The Selective Isolation of the Uterine Oestradiol–Receptor Complex by Binding to Oligo(dT)–Cellulose

THE MEDIATION OF AN ESSENTIAL ACTIVATOR IN THE TRANSFORMATION OF CYTOSOL RECEPTOR

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The [^3H]oestradiol–receptor complex was selectively isolated from rat uterus cytosol by column chromatography on oligo(dT)–cellulose. Optimal conditions are described for the binding of the complex to oligo(dT)–cellulose, which is shown to be similar to its binding to DNA–cellulose. The cytosol complex has an apparent mol.wt. of 50 000–60 000 in high salt concentrations, as determined by Sephadex G-100 chromatography. This corresponds to the 4S cytoplasmic oestradiol receptor. In binding to oligo(dT)–cellulose the receptor is transformed into a form with an apparent mol.wt. of 100 000–120 000, corresponding to the 5S nuclear receptor complex. This transformation mimics the conversion *in vivo* of the cytoplasmic oestradiol receptor into the nuclear form. The binding of the complex to oligo(dT)–cellulose as a 5S nuclear form is unequivocally demonstrated to require the mediation of an activating factor present in the cytosol. This requirement for an activating factor is discussed in relation to reports that nuclear binding of the oestradiol–receptor complex is not dictated solely by the availability of the cytoplasmic oestradiol receptor.

It is well established that one of the ways in which oestradiol influences metabolic activity in uterine tissues is by activating the synthesis of specific mRNA species. As with other steroids, the action of the oestradiol is thought to be mediated by a specific receptor present in the cytosol of target tissues (O'Malley & Means, 1974; King & Mainwaring, 1974). This receptor, after complexing with the steroid, is translocated to the nucleus in a step requiring conversion of the receptor from a 4S into a 5S form. Both forms have been characterized by sucrose-density-gradient centrifugation (Jensen & DeSombre, 1972). In the nucleus the 5S form associates with chromatin, and DNA has been shown to be an essential component in the binding (Shyamala, 1971). It is unresolved whether *in vivo* the conversion occurs in the cytoplasm (Jensen *et al.*, 1968) or in the nucleus (Jungblut *et al.*, 1970).

The oestradiol receptor will bind to DNA (King & Gordon, 1972; Clemens & Kleinsmith, 1972) and to a variety of polynucleotides (Sluyser *et al.*, 1974; Yamamoto & Alberts, 1974). DNA–cellulose chromatography has been used for the isolation of the oestradiol–receptor complex (Yamamoto & Alberts, 1972, 1975; Yamamoto, 1974). Binding of the complex to the DNA–cellulose was achieved by incubation in 0.15M-KCl, and the bound complex was

released from the DNA by 0.4M-KCl. This salt concentration also discharges the nuclear receptor from chromatin. In binding to the DNA–cellulose, the complex is transformed from a 4S into a 5S form indistinguishable from the nuclear 5S receptor observed *in vivo* (Yamamoto & Alberts, 1972), and these studies have led to the proposal that the conversion step *in vivo* and *in vitro* is the result of association of a factor with the receptor complex (Yamamoto, 1974). This proposal has, however, remained a matter of debate in the absence of a clear demonstration of the presence of such a factor (Buller & O'Malley, 1976).

In the present paper we report the isolation of the oestradiol–receptor complex in the 5S form by chromatography of uterine cytosol on oligo(dT)–cellulose, and we show that the conversion of the 4S into the 5S form *in vitro* does involve the participation of an essential activating factor present in uterine cytosol.

Experimental

[2,4,6,7(n)- ^3H]Oestradiol-17 β (85 Ci/mmol) was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Oligo(dT)–cellulose was obtained from Collaborative Research, Koch–Light Laboratories, Colnbrook, Bucks., U.K. Sephadex G-100 (fine grade) was from Pharmacia (G.B.) Ltd., London

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W.5, U.K. All reagents were AnalaR grade. Glassware was siliconized with Siliclad (ARH Horwell Ltd., Kilburn, London N.W.6, U.K.) to prevent adsorption of oestradiol receptor on the glass. Female Wistar rats were either bred in our laboratory or purchased from Charles Rivers Ltd., Margate, Kent, U.K.

DNA-cellulose

DNA-cellulose was prepared by the method of Alberts & Herrick (1971), by using denatured rat liver DNA and Whatman chromatography-grade CC31 cellulose (Whatman Biochemicals Ltd., Maidstone, Kent, U.K.). About 1.5mg of DNA was bound per g of cellulose as determined by the diphenylamine reaction (Burton, 1956).

