Uptake of Oestradiol by the Rabbit Hypothalamus

SPECIFICITY OF BINDING BY NUCLEI *IN VITRO*

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The binding of [6,7-³H]oestradiol-17 β to subcellular fractions of the hypothalamus and the cerebellum of the rabbit was studied *in vitro*. Uptake of steroid was higher in hypothalamic nuclei than in cerebellar nuclei. Lower binding was observed in other fractions of both tissues. After dialysis of the fractions, hypothalamic nuclei retained a high percentage of oestradiol whereas cerebellar nuclei lost most of the bound steroid. Supernatant fractions of both tissues retained a significant proportion of label after dialysis and after gel filtration on Sephadex G-200. No specific binding was observed in these fractions when subjected to sucrose-density-gradient centrifugation. Purification of nuclei followed by incubation with labelled oestradiol in the absence of the supernatant fraction resulted in loss of binding of steroid by hypothalamic nuclei. Incubation of the purified hypothalamic nuclei with supernatant fraction maintained the binding specificity of hormone retention.

Evidence indicating the presence of specific receptors for oestradiol in the hypothalamus has been obtained in several laboratories after injection of the labelled steroid *in vivo* (Eisenfeld & Axelrod, 1965, 1966; Kato & Vilee, 1967*a,b*; Pfaff, 1968). The binding sites appear to be of limited capacity and high affinity as are those of the uterus (Jensen & Jacobson, 1962) where characteristically the nucleus takes up a large percentage of administered hormone (Jensen *et al.* 1968; Shyamala & Gorski, 1969). It was decided to determine whether the subcellular distribution of labelled steroid exhibits a pattern in the hypothalamus comparable to that observed in the uterus. Since retention of oestradiol is greater in target tissues, we have also studied the continued association of hormone with the tissue fractions by using a dialysis technique. Particular attention was paid to the role of the cytoplasm in mediating the uptake and retention of steroid in the nucleus.

MATERIALS AND METHODS

Tissue incubation and fractionation. Hypothalami and sections of cerebellum from immature female rabbits (Pel Freeze Biologicals Inc., Rogers, Ark., U.S.A.) were cut into small pieces (1-2 mm) and incubated for 1 h

at 37°C with 0.1 nM-[6,7-³H]oestradiol-17 β (chromatographically pure, 40 Ci/mmol; New England Nuclear Corp., Boston, Mass., U.S.A.) in 0.05 M-phosphate buffer, pH 7.4, containing 0.25 M-sucrose. In certain experiments as noted, 0.1 nM- or 10 nM-oestradiol, cortisol, progesterone, or testosterone (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was added. Tissues were then rinsed for 5 min in buffer; the latter was removed by decantation and filtration. Subcellular fractions were prepared by centrifugation by using the method of 'equilibrium fractionation' (De Venuto & Chader, 1966) in a buffer containing 0.32 M-sucrose, 1 mM-MgCl₂ and 1 mM-phosphate buffer, pH 6.5 (Løvtrup-Rein & McEwen, 1966).

Dialyses. After determination of protein by the method of Lowry, Rosebrough, Farr & Randall (1951) and radioactivity measurement in a Packard scintillation spectrometer (Kato & Vilee, 1967*b*), the fractions were adjusted to equal protein concentrations (15-20 mg/ml), placed in dialysis tubing (Armour Pharmaceuticals, Chicago, Ill., U.S.A.) dialysed individually against 50 vol. of buffer for 24 h at 4°C with gentle shaking and re-assayed for ³H content. Relatively high concentrations of protein were placed in the dialysis sacs to minimize the known binding of oestradiol to dialysis membranes (Talwar, Sopori, Biswas & Segal, 1968). Before use, dialysis tubing was boiled in 5% (w/v) Na₂CO₃, soaked in 0.05 M-EDTA and 0.01 M-ascorbate, and washed for 48 h in several changes of distilled water.

