The Binding of ^3H -Labelled Oestradiol- and Progesterone-Receptor Complexes to Hypothalamic Chromatin of Male and Female Sheep

By BARRY N. PERRY and ANTONIO LOPEZ*
A.R.C. Meat Research Institute, Langford, Bristol BS18 7DY, U.K.

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Chromatin isolated from hypothalamic nuclei of sexually mature entire male and female sheep was linked to cellulose in u.v. light. The saturation binding of ^3H -labelled oestrogen- and progesterone-receptor complexes, prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation from the 105 000g supernatant of hypothalamic cytosol, was then measured *in vitro* in 0.15M-KCl. Saturation binding was also measured after extraction of histones and masking acidic proteins. Salt+urea was observed to be more effective than guanidine hydrochloride in unmasking receptor acceptor sites, and the binding of labelled receptor complexes to de-histonezed unmasked chromatin was shown to be largely resistant to 0.4M-KCl extraction. Whereas extents of receptor-complex binding were similar to published values for comparable preparations of hen oviduct chromatin, no sex-related difference was observed. However, binding of progesterone-receptor to chromatin was greater than that of oestradiol-receptor. Binding also increased more after removal of histones and masking acidic proteins, suggesting the presence of a greater number of progesterone-receptor acceptor sites in hypothalamic chromatin than of oestradiol-receptor acceptor sites. The failure to demonstrate a sex-related difference in oestradiol-receptor binding to hypothalamic chromatin *in vitro* is discussed.

Developmental biologists and endocrinologists have recognized that steroid hormones secreted during critical stages of foetal or perinatal development influence subsequent patterns of sexual behaviour and growth. Thus testosterone, secreted by the immature testis, brings about neural masculinization of the male, but, paradoxically, the route is thought to involve aromatization of androgen to oestrogen (for reviews see Forest & Bertrand, 1974; McEwen, 1976). Adrenal progesterone may also influence the process (Shapiro *et al.*, 1976).

One consequence of normal male sexual differentiation and of artificial masculinization of females, by using testosterone or oestrogen, is the subsequent inability of target-cell nuclei to bind oestrogen (McGuire & Lisk, 1969; Vertes & King, 1971; Shyamala *et al.*, 1974; Lobl, 1975). Thus, in a sense, target tissue takes on the steroid insensitivity of non-target tissue.

It has been demonstrated that target and non-target tissue differ in progesterone sensitivity because non-target-tissue nuclear chromatin possesses acidic proteins that mask the chromosomal-protein progesterone-receptor acceptor sites (Spelsberg *et al.*, 1976). It has also been suggested by Webster *et al.* (1976) that the number of available acceptor sites in target tissue changes during development or in different physiological states because of such mask-

ing, the consequence of which is to alter gene expression.

It occurred to us that the observed sex-related differences in nuclear binding of oestrogen could be explained by this phenomenon, and that the proportion of steroid-labelled receptor binding to chromatin that is resistant to 0.4M-KCl extraction, which Clark *et al.* (1976) believe is the biologically most important form of oestrogen-receptor association with nuclei, could be assessed.

Our measurements of oestrogen- and progesterone-receptor-complex binding to hypothalamic chromatin from males and females of one domestic mammal, at a physiologically meaningful KCl concentration, which assess 0.4M-KCl-resistant binding, are presented below.

Materials and Methods

Tissue sources and subcellular fractions

Hypothalami used for the preparation of chromatin were obtained from male and female sheep reared at the Institute. Hampshire, South Down, Scottish Blackface and North Country Cheviot sheep were slaughtered at 224 ± 7 days of age, in groups weekly from 5 October to 9 November, i.e. within the normal breeding seasons for the breeds that are seasonally polyoestrous (Dziuk, 1973). Females were examined for sexual maturity by inspection of ovaries

* Present address: Catedra de Bioquímica y Tecnología de los Alimentos, Facultad de Veterinaria, Leon, Spain.

for corpora lutea, and males by microscopic examination of testes to assess spermatogenic competence. Nine groups that comprised six animals each were slaughtered, and breeds were comparably distributed within those male and female groups killed on the same day. Whole brain chromatin was isolated and limited quantities of [^3H]steroid-labelled cytosol receptor were prepared from random animals from the same source. For between-sex comparisons of [^3H]oestradiol- and [^3H]progesterone-labelled receptor binding, [^3H]steroid-labelled receptors were isolated from a single preparation of hypothalamic cytosol (105000g supernatant) obtained from 62 Welsh Mountain castrated male and female lambs slaughtered at a local commercial abattoir.

Brains were removed as soon as possible after electrical stunning and exsanguination. The hypothalamus was defined as that tissue lying ventral to a horizontal slice of 5–6mm depth taken anteriorly from a vertical plane 1mm posterior to the mammillary bodies to a vertical plane 2mm anterior to the optic chiasma and laterally by vertical planes of incision parallel to the mid-line along the fissures delineating lateral hypothalamus from temporal lobe (Barracrough, 1973). All subsequent operations unless otherwise stated were carried out at 0–4°C.