Preparation of uterine cytosol

Intact female rats aged 55–70 days were used instead of immature (19–25 days) rats (Yamamoto & Alberts, 1972) to obtain increased yield of oestradiol receptor capable of binding to either oligo(dT)-cellulose or DNA-cellulose, since it has been shown that, *in vivo*, more binding of oestradiol occurs in mature than in immature rats, and that ovariectomy decreased the amount of nuclear binding of the oestradiol-receptor complex (King *et al.*, 1971). The rats were anaesthetized with chloroform and killed by decapitation. The uterine horns were excised, stripped of fat and placed in ice-cold TED buffer [10mM-Tris/HCl(pH7.6)/1.5mM-EDTA/1mM-dithiothreitol]. All subsequent operations were carried out at 4°C. Weighed uteri were finely chopped and homogenized in 3 vol. of TED buffer (in certain experiments containing 0.15M-KCl) by using a type X1020 homogenizer (The Scientific Instrument Centre, London W.C.1, U.K.) in two bursts of 5 s at mark 4 with constant cooling. The homogenate was centrifuged in the MSE Ti 60 rotor at 200000g for 90 min. In initial experiments the supernatant fraction (cytosol) was made 5 nM with [³H]oestradiol containing a 250-fold excess of testosterone as originally recommended by Yamamoto & Alberts (1972) to decrease non-specific binding. Subsequently it was found that the omission of testosterone did not affect receptor binding of [³H]oestradiol, and later experiments were performed without the addition of testosterone. The labelled cytosol was either used immediately after preparation or stored frozen at -20°C.

Determination of [³H]oestradiol-receptor complex

Samples (0.1–0.5 ml) of the [³H]oestradiol-labelled cytosol were chromatographed on columns (15 cm × 0.6 cm) of Sephadex LH-20, essentially as described

by Ginsburg *et al.* (1974). Fractions (0.2 ml) were collected and assayed for radioactivity. Macromolecular-bound radioactivity corresponding to [³H]oestradiol-receptor complex is eluted with the void volume, and is clearly separated from free [³H]oestradiol, which is retarded by the gel. Measurements of total uterine cytoplasmic receptor obtained by this method are in good agreement with the majority of published values.

Oligo(dT)-cellulose and DNA-cellulose chromatography

Labelled uterine cytosol (0.1–2 ml) prepared in either TED buffer or 0.15M-KCl/TED buffer were incubated on 500mg oligo(dT)-cellulose or DNA-cellulose columns at 4°C by recycling for 0.5–2 h at a flow rate of approx. 16 ml/h by using a DeSaga peristaltic pump (Camlab Ltd., Cambridge, U.K.). Unbound radioactivity was washed out with 10 ml of the low-salt TED buffer, and the columns were eluted with 10 ml of 0.4M-KCl/TED buffer followed by 10 ml of 1.2M-KCl/TED buffer. Fractions (1 ml) were collected (see Fig. 1).

Salt-gradient elution of uterus oestradiol receptor from oligo(dT)-cellulose and DNA-cellulose

Uterine cytosol (1 ml) in TED buffer containing 5 nM-[³H]oestradiol was incubated on a 500mg column of oligo(dT)-cellulose (Fig. 2a) or DNA-cellulose (Fig. 2b). The columns were washed with 5 vol. of TED buffer and eluted in a two-phase linear salt gradient of 0–0.6M-KCl/TED buffer (45 ml), followed by 0.6–1.2M-KCl/TED buffer (20 ml). Fractions (1 ml) were collected from each column and assayed for radioactivity. The linearity of the gradient was monitored by potassium determinations on selected fractions of the eluent by using an IL 143 flame photometer (Instrumentation Laboratories, Altrincham, Cheshire, U.K.).

Chromatography of uterus oestradiol receptor on Sephadex G-100

Samples of labelled cytosol were chromatographed at 4°C on a column (78 cm × 1.6 cm) of Sephadex G-100 previously equilibrated with TED buffer or 0.4M-KCl/TED buffer as appropriate. The flow rate was maintained at 10–12 ml/h. The E_{280} of the eluate was continuously measured by using an ISCO UA-4 absorbance monitor (Fisons Ltd., Crawley, Sussex, U.K.). Fractions (1 ml) were collected. The columns were calibrated with the following marker proteins: (1) Blue Dextran 2000 (mol.wt. 2000000); (2) bovine serum albumin (dimer) (mol.wt. 134000); (3) transferrin (mol.wt. 90000); (4) bovine serum albumin (monomer) (mol.wt. 67000); (5) ovalbumin (mol.wt. 43500).

Sucrose-density-gradient centrifugation

Samples (0.1 ml) of uterine [^3H]oestradiol-receptor complexes in 0.4M-KCl/TED buffer were centrifuged on 4 ml of a 5–15% (w/w) sucrose gradient in the same buffer in the MSE 6 $\times$ 4.2 SW 65 Ti rotor at 300000g_{av} for 16 h at 4°C. After centrifugation, E_{280} was monitored with an ISCO UA-5, and fractions (0.1 ml) were collected with an ISCO 640 gradient fractionator (Fisons Ltd., Loughborough, Leics., U.K.). The following marker proteins (with sedimentation coefficients) were used: bovine serum albumin (4.6S) and γ -globulin (7S). Bacterial alkaline phosphatase (6.8S) was used as an internal marker as described by Yamamoto & Alberts (1972). All proteins were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Radioactivity of the fractions was determined as described below.