Nuclear studies. After tissue incubation with [6,7-³H]-oestradiol, nuclei prepared as above were further purified by density-gradient centrifugation through 2.39 M-sucrose for 1 h at 25000*g* in the Spinco model L2 ultracentrifuge (Løvtrup-Rein & McEwen, 1966). Samples of

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purified nuclei and of the 105000g supernatant fractions were dialysed for 24 h at 4°C by a multiple dialysis technique (De Venuto & Chader, 1966) with all dialysis bags in a single flask containing a total of 50 vol. of buffer. Slight gelation of nuclei was occasionally observed during the dialyses.

In some experiments, nuclei were first purified and then incubated with labelled oestradiol in buffer or in the supernatant fraction. Nuclei were washed and re-isolated by centrifugation at 700g for 10 min, dialysed individually against 50 vol. of buffer for 24 h at 4°C and resampled for radioactive content.

Oestradiol metabolism studies. Hypothalamic tissue was homogenized (271 mg/ml) in 0.05 M-sodium phosphate buffer, pH 7.4, containing 0.25 M-sucrose and incubated for 1 h at 37°C with 0.085 µg of [6,7-³H]-oestradiol (12.5 µCi). At the end of the incubation, 250 µg each of carrier oestradiol, oestrone and oestriol were added and the mixture was extracted four times with 10 ml of methylene chloride. The extract was evaporated to dryness and then dissolved and partitioned between hexane and 90% (v/v) methanol to remove phospholipids and sterols. The methanol fraction was subjected to t.l.c. in a system (chloroform-acetone, 9:1, v/v) that separates oestrone (R_F 0.471), oestradiol (R_F 0.265) and oestriol (R_F 0.00). The radioactivity measured in a strip scanner appeared in a single peak coincident with the carrier oestradiol. The areas corresponding to the added oestrone and oestriol were scraped from the plates, eluted and counted for radioactivity; no radioactivity was detected. The oestradiol area was eluted, authentic oestradiol was added and the mixture was recrystallized to constant specific radioactivity.

Gel filtrations. After tissue incubation with [6,7-³H]-oestradiol, the supernatant fraction was prepared by homogenizing the tissue and centrifuging the homogenate at 105000g for 1 h. Portions (0.3 ml) of the fractions were subjected to gel filtration at 4°C on a column (0.9 cm × 17 cm) of Sephadex G-200. Elution was with 0.05 M-phosphate buffer, pH 7.4. The protein content of the eluate fractions was estimated at 280 nm with a Zeiss PMQ II Spectrophotometer.

Sucrose-density-gradient centrifugation studies. Samples of hypothalamus and cerebellum were homogenized in tris-EDTA buffer (0.01 M-tris, 1.5 mM-EDTA, pH 7.4) at a concentration of approx. 200 mg of protein/ml. The

supernatant fractions were isolated by ultracentrifugation at 105000g for 1 h and incubated with 2 ng of [6,7-³H]-oestradiol each at 4°C for 5 min. Samples (0.2 ml) were layered on to 4.6 ml sucrose gradients at 4°C (5–20%) and centrifuged for 16 h at 36000 rev./min in a Spinco model LB-65 ultracentrifuge with a SW-50.1 rotor. Samples of uterine cytoplasm containing the specific 8S oestradiol-protein complex and of bovine serum albumin (4.7S) were used as markers in the ultracentrifugation system. Centrifuge tubes were finally pierced and 0.13 ml samples were collected and assayed for radioactivity.

RESULTS

Subcellular binding of oestradiol. The distributions of radioactivity in the subcellular fractions of hypothalamus and cerebellum before and after dialysis are summarized in Table 1. The specific radioactivities of the nuclear fractions were high in both tissues with highest binding consistently being seen in the hypothalamic nuclear fraction. The nuclear fraction of the hypothalamus retained a large percentage of its radioactivity after dialysis whereas the cerebellar fraction lost most of its bound radioactivity within 24 h. The binding of steroid to the mitochondrial and microsomal fractions was lower than the binding to nuclei in both tissues and was virtually eliminated after dialysis. Binding of oestradiol in the supernatant fractions was also lower than in the nuclear fractions but dialysis eliminated only about one-third of the bound radioactivity.