Preparation of chromatin

Hypothalami were finely chopped with scissors in buffer A (0.32M-sucrose, 5mM-MgCl₂, 25mM-KCl, 50mM-Tris/HCl, pH 7.5, 20mM-2-mercaptoethanol). After homogenization by hand with a Teflon/glass homogenizer (Jencons Scientific, Hemel Hempstead, Herts., U.K.) the material was filtered through cheesecloth (two layers) and centrifuged at 2000g for 10min. The pellets were resuspended with buffer A, and an equal volume of buffer B (2.4M-sucrose, 1.5mM-MgCl₂ and 10mM-Tris/HCl, pH 7.5) was added. The material was re-homogenized and centrifuged at 20000g for 10min, after which the floating debris was removed, re-homogenized in the supernatant and re-centrifuged. The combined nuclear pellets were then re-homogenized in 2vol. of buffer B+1 vol. of buffer A and centrifugation was repeated.

After resuspension of the pellets by gentle homogenization into the same buffer Triton X-100 was added (200 μl /100ml). Gentle homogenization was repeated to ensure complete mixing, and before centrifugation the solution was underlaid with Triton-free sucrose buffer of marginally greater density.

The resulting pale translucent nuclear pellet, obtained in apparently good yield and free of erythrocyte (haemoglobin) contamination, was, in early preparations, stored overnight in glycerol buffer (Spelsberg *et al.*, 1974). In later preparations chromatin was prepared immediately by gently resuspending

and extracting the nuclear pellets, twice with 80mM-NaCl/20mM-EDTA/10mM-sodium phosphate buffer (pH 6.3), twice with 0.35M-NaCl and twice with 2mM-Tris/HCl (pH 7.5)/0.1mM-EDTA, with centrifuging between each extraction at 6000g for 5min. At this stage chromatin was stored at –20°C in 2mM-Tris/HCl (pH 7.5)/0.1mM-EDTA.

Linking of chromatin to cellulose (after Litman, 1968)

Preparation of chromatin–cellulose differed slightly between groups. For the first two groups of male and female (slaughtered 5 October and 12 October) hypothalami and whole brain, chromatin suspended in 2mM-Tris/0.1mM-EDTA, pH 7.5, was homogenized gently with 2g of acid-washed cellulose (Whatman CC-41; Whatman, Maidstone, Kent, U.K.). After this the material was stirred magnetically for 10–15 min and then dialysed against 3 $\times$ 2 litres of deionized water over 2h. The dialysed solution was then frozen over liquid N₂ and freeze-dried. Freeze-dried material was then suspended in 30–50ml of ethanol and stirred for 30min, after which it was exposed with gentle stirring to u.v. irradiation by using a Minera-light (Shandon SAA R 0051) lamp, emitting 155 $\mu\text{W}/\text{cm}^2$ at 46cm. Irradiation was continued for 10min at 10cm distance. Ethanol was then removed by suction and the linked chromatin was thoroughly washed with 2mM-Tris/0.1mM-EDTA buffer, pH 7.5. The freeze-dried material was stored dry at room temperature (15–18°C).

For subsequent male and female groups 1mM-NaHSO₃ was added to the dilute 2mM-Tris/0.1mM-EDTA (pH 8.3) storage buffer and, before addition of cellulose, chromatin was suspended in 6mM-Tris/HCl/0.1mM-EDTA/1mM-NaHSO₃/20mM-2-mercaptoethanol buffer, pH 7.5. The mixtures were centrifuged at 6000g for 5min rather than dialysed to remove excess buffer before freezing and freeze-drying, to speed preparation. The final 2mM-Tris/0.1mM-EDTA wash buffer, pH 7.5, also contained 1mM-NaHSO₃ and 20mM-2-mercaptoethanol in an attempt, with more rapid preparation, to improve protein stability.

Preparation of steroid-labelled high-affinity cytosol receptors

Hypothalami were homogenized in 0.32M-sucrose/10mM-Tris/HCl (pH 7.4)/100mM-2-mercaptoethanol, centrifuged at 38000g for 10min and the supernatant was centrifuged at 105000g for 1h. ^3H -labelled steroid (1–10nM final concentration) was added by evaporating a solution under N₂ gas, in ice, and incubating steroid+cytosol for 90min with stirring. In some early studies [^3H]diethylstilboestrol (code TRK 472) was used instead of [^3H]oestradiol (code TRK 322). These compounds and [^3H]progesterone

(code TRK 413) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Oestradiol and progesterone were used without further purification within 7 days of receipt, after storage at +2°C.

Oestrogen-labelled receptor was precipitated by adding cold saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 7.2) to 25% saturation (for progesterone, 35% saturation) over a 15min period. Mixtures were stirred for 30min longer and then centrifuged in appropriate portions at 10000g for 15–20min before storage of pellets at –30°C.

Before use pellets were dissolved in 0.15M-KCl/0.5mM-EDTA/50mM-2-mercaptoethanol/5mM-Tris/HCl (pH 7.5) and dialysed against three changes of 300ml of the same buffer over 2.5–3.0h. The dialysed solutions were then centrifuged briefly (10000g for 5min) to remove any sediment and small samples were removed for assay of specific radioactivity (c.p.m./mg of protein).

