7-³H]oestradiol-17 β -binding sites in uterine supernatant*

Uterine supernatant was labelled with different concentrations of [6,7-³H]oestradiol-17 β and the amount of bound [6,7-³H]oestradiol-17 β was determined by protamine precipitation. The binding constants were calculated by the method of Scatchard (1949). Results represent the mean $\pm$ S.D. of three to six measurements.

Hormone status	$10^{11} \times K_D$ (mol/l)	$10^{-13} \times$ Number of binding sites (mol)		
		Per uterus	Per mg of DNA	Per mg of protein
Mature rats				
Intact	15.2 $\pm$ 5.3	26.0 $\pm$ 7.1	60.2 $\pm$ 32.7	2.8 $\pm$ 1.4
Ovariectomized 1 week	8.9 $\pm$ 3.5	14.9 $\pm$ 1.9	32.2 $\pm$ 7.4	2.2 $\pm$ 1.6
Ovariectomized 3 weeks	7.0 $\pm$ 1.2	8.7 $\pm$ 2.1	25.9 $\pm$ 8.5	3.2 $\pm$ 1.8
Hypophysectomized 1 week	5.8 $\pm$ 3.2	9.0 $\pm$ 3.2	10.7 $\pm$ 6.8	2.0 $\pm$ 0.8
Adrenalectomized 1 week	11.9 $\pm$ 5.9	21.1 $\pm$ 5	56.0 $\pm$ 27.2	2.3 $\pm$ 0.4
Immature rats	8.2 $\pm$ 2.0	3.3 $\pm$ 1.5	21.6 $\pm$ 5.1	2.8 $\pm$ 0.5

natant for [6,7-³H]oestradiol-17 β (method 2) have been calculated by the protamine method after hormone manipulation of the host animal (Table 3). None of the treatments affected K_D . The number of binding sites per mg of supernatant protein was remarkably constant in the different groups whereas the number per uterus or per mg of DNA increased when the animal reached maturity and decreased after ovariectomy. Adrenalectomy had no effect on any of the parameters but hypophysectomy decreased the number of binding sites per uterus or per mg of DNA to a greater extent than did ovariectomy for the same period.

Comments on the [6,7-³H]oestradiol-17 β -binding components in uteri from mature rats. The protamine-precipitable material in the 4S region of uterine cytoplasm from mature rats was noteworthy, as it was not present in uteri from ovariectomized, hypophysectomized or immature animals (Fig. 1b). It is unlikely to be due to serum components, as these were not precipitated by protamine (Table 2) and it did not bind [7α -³H]testosterone or [1,2-³H]-cortisol (Fig. 1c). Some progesterone was bound in this region of the gradient but it is not known if this is attached to the same protein as the oestradiol-17 β .

Both the 8S and 4S receptors were precipitated by protamine and the Scatchard plot derived from mature uterine cytoplasm indicated the presence of only one tight-binding component (Fig. 2). This suggested that the two receptors may be related to each other. To study this further, the effect of a number of reagents on oestradiol-17 β binding was studied. *N*-Ethylmaleimide and *N*-bromosuccinimide treatment decreased the amount of tritium radioactivity precipitated by protamine by 54 and 61% respectively as well as decreasing the amount of ³H in both the 8S and 4S regions

of the sucrose gradients. Neither of these reagents affects the interaction of acidic proteins with polycations (R. J. B. King, J. Gordon & A. W. Steggles, unpublished work). Treatment of uteri with either 100nM-oestradiol-17 β or 10 μ M-U-11,100A (1-[2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]-ethyl]pyrrolidine hydrochloride, given by Upjohn Co., Kalamazoo, Mich., U.S.A.) by method 1 before labelling with 1nM-[6,7-³H]oestradiol-17 β decreased the amount of ³H radioactivity precipitated by protamine by 79 and 67% respectively and also decreased both the 8S and 4S components (Fig. 1d). When a [6,7-³H]-oestradiol-17 β -labelled 10⁵g uterine supernatant from ovariectomized rats was kept at 4°C for 7 days there was no change in the percentage precipitated by protamine but tritium was present in the 4S region of the gradient, which suggested that this material could be formed from the 8S material without loss of binding capacity.

The sedimentation profile of supernatant from mature uteri (labelled by method 1) was not altered by fourfold dilution with water, which suggested that the 4S component was not the same as that formed by salt dissociation of the 8S component.

To see if the 4S binding decreased when oestradiol entered the nucleus, uteri from mature rats were incubated with 1nM-[6,7-³H]oestradiol-17 β for 1h at either 0°C (group A) or 37°C (group B). Both groups were then kept at 4°C for 1h. A 10⁵g pellet and supernatant were prepared from each group. Sucrose-gradient analysis of the supernatant showed that both the 8S and 4S binding was lower in group B than in group A (Fig. 3a). The total ³H radioactivity in the uteri of groups A and B was similar (3.7×10^5 and 3.4×10^5 d.p.m. respectively) but in group A, 51% of the ³H radioactivity was in the 10⁵g supernatant as compared with 22% in

Fig. 3. Sedimentation profiles of uteri labelled with 1 nM-[6,7- 3 H]oestradiol-17 β by method 1. The gradients were centrifuged for 16 h at 5°C at 125000 g_{av} in the Spinco SW65 rotor. (a) Supernatant after labelling at 4°C (—) and 37°C (----). (b) Extract in 0.3M-KCl of nuclei after labelling at 4°C (—) and 37°C (----).

group B. The decrease in supernatant binding was accompanied by an increase in the nuclear binding (Fig. 3b).