Assay for factor promoting binding of oestradiol-receptor to oligo(dT)-cellulose

The rationale for the assay is described in the Results and Discussion Section. The assay requires mixing two populations of oestradiol-receptors, one prepared from normal cytosol containing receptor masked by the addition of unlabelled oestradiol and the other containing [^3H]oestradiol-receptor with a decreased ability to bind to oligo(dT)-cellulose. The capacity of the different fractions to bind to oligo(dT)-cellulose is determined by a prior analysis. A subsequent measurement of the binding capacity of the mixed fractions will demonstrate whether, in binding to oligo(dT)-cellulose, the different populations of oestradiol-receptors require the presence of an essential (common) factor for which they compete.

Two methods were used to prepare fractions with a decreased capacity to bind to oligo(dT)-cellulose. The first utilized the observation that, after exhaustive extraction of normal uterine cytosol on oligo(dT)-cellulose, a fraction of the oestradiol-receptor remains unbound. This decreased-binding fraction was used in experiments typified by Table 4 (Expt. A). The alternative method was based on the difference between the published mol.wt. of the 4S form (61 000) and the 5S form (105 000), which was attributed to the activator which thus had a presumed mol.wt. of 44 000 (calculations derived from Yamamoto & Alberts, 1972). By comparison, the receptor in low-salt buffer (i.e. the 8S form) has a mol.wt. of 240 000 (Puca *et al.*, 1971). We therefore subjected uterus cytosol to chromatography on Sephadex G-100 in low-salt buffer (as shown in Fig. 3a) to separate receptor from the activator, which would be expected to be retained within the gel. The receptor present in the first few millilitres of the excluded material served as the source of receptor with a decreased binding

capacity and used in experiments illustrated in Table 4 (Expt. B).

Determination of radioactivity

Fractions from sucrose density gradients were assayed in 5 ml and all other samples were assayed in 10 ml of scintillation 'cocktail' containing 10% (v/v) Bio-Solv BBS3 (Beckman RIIC Ltd., Hitchin, Herts., U.K.) and 4.5 g of butyl-PBD [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole; Ciba Ltd., Hordsham, Sussex, U.K.] in 1 litre of toluene, by using a Beckman LS 230 spectrometer (1000 c.p.m. $\equiv$ 14.3 fmol of [^3H]oestradiol).

Results and Discussion

Binding of oestradiol-receptor complex to oligo(dT)-cellulose

A labelled oestradiol-receptor complex is produced by incubation of uterine cytosol with [^3H]oestradiol at 0–4°C for 90–120 min. The amount of labelled complex determined by exclusion chromatography on Sephadex LH-20 (Ginsburg *et al.*, 1974) represents a minimal estimate of the receptor content, owing to the occupation of some receptor sites by endogenous oestradiol. However, the uterine content of oestradiol in adult female rats is always very low compared with the concentration of receptor in the tissue (Eisenfeld, 1967; Feherty *et al.*, 1970), so that the error introduced into the estimation of receptor content is very small. The experiments described in this paper do not depend on estimates of the absolute amounts of receptor, and so are unaffected by this consideration. When the labelled complex is incubated with oligo(dT)-cellulose in TED buffer a proportion is bound. As shown in Fig. 1, the bound oestradiol receptor can be eluted from the column containing oligo(dT)-cellulose with 0.4M-KCl/TED buffer, which normally discharges bound receptor from the nucleus. Residual bound radioactivity can be further eluted with 1.2M-KCl/TED buffer. Similar results are obtained if DNA-cellulose is used instead of oligo(dT)-cellulose.

In an initial binding experiment (Table 1a), about 30% of the labelled complex was bound to oligo(dT)-cellulose, and eluted from the column by increasing the salt concentration. Almost all of the unbound complex was recovered on analysis of the non-binding low-salt eluate on Sephadex LH-20. About 85% of the bound labelled complex was eluted by 0.4M-KCl and the remaining 15% by 1.2M-KCl. The low-salt eluate, when reincubated on a second column of oligo(dT)-cellulose, still contained receptor that would bind to oligo(dT)-cellulose. This binding corresponded to 10% of the labelled complex. In subsequent experiments we were able to bind 85% of the

amount of receptor capable of binding to oligo(dT)-cellulose after a single incubation. Re-incubation of the low-salt eluate resulted in the binding of less than 5% of the labelled complex. As a routine, we obtained a binding of between 35 and 60% of the cytosol receptor to the oligo(dT)-cellulose. Intact animals were used without regard to the state of their oestrous cycle, which might be responsible for this variation. Similar results were also obtained with DNA-cellulose chromatography (Table 1b).