Extraction of chromatin–cellulose, steroid–receptor–complex binding and radioactivity assay

A variety of solvents were used to extract chromatin–cellulose preparations within the following general scheme. Triplicate samples of dry chromatin–cellulose were weighed into glass tubes and hydrated with 2mM-Tris/HCl/0.1mM-EDTA/1mM- NaHSO_3 /20mM-2-mercaptoethanol, pH 7.5. Samples were then extracted twice with over 20vol. of the solvent, washed three times with 2mM-Tris/HCl (pH 7.5)/0.1mM-EDTA (for guanidine hydrochloride extraction) or 10mM-Tris/HCl (pH 7.5) (for NaCl and NaCl+urea extraction) and then with 5mM-Tris/HCl (pH 7.5)/0.5mM-EDTA/20mM-2-mercaptoethanol/0.15M-KCl. Labelled receptor was then added and samples were incubated for 90min. Unbound radioactivity was removed by washing three times with dilute 2mM-Tris/0.1mM-EDTA (pH 7.5). At this stage appropriate samples were extracted twice with 0.4M-KCl in KH_2PO_4 buffer (pH 7.2, 20mM) containing 1mM- NaHSO_3 and 5mM-2-mercaptoethanol, and washed with dilute 2mM-Tris/0.1mM-EDTA (pH 7.5) to remove excess KCl. All samples, including cellulose blanks taken through the extraction and binding procedures, were then transferred to polythene counting vials as follows.

To the pelleted chromatin–cellulose 1ml of NCS solubilizer (Hopkin and Williams, Romford, Essex, U.K.) was added. Samples were then incubated, with intermittent mixing, at 37°C for 60min, after which 110 μ l of 6M-HCl was added to each. This mixture was then transferred quantitatively by pipette to scintillation vials with 3 $\times$ 4ml washes of scintillator (Insta-Gel; Packard Instrument Co., Slough, U.K.). Samples were counted for radioactivity for 10min after allowing temperature equilibration. There was no evidence of chemiluminescence.

Chemical analyses of chromatin–cellulose

Samples of chromatin–cellulose, as prepared or subjected to various extraction procedures, were analysed for DNA and for total, acidic and histone protein. Protein was measured by the method of Lowry *et al.* (1951) by using bovine serum albumin (Sigma Chemical Co., Poole, Dorset, U.K.) as standard for total and acidic protein, and calf thymus histone (Sigma, type 11-s) for histone. Total protein was extracted with 0.5M-NaOH at 37°C for 60min. Histone was extracted with either 0.4M- H_2SO_4 (Spelsberg *et al.*, 1976) or 0.2M-HCl (Marushige & Bonner, 1966) at 2°C for 30min, and acidic protein was subsequently extracted with 0.1M-NaOH (Spelsberg *et al.*, 1976) at 37°C for 60min. DNA was assayed by using the Giles & Myers (1965) modification of the diphenylamine reaction (Burton, 1956) by using highly polymerized calf thymus DNA (BDH Chemicals, Poole, Dorset, U.K.) as standard. Hydrolysis of DNA was carried out at 80°C for 15min in 0.6M- HClO_4 .

Results

Identification of appropriate assay conditions

(a) *Effect of guanidine hydrochloride extraction of chromatin on [^3H]diethylstilboestrol–receptor binding.* We were unable to reproduce the protocol of Spelsberg *et al.* (1976) that, by sequential removal of histone and acidic proteins with increasing concentrations (0–8M) of guanidine hydrochloride, allowed, in principle, estimation of both the number of acceptor sites in chromatin and the degree of masking from measurement of peak binding. [^3H]Diethylstilboestrol-labelled receptor and chromatin prepared from either the whole brain of a female sheep or from hypothalami of male and female sheep were used. No increased binding of receptor was observed when chromatin was extracted with 4–7M-guanidine hydrochloride (Fig. 1a), but the extent of initial binding was close enough to that reported for progesterone–receptor binding by Spelsberg *et al.* (1976) to be accounted for by differences in receptor specific radioactivity and by too low a ratio of receptor to DNA for binding at saturation (Fig. 1b). The retention of chromatin (DNA) to cellulose during guanidine hydrochloride extraction was satisfactory (Table 1). The failure to unmask oestrogen acceptor sites in chromatin with guanidine hydrochloride was attributed to the considerably higher concentration of protein in our chromatin (Table 2) than that in hen oviduct chromatin used by Spelsberg *et al.* (1976), although our values were comparable with those of chromatin prepared from rat ventral prostate by Davies & Griffiths (1974).

(b) *Effect of salt+urea extraction of chromatin on oestrogen–receptor binding.* The problems encountered

Fig. 1. Binding of $[^3H]$ diethylstilboestrol-receptor complex to guanidine hydrochloride-extracted chromatin-cellulose. In (a) the binding of $[^3H]$ diethylstilboestrol-receptor complex to female whole-brain chromatin (Δ) is compared with that from hypothalami of male ($\bullet$, 5 October; $\circ$, 12 October) and female ($\blacksquare$, 5 October; $\square$, 12 October) sheep. Assay precision is represented by bars showing the range in triplicate observations for whole-brain chromatin. Although similar quantities of chromatin (approx. $120 \mu\text{g}$ of DNA/sample) were assayed the specific radioactivity of the receptor solution added to whole-brain chromatin ($1498 \text{ c.p.m.}/\mu\text{g}$ of protein; $94 \mu\text{g}$ in $300 \mu\text{l}$ /sample) was higher than that added to hypothalamic chromatin ($295 \text{ c.p.m.}/\mu\text{g}$ of protein; $800 \mu\text{g}$ in $300 \mu\text{l}$ /sample). In (b) $15\text{--}16 \text{ mg}$ samples of 4 M -guanidine hydrochloride-extracted whole-brain chromatin-cellulose were assayed in $700 \mu\text{l}$ final volume (as in a) with samples of the receptor solution prepared for use with hypothalamic chromatin. Interposed data for whole-brain chromatin (Δ) obtained in (a) with 150 and $300 \mu\text{l}$ of receptor solution show that in these assays binding was not measured at saturation. In (a) and (b) guanidine hydrochloride was dissolved in 10 mM -Tris/HCl ($\text{pH } 8.5$)/ 100 mM -2-mercaptoethanol/ 5 mM - NaHSO_3 buffer.