In other experiments, tissues were incubated with [6,7-³H]oestradiol and nuclei were purified by sucrose-density-gradient centrifugation to reduce the possibility of contamination by cytoplasmic components. As seen in Table 2 the same pattern emerged before dialysis as that observed previously, i.e. hypothalamic nuclei bound more oestradiol than cerebellar nuclei. After the nuclear and supernatant fractions had been subjected to multiple dialysis, the highest radioactivity was found in the

Table 1. *Distribution of radioactivity in subcellular fractions of hypothalamus and cerebellum after incubation of tissue with [6,7-³H]oestradiol*

The results are expressed as c.p.m./mg of protein and are mean values ± s.e. from duplicate samples in three experiments. Dialysis was for 24 h; each sample was dialysed individually against 50 vol. of buffer.

Fraction	Radioactivity (c.p.m./mg of protein)			
	Hypothalamus		Cerebellum	
	original	dialysed	original	dialysed
Nuclear	572 ± 34	437 ± 29	421 ± 38	156 ± 11
Mitochondrial	207 ± 14	9 ± 2	188 ± 5	15 ± 8
Microsomal	247 ± 22	43 ± 17	220 ± 27	27 ± 6
Supernatant	389 ± 16	205 ± 26	293 ± 20	192 ± 46

Table 2. *Radioactivity in purified nuclear and supernatant fractions of hypothalamus and cerebellum after incubation of tissue with [6,7-³H]oestradiol*

The results are expressed as c.p.m./mg of protein and are mean values $\pm$ s.e. from duplicate samples in three experiments. Dialysis was for 24 h; all samples were dialysed in the same flask against a total of 50 vol. of buffer.

Fraction	Radioactivity (c.p.m./mg of protein)			
	Hypothalamus		Cerebellum	
	original	dialysed	original	dialysed
Nuclear	745 $\pm$ 21	592 $\pm$ 33	507 $\pm$ 43	132 $\pm$ 26
Supernatant	373 $\pm$ 47	284 $\pm$ 19	302 $\pm$ 29	195 $\pm$ 31

Table 3. *Effect of the supernatant fraction on the uptake and retention of [6,7-³H]oestradiol by purified nuclei of hypothalamus and cerebellum*

The results are expressed as c.p.m./mg of protein and are mean values $\pm$ s.e. from triplicate samples in two experiments. Nuclei were incubated with [6,7-³H]oestradiol in buffer or with supernatant fraction and then re-isolated by centrifugation. Dialysis was for 24 h; each sample was dialysed individually against 50 vol. of buffer.

Fraction	Radioactivity (c.p.m./mg of protein)			
	Hypothalamus		Cerebellum	
	original	dialysed	original	dialysed
Nuclei incubated in buffer	897 $\pm$ 41	148 $\pm$ 13	912 $\pm$ 56	103 $\pm$ 15
Nuclei incubated in supernatant	644 $\pm$ 29	568 $\pm$ 26	561 $\pm$ 34	189 $\pm$ 19

hypothalamic nuclei with supernatant fractions losing proportionately less radioactivity than the cerebellar nuclei. In these studies all of the fractions were dialysed against each other to allow for competition between the fractions for labelled oestradiol and thus increase specificity of binding.

Oestradiol binding to purified nuclei. In marked contrast with these results, when purified nuclei from the hypothalamus and cerebellum were incubated in buffer containing [6,7-³H]oestradiol, there was no difference between binding by the two nuclear fractions before or after dialysis (Table 3). Hypothalamic nuclei incubated in the supernatant fraction, however, showed the usual pattern of prolonged retention of radioactivity throughout dialysis indicating the importance of soluble cytoplasmic components in mediating nuclear binding of hormone in the hypothalamus.