Heating cytosol extracts at 65°C for 10 min, which abolishes activity of the high-affinity oestradiol receptor (Puca *et al.*, 1972), caused a decrease of more than 95% of the amount of radioactivity retained on the oligo(dT)-cellulose. In other control experiments, the binding of [³H]oestradiol alone to oligo(dT)-cellulose, and of labelled uterine cytosol to blank cellulose, were measured. Although free oestradiol was slightly retarded, resulting in tailing of radioactivity in the eluate, no extra oestradiol was eluted when the salt concentration was increased to 0.4M-KCl and to 1.2M-KCl. Binding of the receptor to blank cellulose represented less than 2% of the binding to oligo(dT)-cellulose. The binding of the radioactive oestradiol-receptor complex to oligo(dT)-cellulose (500mg) was proportional to the input up to a maximum measured receptor capacity of 1500fmol (110000 c.p.m.) of [³H]oestradiol. The volume applied varied from 0.1 to 2ml of the cytosol prepared from a 25% (w/v) homogenate of uterine tissue. Storage of cytosol at -20°C for 3-4 weeks resulted in a loss of about 20% of the receptor binding.

A greater extent of binding was achieved at 4°C than at 18°C. At 4°C maximal binding occurred within 30 min (Table 2). Prolonged exposure of the receptor complex to the higher temperature resulted in lowered binding to oligo(dT)-cellulose. This is seen as a decrease of about 50% on extending the incubation from

Fig. 1. *Oligo(dT)-cellulose chromatography of oestradiol-receptor*

Uterine cytosol (0.5ml) in 10mM-Tris/HCl (pH 7.6)/1.5mM-EDTA/1mM-dithiothreitol (TED buffer) containing 5nM-[³H]oestradiol was incubated on a 250mg column of oligo(dT)-cellulose at 4°C (for details, see the Experimental section). Unbound [³H]oestradiol was washed out with 10ml of TED buffer. Bound [³H]oestradiol-receptor was eluted with 0.4M-KCl/TED buffer. After 10ml of this buffer a further elution was achieved with 10ml of 1.2M-KCl/TED buffer. The arrows represent the initiation of the salt elutions. The first two 1ml fractions contained radioactivity in excess of the ordinate scale. Dashed lines indicate a parallel experiment performed with blank cellulose instead of oligo(dT)-cellulose.

Table 1. *Binding of oestradiol receptor to oligo(dT)-cellulose*

(a) [³H]Oestradiol-labelled uterine cytosol (2ml in TED buffer) was incubated on a 500mg column of oligo(dT)-cellulose; after the wash with TED buffer, bound receptor was eluted with 0.4M-KCl/TED buffer and 1.2M-KCl/TED buffer as described in Fig. 1. The initial wash in TED buffer was successively incubated on a second and third column. (b) A 0.4ml sample of the labelled cytosol was incubated with a column of either DNA-cellulose or oligo(dT)-cellulose for a comparison of the binding of the receptor to these two matrices. Bound receptor was eluted as described above. Total macromolecular-bound radioactivity corresponding to the [³H]oestradiol-receptor was determined by Sephadex LH-20 chromatography as described in the Experimental section.

Chromatography column	Total macromolecular-bound radioactivity in cytosol (c.p.m.)	Column-bound radioactivity (c.p.m.)	
		0.4M-KCl eluate	1.2M-KCl eluate
(a) Oligo(dT)-cellulose			
First incubation	420000	106900	22100
Second incubation	244000	42000	8700
Third incubation	—	10300	2200
(b) Oligo(dT)-cellulose	87000	26600	5700
DNA-cellulose	87000	25800	900

30 to 90 min. Subsequent experiments were therefore performed at 4°C.

Comparison of the binding of receptor to oligo(dT)-cellulose and to DNA-cellulose

The interaction of the oestradiol-receptor complex with the oligo(dT)-cellulose appears to parallel its binding to DNA-cellulose (Table 3). The same fraction of the oestradiol-receptor complex binds to either DNA-cellulose or oligo(dT)-cellulose, since no significant increment in binding was shown when the initially unbound fraction was recycled on heterologous columns. Thus, after an initial binding of about 30% of labelled receptor complex on oligo(dT)-cellulose, only a further 4–5% was bound on a second column of either oligo(dT)-cellulose or DNA-cellulose. Similar results were obtained if the initial binding was achieved on DNA-cellulose.