Table 1. The effect of guanidine hydrochloride extraction on the DNA content of whole-brain chromatin-cellulose. Three replicate samples (approx. 15 mg each) were hydrated in 2 mM -Tris/ 0.1 mM -EDTA ($\text{pH } 7.5$), extracted twice with guanidine hydrochloride in 10 mM -Tris/HCl ($\text{pH } 8.5$)/ 100 mM -2-mercaptoethanol/ 5 mM -sodium bisulphite (or buffer only) and washed three times with 2 mM -Tris/ 0.1 mM -EDTA ($\text{pH } 7.5$) before analysis as described in the Materials and Methods section.

Concentration of guanidine hydrochloride in extractant (M)	Mean DNA content and range for triplicate samples ($\mu\text{g}/\text{mg}$ of chromatin-cellulose)
0	9.17 (8.4–10.2)
3	9.08 (8.2–9.9)
4	9.08 (8.8–9.4)
5	8.40 (8.2–8.6)
6	9.60 (8.9–10.3)
7	8.60 (8.0–9.2)

with guanidine hydrochloride as an extractant led us to investigate receptor binding by whole-brain chromatin when extracted by the salt+urea procedure of Webster *et al.* (1976). The same preparation of $[^3H]$ diethylstilboestrol-receptor was used although it was now 10 days old, but measurements of the binding capacity of rat hypothalamic-cytosol oestrogen-receptor after different periods of storage, by using the Sephadex LH-20 assay of Barley *et al.* (1974), indicated this would be satisfactory. This was confirmed by a marked increase in binding that followed extraction of histone and masking acidic protein fractions $\text{AP}_1 + \text{AP}_2$ (Table 3). Nevertheless, both the extent and proportionate increase in binding was still low, suggesting that unmasking of chromatin was still incomplete. Therefore we proceeded to identify a buffer that maximized receptor binding by using hypothalamic chromatin and samples of one oestradiol-labelled receptor preparation.

Varying the ionic strength and pH of buffers used,

Table 2. *Chemical composition of chromatin-cellulose preparations studied*

Data are mean values for three replicates. Samples weighed 15–30mg each and were hydrated in 2mM-Tris/0.1mM-EDTA (pH7.5) before analysis by using procedures described in the Materials and Methods section.

Date of preparation	Sample origin	Sex	Content ($\mu\text{g}/\text{mg}$ of chromatin-cellulose)				Total protein/ DNA	Histone protein/ DNA	Acidic protein/ DNA
			Total protein	Histone protein	Acidic protein	DNA			
21 September	Brain	F	25.40	7.48	9.48	9.17	2.77	0.82	1.03
5 October	Hypothalamus	F	19.52	3.41	9.54	5.34	3.65	0.64	1.79
5 October	Hypothalamus	M	24.30	3.61	10.81	6.22	3.91	0.58	1.74
12 October	Hypothalamus	F	15.86	2.52	7.80	4.87	3.26	0.52	1.60
12 October	Hypothalamus	M	16.59	2.31	8.22	3.94	4.21	0.55	2.09
19 October	Hypothalamus	F	13.50	2.86	6.19	2.27	5.95	1.25	2.73
26 October	Hypothalamus	F	14.80	2.86	6.03	2.17	6.82	1.32	2.78
26 October	Hypothalamus	M	15.90	2.17	6.05	1.78	8.93	1.22	3.40
2 November	Hypothalamus	F	12.90	3.27	6.05	2.54	5.08	1.28	2.38
9 November	Hypothalamus	M	12.50	1.93	4.76	2.76	4.53	0.70	1.72

Table 3. *Binding of [^3H]diethylstilboestrol-labelled receptor to whole-brain chromatin-cellulose extracted by the scheme of Webster et al. (1976)*

Receptor solution (300 μl) was incubated in a total volume of 700 μl .

	Range in triplicate sample weight (mg)	Range in sample radioactivity (counts/20min)	Mean radioactivity (counts/20min)	$10^{-5} \times$ Mean sample binding of [^3H]diethylstilboestrol-receptor complex (c.p.m./mg of DNA)
Cellulose	36.2–45.8	206 194–236 406	221 300	—
Chromatin-cellulose	10.2–10.9*	329 204–367 348	348 276	0.645
De-histionized chromatin-cellulose	10.6–10.9*	397 293–390 229	388 761	0.865
De-histionized unmasked chromatin-cellulose	10.1–11.1*	434 842–464 133	449 337	1.179

* With 25–30mg of carrier cellulose per sample added.

within the range pH6.0–8.3, showed that guanidine hydrochloride was always less efficient in unmasking chromatin than NaCl+urea (Fig. 2), as we found with whole-brain chromatin. However, 3M-NaCl+5M-urea was as effective and more rapid than extraction with 2M-NaCl followed by extraction with 2M-NaCl+5M-urea, and maximal unmasking of oestrogen acceptor sites was achieved at pH7.2 (Fig. 3). We attribute the lower oestradiol-receptor binding after extraction at pH6.0 to dissociation of the acidic acceptor-protein fraction from chromatin in agreement with the decline in progesterone-receptor binding of chromatin extracted with increasing guanidine hydrochloride concentration above 4–5M (Spelsberg *et al.*, 1976).