Oestradiol metabolism studies. No detectable metabolism of [6,7-³H]oestradiol occurred when incubated with rabbit hypothalamic tissue at 37°C for 1 h. In contrast, Kato & Vilee (1967a) found that after injection of [6,7-³H]oestradiol in the rat *in vivo*, approximately 80% of the radioactivity extracted from the hypothalamic area was oestradiol.

Specificity of oestradiol binding. When hypothalamic tissue was incubated with 0.1 nM-[6,7-³H]-oestradiol in the presence of 10 nM unlabelled oestradiol, radioactivity in the isolated nuclear fraction was decreased by 56%. The total radioactivity found in the nuclear fraction of brain tissue was decreased by only 2% when incubated in a similar manner. No significant decrease in total radioactivity was observed in other isolated subcellular fractions. No decrease in binding to the hypothalamic fractions was observed when 10 nM-cortisol, -testosterone or -progesterone were added to the initial incubations, indicating that these steroids do not compete with oestradiol for binding sites within the fractions. This does not eliminate the possibility that metabolites such as 20 α -hydroxypregn-4-en-3-one or 17 β -hydroxy-5 α -androst-3-one may compete with oestradiol *in vivo* for these binding sites.

Table 4 shows the percentage of radioactivity remaining after dialysis of subcellular fractions isolated from tissue incubated with [6,7-³H]-oestradiol in the presence of unlabelled oestradiol as compared with control fractions of tissues incubated with labelled oestradiol alone. Retention of [6,7-³H]oestradiol in hypothalamic nuclei was decreased by 34 and 72% by incubation in

Table 4. *Distribution of radioactivity after dialysis of subcellular fractions of hypothalamus and cerebellar tissue incubated with 0.1nM-[6,7-³H]oestradiol and either 0.1nM or 10nM unlabelled oestradiol*

The results are expressed as average percentages of radioactivity remaining in the fraction as compared with that in the control fraction where no unlabelled oestradiol was present during the original incubation. Dialysis was for 24 h; each sample was dialysed individually against 50 vol. of buffer.

Fraction	Radioactivity remaining (% of control)			
	Hypothalamus		Cerebellum	
	+0.1nM-oestradiol	+10nM-oestradiol	+0.1nM-oestradiol	+10nM-oestradiol
Nuclear	66 ± 3	28 ± 6	104 ± 18	105 ± 5
Mitochondrial and microsomal	94 ± 10	90 ± 28	72 ± 17	74 ± 12
Supernatant	61 ± 10	61 ± 21	110 ± 65	71 ± 22

Fig. 1. Gel filtration of the supernatant fraction of hypothalamus and cerebellum after incubation of the tissue with [6,7-³H]oestradiol. Samples (0.3ml) of the 105000g supernatant of (a) hypothalamus and (b) cerebellum were applied to columns (0.9 cm × 17 cm) of Sephadex G-200. Elution was with 0.05M-phosphate buffer, pH 7.4, at 4°C. ●, $E_{280}^{0.5\text{cm}}$; ○, radioactivity (c.p.m.).

0.1nM- and 10nM- unlabelled oestradiol respectively whereas retention of radioactivity in cerebellar nuclei did not show this displacement pattern. Radioactivity in the hypothalamic supernatant fraction was decreased somewhat by the presence of unlabelled oestradiol but not in the same dose-related fashion observed in the nuclear fraction. Other fractions of hypothalamus and brain appeared to be relatively unaffected by the presence of unlabelled steroid in the incubation.

Studies on the supernatant fractions. To characterize further the substances responsible for cytoplasmic binding, the supernatant fractions were subjected to gel filtration on Sephadex G-200 (Fig. 1). The total bound radioactivity was somewhat higher in the hypothalamic supernatant fraction (84%) than in the cerebellar supernatant (67%).