Further similarities between the binding of the oestradiol-receptor complex to DNA-cellulose and oligo(dT)-cellulose are observed in the response of the bound complex to the effects of increasing salt concentrations (Fig. 2). Initial binding of the receptor was achieved by chromatography of labelled uterine cytosol. After extensive washing with TED buffer, the radioactive complex was eluted from the columns containing bound receptor with an increasing gradient of KCl. The complex began to be eluted from both columns of oligo(dT)-cellulose or DNA-cellulose at a salt concentration of about 0.22M-KCl. Maximal elution was achieved with a salt concentration of around 0.25–0.35M-KCl. On oligo(dT)-cellulose there was a fraction that appeared to be more strongly bound, being eluted at a salt concentration of between 0.45 and 0.5M-KCl, and seen as a shoulder in the elution profile. In other experiments this more tightly bound fraction occasionally appeared as a separate peak of radioactivity. The receptor binding to DNA-cellulose contained a similar but less marked tightly bound fraction.

The oestradiol-receptor complex is bound to oligo(dT)-cellulose as a 5S form

We have used chromatography on Sephadex G-100 to investigate the nature of the oestradiol-receptor complex in different salt concentrations, as well as its alteration on reacting with oligo(dT)-cellulose (Fig. 3). In low-salt buffer the labelled oestradiol-receptor complex was excluded from the column of Sephadex G-100. This is consistent with its being in the high-molecular-weight polymeric form designated 8S as a result of its sedimentation characteristics in sucrose density gradients (O'Malley & Means, 1974; Jensen & DeSombre, 1972). In the presence of high-salt (0.4M-KCl) buffer the labelled complex was retained within the column of Sephadex G-100 and was eluted as a component with twin peaks of radioactivity corresponding to a mol.wt. of 50000–60000. This form corresponds to the 4S cytosol receptor described in the literature (O'Malley & Means, 1974; Jensen & DeSombre, 1972). The oestradiol-receptor complex

Table 2. Conditions for binding of oestradiol receptor to oligo(dT)-cellulose

Identical samples of [³H]oestradiol-labelled uterus cytosol (0.5 ml) were incubated on oligo(dT)-cellulose columns under the various conditions indicated. The columns were washed with TED buffer; radioactivity corresponding to bound oestradiol receptor was eluted with 0.4M-KCl/ and 1.2M-KCl/TED buffers as described in Fig. 1.

Temperature (°C)	Incubation time (min)	Oligo(dT)-cellulose-bound radioactivity (c.p.m.)	
		0.4M-KCl eluate	1.2M-KCl eluate
4	30	25600	3700
	90	22100	2900
18	30	18000	2500
	90	8200	3000

Table 3. Comparison of the binding of oestradiol-receptor to oligo(dT)-cellulose or DNA-cellulose

Duplicate samples of labelled cytosol (0.8 ml) containing 53500 c.p.m. of [³H]oestradiol-receptor were incubated on two oligo(dT)-cellulose or two DNA-cellulose columns. For each pair the unbound low-salt eluates were re-incubated on either an oligo(dT)-cellulose or a DNA-cellulose column. All columns were subsequently eluted with 0.4M-KCl/ and 1.2M-KCl/TED buffer to assay for bound material. The Table shows total eluted radioactivity from each column.

First column	Column-bound radioactivity (c.p.m.)	Receptor bound (%)	Second column	Column-bound radioactivity (c.p.m.)	Receptor bound (%)
1. Oligo(dT)-cellulose	18500	35	(a) Oligo(dT)-	2790	5.2
			(b) DNA-	2210	4.1
2. DNA-cellulose	17000	32	(a) Oligo(dT)-	2290	4.2
			(b) DNA-	1960	3.6

Fig. 2. Salt-gradient elution of oestradiol-receptor from oligo(dT)-cellulose and DNA-cellulose

Uterine cytosol (1 ml) containing [^3H]oestradiol-receptor was incubated on a column of oligo(dT)-cellulose (a) or DNA-cellulose (b). The columns were washed with TED buffer and eluted in a two-phase linear salt gradient of 0–0.6 M-KCl/TED buffer followed by 0.6–1.2 M-KCl/TED buffer (----). For further details, see the Experimental section.

binding to oligo(dT)-cellulose and eluted by 0.4 M-KCl is clearly differentiated from the low-salt (8 S) and high-salt (4 S) forms, eluted on Sephadex G-100 at a position intermediate between these two forms. This bound form has an apparent mol.wt. of 100000–120000 and corresponds to the 5 S nuclear form.

The low yield of labelled 5 S oestradiol-receptor complex in this experiment (Fig. 3b) is readily explained by dissociation of the [^3H]oestradiol-receptor complex during chromatography on Sephadex G-100, and subsequent adsorption of [^3H]oestradiol by the Amicon concentrator; it seems highly improbable that this would selectively affect the 4 S form (if it were present), which has the same dissociation constant as the 5 S form (Sala-Trepat & Reti, 1974). After exhaustive chromatography of labelled cytosol on oligo(dT)-cellulose, the unbound material (containing 50–60% of the original amount of receptor) had an elution volume on Sephadex G-100 chromatography identical with that of the 4 S receptor shown in Fig. 3(a), that is, no 5 S receptor was present.