The protein concentration of oestradiol-receptor necessary to attain saturation binding is established in Fig. 4 for the previous sample of chromatin investigated and for a further two samples assayed by using a lower volume of receptor (550 μl rather

than 1100 μl), but of higher protein concentration (3.6 rather than 2.6mg/ml). In subsequent assays receptor was used at protein concentrations above 3.6mg/ml (Table 4).

Chemical composition of salt+urea extracted chromatin

Neither 2M-NaCl nor 3M-NaCl+5M-urea extracted DNA from chromatin-cellulose (Table 5); therefore no systematic error in receptor-binding (c.p.m./mg of DNA) estimation would arise from the fact that we did not measure DNA directly in the samples to which labelled receptor was bound. However, variability in protein and DNA values was often reflected in results for receptor binding, probably owing to heterogeneous attachment of chromatin to cellulose. Latterly we used small (approx. 10mg) samples of chromatin-cellulose, which would increase sampling errors. Also chroma-

Fig. 2. Binding of $[^3\text{H}]$ oestradiol-receptor complex to female hypothalamic chromatin

Two assays are shown. In the first chromatin (female, 26 October) was extracted with either 2M-NaCl followed by 2M-NaCl+5M-urea, pH 6.0 (○), 3M-NaCl+5M-urea, pH 8.3 (△), or 4M-guanidine hydrochloride, pH 6.0 (□), before incubation with receptor solution (<800 μl) in 1100 μl final volume. In the second the same chromatin preparation was extracted with 4M-guanidine hydrochloride, pH 6.0 (□), pH 7.2 (■), 5M-guanidine hydrochloride, pH 6.0 (▽), pH 7.2 (●), pH 8.3 (●), 3M-NaCl+5M-urea, pH 6.0 (▲), pH 7.2 (▼), before incubation with 1100 μl of receptor solution. The ranges of estimates for triplicate samples are shown as vertical bars, except for the closely grouped samples (4–5M-guanidine hydrochloride). Solution pH refers to that of 100mm-solvent buffers containing 10mm- NaHSO_3 and 2ml of 2-mercaptoethanol/litre measured at 17°C. Extraction salts (guanidine hydrochloride; NaCl+urea) were dissolved in these at a final buffer concentration of 50mm. Buffers at pH 6.0 and 7.2 were $\text{KH}_2\text{PO}_4/\text{KOH}$ and at pH 8.3, Tris/HCl.

tin is difficult to suspend finely and subsample (P. Davies & P. Thomas, personal communication) and was linked to cellulose of small particle size. We avoided possible detrimental effects on chromatin during isolation of over-extraction or excessive shear forces through homogenization. Furthermore, homogenized tissue was only coarsely screened through cheesecloth; finer nylon, Dacron or silk screens were

Fig. 3. Effect of pH on the binding of $[^3\text{H}]$ oestradiol-receptor complex to 3M-NaCl+5M-urea-extracted chromatin-cellulose

Symbol ▲ refers to samples of extracted chromatin incubated with 1100 μl of receptor solution (1.12mg of protein/ml), and ● to samples incubated with 1100 μl of receptor solution (2.6mg of protein/ml). Vertical bars represent the ranges for three replicates of analysis.

not used subsequently. With two samples (M 26/10, F 26/10) this was inadvertently omitted. These two samples possessed the highest ratios of total protein/DNA (8.93 and 6.82 μg of total protein/μg of DNA respectively) (see Table 2) and more protein remained after 3M-NaCl+5M-urea extraction (see Table 5).

Therefore contamination, particularly with connective-tissue protein, would explain the failure of acidic protein and histone protein analyses to summate and correspond to values for total protein (see Table 2). We ascertained those conditions that did not allow NaHSO_3 , 2-mercaptoethanol, NaOH, H_2SO_4 , HCl or DNA to interfere with colour development in the Lowry *et al.* (1951) procedure. Also the high proportion of total protein, but minimal quantity of histone protein, remaining in de-histon-

Fig. 4. Saturation binding of [³H]oestradiol-receptor complex to hypothalamic chromatin previously extracted with 3M-NaCl+5M-urea (pH7.2)

Samples of hypothalamic chromatin coded ● (female, 26 October; 20–25 μg of DNA/sample) were incubated with different volumes of receptor solution in 1100 μl final volume. Samples ▲ (male, 26 October; 15–21 μg of DNA/sample) and Δ (female, 19 October; 20–25 μg of DNA/sample) were incubated with 550 μl of receptor solution without further dilution. Vertical bars represent the ranges for three replicates of analysis.

ized unmasked chromatin (Table 5) can be accounted for by this.