DISCUSSION

Evidence from other laboratories has indicated that binding of oestradiol-17 β to proteins in the 4–6S region of sucrose gradients was non-specific (Toft *et al.* 1967). In our experiments, protamine precipitated bound oestradiol-17 β from this region of the gradients as well as the 8S material so it is necessary to consider the evidence that the material precipitated by protamine was related to specific, rather than non-specific, binding of the hormone. Our method of labelling the tissues removed a large part of the non-specific material, which explains the absence of non-specific binding of [6,7- 3 H]-oestradiol-17 β in immature uterus at high concentrations of [6,7- 3 H]oestradiol-17 β . [6,7- 3 H]Oestradiol-17 β equilibrated with serum was not precipitated by protamine so the 4S component is unlikely to be due to non-specific binding, although one cannot rule out the possibility of selective uptake of the serum oestradiol-17 β -binding protein by the mature uterus (Peterson & Spaziani, 1969). This is, however, unlikely as the evidence suggests that the oestradiol-17 β -binding protein in serum is similar to the testosterone-binding globulin (Rosner & Deakins, 1968) and our experiments indicated no binding of testosterone. No binding of cortisol was detected but a small amount of progesterone sedimented in the 4–6S region of the gradient. This corresponded to some precipitation with protamine but it is not known if this is due to the binding of progesterone and oestradiol-17 β to the same protein. It is possible that there is a different salt concentration in the mature and immature uteri and that the

4S material is due to salt dissociation of the 8S component (Erdos, 1968). This is unlikely, as dilution with water did not convert the 4S material into 8S material. This indicates that the 4S receptor described in this paper is different from the 4S material formed by salt treatment of the 8S receptor. The salt-dissociated 4S material is not precipitated by manganese chloride whereas the 4S material in mature cytoplasm is (R. J. B. King, J. Gordon & A. W. Steggles, unpublished work), which further suggests that the two 4S components are not the same.

As protamine, *N*-ethylmaleimide, *N*-bromosuccinimide, unlabelled oestradiol-17 β and U-11,100A all affected both the 8S and 4S components, they must be related. The 4S receptor may be a stabilized form of the salt-dissociated 4S receptor. Ca^{2+} can produce this effect (De Sombre, Puca & Jensen, 1969), but the presence of Ca^{2+} is unlikely to explain our results, as the medium contained EDTA.

The results can best be explained by the presence of a specific 4S oestradiol-17 β -binding protein in the cytoplasm of mature animals. The Scatchard binding plot derived from both the protamine and sucrose-gradient results indicated only one binding component under conditions where both 8S and 4S proteins were labelled, which suggests that they both have similar dissociation constants. The 4S component disappeared when pituitary or ovarian hormones were removed. The number of binding sites either per uterus or per mg of DNA was also decreased by these treatments but hypophysectomy had a greater effect than ovariectomy. Measurements on tissues from mature animals should be taken as minimal values, as they make no allowance for the endogenous oestrogens, which would depress

the binding of [6,7- 3 H]oestradiol. Hypothalamic regions may also be involved in uterine receptor formation (Vertes & King, 1969). None of the endocrine treatments affected the number of binding sites per mg of supernatant protein. These results could be explained if the synthesis of the 4S protein was under ovarian and pituitary control such that, as uterine protein synthesis decreased, so did 4S-protein synthesis. The greater effect of hypophysectomy as compared with ovariectomy might be due to either growth hormone (Korner, 1968) or insulin (Wool *et al.* 1968) effects on ribosomal protein synthesis. The large variation in the number of binding sites per uterus or per mg of DNA in mature and adrenalectomized animals might be due to changes in the number of receptor sites with the oestrous cycle.

As [6,7- 3 H]oestradiol bound to both the 8S and 4S receptors appears to be transferred to the nucleus, our experiments do not indicate which is the more important receptor *in vivo*, but it is noteworthy that oestradiol-4S receptor stimulates nuclear RNA synthesis *in vitro* whereas oestradiol-8S receptor does not (Beziat, Guilleux & Mousseron-Canet, 1970).

The evidence presented above suggests that protamine can be used to study the binding of [6,7- 3 H]oestradiol to specific receptors in uterine cytoplasm, and we have also used it to distinguish between specific and non-specific uptake of [6,7- 3 H]-oestradiol by different parts of rat brain (Vertes & King, 1969). The values of K_D and n obtained by the protamine method are in reasonable agreement with our values obtained by sucrose-gradient analysis and with those obtained by other authors.

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