Further evidence that the oestradiol receptor will not bind to oligo(dT)-cellulose as a 4 S receptor, but only as the 5 S receptor, was obtained by sucrose-

density-gradient analyses of the different receptors. Thus the oestradiol receptor that bound to oligo(dT)-cellulose and eluted with 0.4 M-KCl/TED buffer had a sedimentation coefficient of 4.7 S (i.e. 5 S), whereas the oestradiol receptor that remained unbound had a sedimentation coefficient of 4 S (Fig. 4).

Requirement for an activating factor in the binding of the oestradiol-receptor complex to oligo(dT)-cellulose

Yamamoto (1974) and Yamamoto & Alberts (1972, 1974) have carried out extensive studies on the 4 S into 5 S conversion based on the binding of the oestradiol-receptor complex to DNA-cellulose almost entirely as the 5 S form. The 5 S form is not a dimer of the 4 S form, as shown by the studies by, for example, Williams & Gorski (1972) and by Yamamoto (1974), who proposes that conversion into the 5 S form requires the binding of another protein to the complex. This proposal was based largely on changes in the sedimentation characteristics of the different receptor forms. The binding factor involved is to be distinguished from the receptor-transforming factor (RT-factor) claimed by Puca *et al.* (1972) to be respon-

Fig. 3. Chromatography of oestradiol-receptor on Sephadex G-100

(a) Uterus cytosol (2ml), containing [^3H]oestradiol-receptor, in either TED buffer (●) or 0.4M-KCl/TED buffer (○) was chromatographed on Sephadex G-100 with the appropriate buffer for elution. Absorbance is not shown. (b) A 2ml sample of the bound [^3H]oestradiol-receptor eluted from oligo(dT)-cellulose by 10ml of 0.4M-KCl/TED buffer was chromatographed in the same buffer. The eluate from Sephadex G-100 was concentrated five-fold on an Amicon CEC 1 concentrator (Amicon Ltd., High Wycombe, Bucks., U.K.) for detection of absorbance. ○, Radioactivity; —, E_{280} . The elution volumes of marker macromolecules with relevant mol.wts. are shown: (1) Blue Dextran 2000, 2000000; (2) bovine serum albumin dimer, 134000; (3) transferrin, 90000; (4) bovine serum albumin monomer, 67000; (5) ovalbumin, 43500.

sible for the production of a cytoplasmic receptor (mol.wt. 60000) from the larger cytoplasmic oestradiol-binding protein (mol.wt. 240000) and which requires Ca^{2+} ions for its activity. Since the cytoplasmic receptor of mol.wt. 50000–60000 can be obtained in the absence of Ca^{2+} (see Fig. 3) it remains to be established what the true function of the 'RT-factor' is.

(a) *Experimental rationale.* Our experiments to demonstrate unequivocally the presence of the activating factor used the observation that the oestradiol-receptor complex also binds to oligo(dT)-cellulose in the 5S form (Fig. 3b), and we have used the binding

Fig. 4. Sucrose-density-gradient analyses of [^3H]oestradiol-receptors

Samples (0.1ml) of the bound [^3H]oestradiol-receptor eluted from oligo(dT)-cellulose with 0.4M-KCl/TED buffer (○) and of unbound [^3H]oestradiol-receptor (●) were subjected to sucrose-density-gradient centrifugation as described in the Experimental section. Before analysis the unbound receptor in the low-salt eluate was first made up to 0.4M-KCl and freed of excess of [^3H]oestradiol by chromatography on Sephadex LH-20. The positions of marker proteins, bovine serum albumin (4.6S) and γ -globulin (7S) are shown by arrows. Samples also contained alkaline phosphatase (6.8S) as an internal marker (Yamamoto & Alberts, 1972). Uterus cytosol [^3H]oestradiol-receptors not subjected to oligo(dT)-cellulose chromatography sedimented in the same position as the unbound receptor (not shown).

technique as a measure of the capacity of the cytosol receptor for conversion from 4S into 5S forms. We first obtained a fraction of cytosol (containing the 4S form) with a decreased ability to convert into the 5S form. This fraction, labelled with [^3H]oestradiol,

Table 4. *Demonstration of an essential activating factor in uterus cytosol*

'Decreased-binding' fractions A and B labelled with 5 nM-[³H]oestradiol were prepared as described in the text. Fresh cytosol was incubated with unlabelled oestradiol (5 nM) to mask the receptors. In a separate experiment the total receptor content of these samples was measured by exclusion on Sephadex LH-20 (*a* and *b*) and the capacity of the unfractionated cytosol to bind to oligo(dT)-cellulose determined (*c*). Three parallel incubations on oligo(dT)-cellulose contained respectively 0.5 ml of decreased-binding fraction (*d*), unlabelled cytosol (0.5 ml) (*e*), and a mixture of 0.5 ml of each (*f*); labelled or unlabelled oestradiol was added as appropriate to give identical concentrations in each sample. Predicted values (*g*) were derived by using the formula given in the text and compensating for the exchange of label: e.g. in Expt. A, predicted result is $860/2030 + 860 \times (470 + 59) + 4$ (calcd. exchange) = 162 fmol.