Binding of oestrogen- and progesterone-labelled receptor complexes to male and female chromatin

Under the optimized conditions we examined the samples of hypothalamic chromatin for sex-related differences in oestradiol-receptor binding. Chromatin, chromatin de-histoneized with 2M-NaCl (pH 6.0), chromatin de-histoneized and unmasked with 3M-NaCl+5M-urea (pH 7.2) and samples of the last extracted with 0.4M-KCl to ascertain the proportion of KCl-resistant high-affinity binding were studied.

The observed ranges in estimates for triplicate samples were no greater than those shown in Figs. 2–5, which are comparable with those of Webster *et al.* (1976). Thus mean data show that oestradiol-receptor binding to chromatin was not systematically affected by initial chromatin composition (Tables 2 and 6), but removal of histones with 2M-NaCl or extraction with salt+urea both consistently increased binding of the receptor complex by comparable amounts. Neither receptor binding by chromatin nor the degree to which binding was increased after extraction was affected by sex (Table 6). Extraction of bound receptor complex with 0.4M-KCl also failed to reveal any sex-related difference.

That KCl-resistant binding was a high and fairly constant proportion of the receptor complex bound to dehistonized unmasked chromatin (range 0.69–0.88), and was greater than for unextracted chromatin, suggests that both masked and unmasked acceptor sites possess similar receptor-binding characteristics and that they are possibly the same or a closely related class of proteins.

The same extraction conditions were used to study progesterone-receptor binding, but, because too little of this receptor complex was prepared to allow the study of all samples and estimation of a saturation

Table 4. Protein concentrations, specific radioactivities and volumes of [³H]oestradiol- and [³H]progesterone-labelled receptor complexes used in binding assays

	Sample no.	Final volume prepared (ml)	Sample volume used (μl)	Protein concentration (mg/ml)	10 ⁻⁵ × Specific radioactivity (c.p.m./mg of protein)
[³ H]Oestradiol-receptor complex	1	35.0	1100	2.6	2.13
	2	16.0	550	3.6	2.58
	3	18.0	650	4.4	2.27
	4	17.0	594	3.9	2.12
	5	12.5	325	6.9	2.13
[³ H]Progesterone-receptor complex	1	15.0	750	5.3	3.50
	2*	20.0	590	—	—

* Protein concentration and specific radioactivity were not measured but five (NH₄)₂SO₄ pellets were used compared with three in 15 ml (sample 1). Therefore a higher protein concentration would have been expected, but of similar specific radioactivity (see the text).

Table 5. Mean concentrations of total and histone protein and DNA in available chromatin-cellulose extracted by the scheme of Webster *et al.* (1976)

Data are expressed as proportions of the values observed in corresponding unextracted samples. For total protein and DNA analyses three replicates of 15–20mg each were used. For histone protein analysis three larger replicates of 30–40mg each were used to increase assay precision. Abbreviations used: M, male; F, female.

	Sex	Date of preparation	De-histoneized chromatin	De-histoneized unmasked chromatin	0.4M-KCl-extracted de-histoneized unmasked chromatin
Total protein	F	5 October	0.67	0.48	—
	F	19 October	0.59	0.44	—
	F	26 October	0.67	0.62†	—
	M	26 October	0.59	0.54†	—
	F	2 November	0.54	0.47	0.39
	M	9 November	0.48	0.38	0.35
Histone protein	F	5 October	0.335*	—	—
	F	19 October	0.104	—	—
	F	26 October	0.094	0.053	—
	M	26 October	0.096	0.052	—
	F	2 November	0.082	—	—
	M	9 November	0.124	—	—
DNA	F	5 October	0.97	1.08	—
	F	19 October	0.94	0.99	—
	F	26 October	1.26	1.20	—
	M	26 October	1.14	0.96	—
	F	2 November	1.07	0.97	0.98
	M	9 November	1.03	0.97	0.97

* Chromatin-cellulose prepared in the absence of NaHSO₃ or 2-mercaptoethanol.

† Tissue homogenates not screened through cheesecloth.

curve (Fig. 5), two samples for each sex were assayed and KCl-resistant binding was measured in only one sample of each. The results (Table 7) showed a similar pattern to oestradiol-receptor binding, the value 0.76 of 0.4M-KCl-resistant binding obtained for the female sample (2 November) being the same as the mean obtained in the oestradiol-receptor studies. More results would be required to establish whether the very high male value (0.96) was significantly different.

It might appear that the number of acceptor sites for the progesterone-receptor complex is greater (Table 7) than for the oestradiol-receptor complex (Table 6), but the former was precipitated at a higher (NH₄)₂SO₄ concentration, which will precipitate more protein. Therefore the difference in specific radioactivity between oestradiol- and progesterone-receptors (Table 4) could be greater than that shown. However, on the contrary, receptor-complex specific radioactivity would not influence the fact that binding of the progesterone-receptor complex to de-histoneized unmasked chromatin, compared with unextracted chromatin, was greater (7.9-fold mean increase) than the corresponding mean increase obtained with the oestradiol-receptor complex (4.9-fold).