The hypothalamic fraction (Fig. 1a) bound a proportionately greater percentage of radioactivity (61%) in the first absorption peak eluted whereas the supernatant fraction of the cerebellum bound a smaller fraction of radioactivity (41%) in this peak (Fig. 1b). This binding is not the same, however, as the specific oestrogen binding in uterine supernatant first described by Toft & Gorski (1966). When portions of the supernatant fractions of hypothalamus and cerebellum were incubated with [6,7-³H]oestradiol and subjected to sucrose-density-gradient centrifugation, the radioactive patterns obtained were identical, with virtually all of the radioactivity remaining at the top of the gradient in each case. No specific binding was observed in the 8S region or other regions of the gradient. Specific 8S receptor for oestradiol has been reported

in the bovine hypothalamus (Kahwanago, Heinrichs & Herrmann, 1969).

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DISCUSSION

The uptake of hormone in nuclei of specific target tissues has generally been considered to be a prerequisite step in the mechanism of action of steroid hormones, e.g. the action of oestradiol in the rat uterus (Jensen *et al.* 1968). Rapid effects of oestradiol on nuclear metabolic activities are well established (Means & Hamilton, 1966). The present study demonstrates that nuclei of the rabbit hypothalamus concentrate oestradiol and retain it over an extended period of dialysis. The presence of an oestradiol-binding species in the supernatant fraction of the hypothalamus (and possibly also in the cerebellum) is indicated, but its function is unknown at present. These data, together with previous studies (Eisenfeld & Axelrod, 1966; Kato & Vilee, 1967*a,b*; McGuire & Lisk, 1968; Kato, Inaba, Kobayashi, & Kobayashi, 1969) substantiate the role of the hypothalamus as a target tissue for oestrogen and also demonstrate the nucleus as a primary site for intracellular binding. The specificity of uptake and retention of oestradiol into the nucleus may, however, reside with cytoplasmic factors rather than with any inherent nuclear mechanism for hormone concentration. If specific oestradiol 'receptors' do exist in the cytoplasmic soluble fraction of the rabbit hypothalamus, they appear to be different from those observed in the uterus.

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REFERENCES

- De Venuto, F. & Chader, G. J. (1966). *Biochim. biophys. Acta*, **121**, 151.
- Eisenfeld, A. J. & Axelrod, J. (1965). *J. Pharmac. exp. Ther.* **150**, 469.
- Eisenfeld, A. J. & Axelrod, J. (1966). *Endocrinology*, **79**, 38.
- Jensen, E. V. & Jacobson, H. I. (1962). *Recent Prog. Horm. Res.* **18**, 387.
- Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W. & DeSombre, E. R. (1968). *Proc. natn. Acad. Sci. U.S.A.* **59**, 632.
- Kahwanago, I., Heinrichs, W. & Herrmann, W. (1969). *Nature, Lond.*, **223**, 313.
- Kato, J., Inaba, M., Kobayashi, T. & Kobayashi, T. (1969). *Acta endocr., Copnh.*, **138** (Suppl.), 193.
- Kato, J. & Vilee, C. A. (1967*a*). *Endocrinology*, **80**, 567.
- Kato, J. & Vilee, C. A. (1967*b*). *Endocrinology*, **80**, 1133.
- Løvtrup-Rein, H. & McEwen, B. S. (1966). *J. Cell Biol.* **30**, 405.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- McGuire, J. L. & Lisk, R. D. (1968). *Proc. natn. Acad. Sci. U.S.A.* **61**, 497.
- Means, A. R. & Hamilton, T. H. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56**, 1594.
- Pfaff, D. W. (1968). *Endocrinology*, **82**, 1149.
- Shyamala, G. & Gorski, J. (1969). *J. biol. Chem.* **244**, 1097.
- Talwar, G. P., Sopori, M. L., Biswas, D. K. & Segal, S. J. (1968). *Biochem. J.* **107**, 765.
- Toft, D. & Gorski, J. (1966). *Proc. natn. Acad. Sci. U.S.A.* **55**, 1574.