	Total receptor (fmol of [³ H]oestradiol bound)		Oligo(dT)-cellulose-bound receptor (fmol of [³ H]oestradiol bound)				
	Unlabelled cytosol (<i>a</i>)	Decreased-binding fraction (<i>b</i>)	Unlabelled cytosol (<i>c</i>)	Decreased-binding fraction (<i>d</i>)	Exchange of label (<i>e</i>)	Mixed sample	
						Measured (<i>f</i>)	Predicted (<i>g</i>)
Expt. A	2030	860	470	59	5	103	162
Expt. B							
(1)	1500	370	360	69	5	94	90
(2)	1290	172	310	5	16	56	51

was then mixed with normal uterine cytosol to which unlabelled oestradiol has been added to mask the receptor molecule. The amount of labelled complex from this mixed sample which would bind to oligo(dT)-cellulose (i.e. convert into 5S) was measured and compared with the amount bound from the decreased-binding fraction alone. A net increase in the radioactivity bound to oligo(dT)-cellulose (after taking into account exchange of label during incubation) would imply the existence of an activating factor, a factor which had been largely removed in the preparation of the decreased-binding fraction.

(b) *Predictive approach.* The format of these experiments makes possible a predictive approach, assuming that the 5S receptor complex consists of a 4S receptor plus activator which are non-interacting in normal cytosol not exposed to oligo(dT)-cellulose. If the decreased-binding fraction contains *y* mol of [³H]oestradiol-receptor with a capacity to bind *n* mol of this to oligo(dT)-cellulose, whereas the unfractionated cytosol contains *z* mol of unlabelled oestradiol-receptor, and has a binding capacity of *m* mol, a mixture of the two fractions will have a binding capacity of (*n+m*); the unlabelled complex and the [³H]oestradiol complex will compete for this so that a maximum of [*y*/(*y+z*)]·(*n+m*) mol of labelled complex will bind to oligo(dT)-cellulose. A comparison of the predicted and measured binding capacity requires the following parameters (Table 4): the total amount of oestradiol-receptor in cytosol with (*a*) normal and (*b*) decreased binding capacity; the amount of receptor in each of these two extracts that will bind to oligo(dT)-cellulose (*c* and *d* respectively); (*e*) the amount of [³H]oestradiol exchanged on to the receptor present in untreated cytosol; (*f*) the amount

of [³H]oestradiol binding to oligo(dT)-cellulose when the fractions with normal and decreased binding abilities are mixed.

The results of the mixing experiments (Table 4) with fractions with limited binding capacity show that obvious increases in binding of label occur as a result of mixing together fractions with limited and with normal binding capacities. For example, as might be expected from the receptor-activator model of the 4S conversion, the capacity of the receptor after separation on Sephadex G-100 [Table 4, Expt. B(2)] to bind to oligo(dT)-cellulose was greatly decreased, from a normal value of 310 to 5 fmol. On mixing, the binding capacity increased 11-fold. The observed increases are in good agreement with those predicted for a receptor-activator model and indicate the presence in normal untreated cytosol of an activating factor essential for the binding of receptor to oligo(dT)-cellulose.

Conclusions

Our results show that in most respects the binding of the oestradiol-receptor complex to oligo(dT)-cellulose is analogous to its binding to DNA-cellulose, and the binding of receptor to both is disrupted to a similar extent by changes in the ionic environment. Further, as with DNA-cellulose chromatography, the 4S form is also converted into the 5S form on chromatography on oligo(dT)-cellulose.

This equivalence of oestradiol-receptor binding to either DNA-cellulose or oligo(dT)-cellulose is consistent with reports that the receptor will interact with rat uterine DNA, *Escherichia coli* DNA and poly[d(A-T)] (Yamamoto & Alberts, 1974) to produce

a 5S form. Sluyser *et al.* (1974) have also demonstrated that the 5S receptor will bind to poly(dT), poly(dA)·poly(dT), poly(dG)·poly(dC) and calf thymus DNA. It is noteworthy that polypurine and polypyrimidine sequences are present in eukaryotic DNA (Shenkin & Burdon, 1974; Birnboim & Straus, 1975). We have used oligo(dT)-cellulose in our investigations for the following reasons: (a) this defined sequence of homopolymers covalently bound to cellulose is commercially available in a purified form; (b) its continued use is possible, since, in contrast with DNA-cellulose, alkali and other strong denaturing agents can be used to regenerate oligo(dT)-cellulose free of protein; (c) Yamamoto & Alberts (1974) have pointed out that some preparations of 'purified' DNA contain natural contaminants (e.g. acidic polysaccharides) which can induce considerable aggregation of receptor.