Discussion

Results reported here for oestradiol- and progesterone-receptor-complex binding to sheep hypothalamic chromatin confirm the deductions of Spelsberg and co-workers (Spelsberg *et al.*, 1976; Webster *et al.*, 1976), studying avian oviduct chromatin, that receptor binding is markedly affected by the chemical composition of chromatin. In our assays, binding of steroid-labelled receptor to 2M-NaCl-extracted chromatin was greater than to unextracted chromatin, whereas with the same extractant binding of progesterone-receptor complex to hen oviduct chromatin was not increased to the same extent (Webster *et al.*, 1976). We can only attribute this difference between studies to the fact that we subsequently washed the extracted chromatin-cellulose with 10mM-Tris/HCl (pH7.5). Similarly we attribute our comparative failure to unmask acceptor sites on chromatin with guanidine hydrochloride to between-study differences in the initial chemical composition of chromatin and the efficiency with which contaminating and/or masking proteins were removed. Thus studies by Spelsberg *et al.* (1976) and Levy *et al.* (1972) show that guanidine hydrochloride is a more efficient chromatin-deproteinizing agent than NaCl, urea,

Fig. 5. Saturation binding of [^3H]progesterone-receptor complex to hypothalamic chromatin

Binding was assayed by using 3M-NaCl+5M-urea (pH 7.2)-extracted chromatin (female, 2 November) and approx. 20 μg of DNA/sample. The volumes of receptor solution added (preparation 1; see Table 4) were not further diluted. The range in triplicate samples is represented by vertical bars.

urea+guanidine hydrochloride or urea+NaCl (see also Chaudhuri, 1973; Webster *et al.*, 1976), but unmasking of chromatin, of the same initial composition from the same tissue, is achieved at a higher protein/DNA ratio with salt+urea (Webster *et al.*, 1976) than when extracted with guanidine hydrochloride (Spelsberg *et al.*, 1976). Therefore, selectivity of protein removal is clearly important.

The good agreement between observed extents of progesterone-receptor complex binding to de-histone unmasked hypothalamic chromatin and comparable data for avian oviduct chromatin (Spelsberg *et al.*, 1976; Webster *et al.*, 1976) strongly suggests that salt+urea extraction overcame the effects of extraneous protein contamination on chromatin-

receptor binding and that comparable classes of binding sites were measured. The same appeared to be the case for oestradiol-receptor complex binding, for we demonstrated that this receptor was of lower specific radioactivity. But the possibility cannot be excluded that hypothalamic chromatin possesses fewer oestradiol-receptor-complex acceptor sites than progesterone-receptor-complex acceptor sites.

Webster *et al.* (1976) deduced that progesterone-receptor-complex binding to chromatin in 0.15M-KCl at saturation represents the highest-affinity classes of binding sites and those with a physiological function. Clark *et al.* (1976) consider that 0.4M-KCl-resistant binding of oestrogen-receptor complex to nuclei represents the biologically most important long-term high-affinity form of nuclear-receptor association. Our observations that the binding of oestradiol- and progesterone-receptor complexes to de-histone unmasked chromatin was largely 0.4M-KCl-resistant establishes a clear association between these phenomena *in vitro* and *in vivo*.

Despite this, no sex difference in chromatin binding of oestradiol- or progesterone-receptor complexes was observed that could be attributed to differential masking of acceptor sites. Therefore it might be concluded that sex-related differences in hypothalamic nuclear oestrogen binding that exist in rats (Vertes & King, 1971) do not occur in sheep, or that some other mechanism that differentiates nuclear-receptor binding from chromatin-receptor binding is responsible. With respect to the former, we know of no data that demonstrate a sex-related difference in hypothalamic nuclear oestrogen binding in sheep. But it is established that several target tissues of androgenized female rats and mice (Shyamala *et al.*, 1974; Lobl, 1975) possess the lower capacity, as of males, to effect nuclear oestrogen retention. Also both androgenized female rats (Barracough & Turgeon, 1974) and androgenized female sheep (Short, 1974) fail to respond to oestrogen stimulation in lutropin secretion, which is a male characteristic. Therefore we consider it unlikely that a species difference accounts for our failure to identify a sex-related difference in oestrogen-receptor-complex binding to chromatin.

On the other hand, failure to observe a sex-related difference in receptor binding could be a direct consequence of the protocol used for assay *in vitro*. For example, De Sombre *et al.* (1972), working with calf uterine oestrogen-receptor complex, observed that $(\text{NH}_4)_2\text{SO}_4$ at 20% saturation brought about conversion of the receptor. Even in the absence of steroid this transformed receptor was capable of stimulating uterine nuclear RNA polymerase, although enhancement of RNA synthesis was greater when steroid was present. They deduced that the physiological role of oestrogen is to promote conversion of extranuclear cytosol receptor into a biologically active form capable of entering the nucleus (binding to acceptor

Table 6. *Binding of [³H]oestradiol-receptor complex to male and female hypothalamic chromatin*

Values in parentheses are expressed as proportions of chromatin values, others are means for triplicate samples. Abbreviations used: M, male; F, female.

Date of sample preparation	$10^{-5} \times$ Mean bound radioactivity (c.p.m./mg of DNA)								Mean chromatin/receptor ratio (μ g of DNA/mg of protein)		Volume of receptor (μ l/sample)	
	Chromatin		De-histoneized chromatin		De-histoneized unmasked chromatin		0.4M-KCl-resistant activity		M	F	M	F
	M	F	M	F	M	F	M	F				
5 October	0.53	0.67	1.55	1.65	2.17	2.58	1.79	1.83	17.9	16.3	594	594
12 October	0.38	0.28	1.33	1.24	2.87	2.30	2.01	1.85	10.8	12.9	650	650
19 October	—	0.79	—	2.16	—	2.70	—	2.38	—	11.4	—	550
26 October	0.93	0.89	2.29	1.76	3.37	4.31*	2.43	—	9.9	7.4	550	1100
26 October	—	—	—	—	—	4.33	—	2.99	—	7.7	—	325
2 November	—	0.54	—	2.12	—	3.43	—	2.53	—	8.6	—	325
9 November	0.51	—	2.32	—	3.18	—	2.36	—	10.0	—	325	—
Means	0.59	0.64	1.88	1.79	2.90 (4.93)	3.07 (4.83)	2.15 (3.66)	2.32 (3.65)				

* One sample only.

Table 7. *Binding of [³H]progesterone-receptor complex to chromatin from male and female hypothalami*

Values in parentheses are expressed as proportions of chromatin values, except for 0.4M-KCl-resistant binding, the values of which are expressed as proportions of values for de-histoneized unmasked chromatin. Others are means for three replicates. Abbreviations used: M, male; F, female.

Date of sample preparation	$10^{-5} \times$ Mean bound radioactivity (c.p.m./mg of DNA)								0.4M-KCl-resistant binding		Volume of receptor (μ l/sample)	
	Chromatin		De-histoneized chromatin		De-histoneized unmasked chromatin		M	F	M	F	M	F
	M	F	M	F	M	F						
26 October	2.63	2.14	14.56 (5.54)	9.24 (4.32)	20.59 (7.83)	16.14 (7.55)	—	—	—	—	590	590
2 November	—	1.17	—	7.05 (6.02)	—	9.08 (7.76)	—	6.91 (0.76)	—	—	—	750
9 November	1.70	—	10.01 (5.90)	—	14.32 (8.44)	—	13.75 (0.96)	—	—	—	590	—
Means	2.16	1.65	12.29 (5.45)	8.14	17.45 (7.90)	12.61						

sites on chromatin) and mediating the hormonal effect.

This is in direct contradiction to the mechanism proposed by Pikler *et al.* (1976), who argue that both steroid and receptor as an activated complex are essential for nuclear binding. But these workers based their conclusions on assessment of the amount of receptor extracted from the nuclear pellet by 0.4M-KCl. Our observations suggest that the proportion of receptor that can be dissociated from acceptor sites by 0.4M-KCl is low, and also that the proportion of total labelled receptor complex added to chromatin that is bound is low. Therefore if the observations of De Sombre *et al.* (1972) apply to the progesterone-

receptor complex this could be an important consideration. For example, O'Malley *et al.* (1976) working with chick oviduct chromatin failed to obtain additive effects of progesterone and diethylstilboestrol on RNA synthesis, although both hormones at the same dose individually effectively stimulated the same number of initiation sites. They reasoned that oestrogen and progesterone may act at similar chromatin sites to enhance transcription.

Hsueh *et al.* (1976) studying rat uteri argue that neither the initial binding of oestrogen to its receptor nor translocation of the steroid-receptor complex to the nucleus is inhibited by progesterone, but their data show that progesterone prevents long-term

nuclear binding of oestrogen-receptor complex. Thus a direct consequence of decreased high-affinity 0.4M-KCl-resistant binding of oestrogen-receptor complex to nuclei (Clark *et al.*, 1976) is diminished cytoplasmic oestrogen-receptor complex synthesis, for the concentration of oestrogen receptor was stimulated by oestradiol in the absence of progesterone. That a delay in progesterone treatment by as much as 8 h after oestradiol treatment also inhibited the oestrogen-stimulated increase in cytosol oestrogen receptor (Hsueh *et al.*, 1976) suggests to us progesterone acts at some additional site(s) to affect the rate of receptor-protein catabolism.

Equally important is the fact that testosterone propionate and the oestrogen antagonist nafoxidine interfere with cytosol-receptor replenishment (Hsueh *et al.*, 1976). This has been confirmed by Charreau & Denti (1975), who studied the concentrations of hypothalamic oestrogen receptor in adult female rats treated with testosterone propionate before slaughter, and by Nicholson *et al.* (1976), who studied the effect of Tamoxifen (ICI 46474), another antioestrogen, on cytoplasmic oestradiol-receptor replenishment in 7.12-dimethylbenz[*a*]anthracene-induced rat mammary-gland tumours.

Hsueh *et al.* (1976) have recognized that the ability of progesterone to antagonize or modify the action of oestrogen through effects on the concentration of cytoplasmic receptor may form the basis of inter-hormonal control mechanisms. However, to our knowledge, possible steric or other interactions between hormone receptors, which are proteins, at the level of chromatin have not been systematically studied. The suggestion from our data and those considered above is that aspects of sexual differentiation and steroid sensitivity that cannot be attributed to marked differences in the degree of chromatin masking might be explained by such phenomena. Steroids can be linked to polystyrene (Vonderhaar & Mueller, 1969) or Sepharose (Hassoux *et al.*, 1977) to produce high-capacity adsorbents; thus the means exist whereby separation of the major classes of steroid receptors can be affected and the interaction of steroid-hormone receptors singly or in combination with chromatin could be studied.

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