We have found that, under certain conditions, the receptor will bind to both DNA- and oligo(dT)-cellulose even in the absence of oestradiol (Hall *et al.*, 1976). This observation is consistent with other reports of the oestradiol-independent production of a 5S receptor (DeSombre *et al.*, 1972) and binding of receptor to chromatin *in vitro* (Chatkoff *et al.*, 1974).

That the 4S form has acquired an additional component in changing into the 5S form cannot be established solely on the basis of separation of these forms of the receptor on gel chromatography (Fig. 3) or by sucrose-density-gradient centrifugation (Yamamoto, 1974), since the separation may be influenced by conformational changes. However, several additional lines of evidence from our work establish the involvement of a cytosol factor in the 4S into 5S conversion. Even after exhaustive chromatography of labelled receptor on the oligo(dT)-cellulose, a substantial proportion remains unbound, strongly suggesting the presence of a limiting factor required for binding. The cytosol receptor obtained by separation on Sephadex G-100 has a decreased ability to bind to oligo(dT)-cellulose, explainable by the partial separation by chromatography of an essential factor. The experiments described in Table 4 can only be interpreted in terms of the essential mediation of a factor in the conversion of the 4S into 5S complex on binding to oligo(dT)-cellulose. The design of the experiments is based on looking for an increase in the binding of labelled receptor from a cytosol sample with limited binding capacity, by allowing it to react with normal components of cytosol (in which the receptor is masked) during chromatography on oligo(dT)-cellulose. Such an increase has been observed. We conclude that a factor in the cytosol is freely available for interactions with oestradiol receptor to produce a 5S form of the receptor, and that this activating factor is probably also responsible *in vivo* for the production of the nuclear 5S receptor.

The existence of such a factor provides an explanation for the numerous reports that nuclear binding of oestradiol is regulated not merely by the availability of the cytoplasmic receptors. In oestradiol-independent mouse mammary tumours high concentrations of cytoplasmic receptors, similar to those from the uterus, are not accompanied by significant nuclear binding of oestradiol. Thus 0.4M-KCl extracts negligible amounts of receptor from tumour nuclei, whereas it extracts large amounts of the 5S nuclear receptor from uteri (Shyamala, 1972). In the female rat hypothalamus (which in the adult specifically binds oestradiol; McEwen *et al.*, 1974; Pfaff *et al.*, 1974), although adult concentrations of the specific cytoplasmic receptors are already present at 20–26 days after birth, adult values for nuclear binding are attained only on the 26th day. This represented a six-fold increase in nuclear binding without a comparable increase in cytoplasmic receptor concentrations (Plapinger & McEwen, 1973). Clearly some other factor(s) mediates the binding of the receptor to the nucleus. We have found (J. White & L. Lim, unpublished work) that the results of oligo(dT)-cellulose binding of hypothalamic cytoplasmic oestradiol receptors, isolated from the developing rat brain, are in very close agreement with the values of nuclear binding of oestradiol *in vivo* obtained by Plapinger & McEwen (1973), showing a sudden increment in the fourth week *post partum*. Oligo(dT)-cellulose chromatography therefore provides a suitable method for investigating developmental factor(s) responsible for tissue-specific response to oestradiol.

It is also known that treatment of neonatal female rats with testosterone (i.e. 'androgenization') results in the loss at adulthood of the characteristic feminine pattern of cyclic release of gonadotropin hormones which regulate the oestrous cycle; these rats instead display a tonic release of the hormones characteristic of the male (Harris, 1964; Gorski, 1971). Vertes & King (1971) found that the anterior hypothalamus of these 'androgenized' females, 28 days after birth, showed decreased nuclear binding of oestradiol, despite having higher than normal concentrations of the cytoplasmic receptor. A corresponding decrease in translocation of the oestradiol receptor into uterine nuclei was also reported to occur in the 'androgenized' rat at 60 days (Lobl, 1975).

It is considered that conversion of the 4S form of the oestradiol receptor into the 5S form is not only necessary for translocation into the nucleus, but that it is only the 5S form that can stimulate RNA synthesis (O'Malley & Means, 1974). It is an intriguing possibility that the activating factor itself can stimulate transcription and that the oestradiol receptor serves to introduce the factor into the nucleus. It remains to be established whether the activating factor is common to all target tissues and whether it can determine the specific hormonal response to oestra-

diol while in the nucleus. In this context, cytoplasmic low-molecular-weight proteins have been shown to stimulate specifically chromatin-bound RNA polymerase (Jacob, 1973). This observation may be of particular relevance to steroid-dependent development of target tissues like uterus and hypothalamus, since it has been demonstrated that the scheduled appearance of a cytoplasmic protein in embryonic tissue, which regulated nuclear transcription, was essential for continued successful development (Brothers, 1976).

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