Oestrogen Receptor of Calf Mammary Gland

PURIFICATION BY USE OF SODIUM BROMIDE AND HEPARIN-SEPHAROSE

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1. Calf mammary-gland cytosol apparently has a single oestrogen receptor capable of auto- and/or hetero-association of varying complexity. Computation of the dissociation constant for oestradiol-17 β gives $K_d = 0.5$ nM. The number of binding sites is 40 fmol/mg of cytosol protein. The oestrogen receptor in the presence of NaBr, a chaotropic salt that inhibits the interaction of receptor with other cytosol components, sediments through sucrose density gradients as a single sharp peak at 4S, and it has a Stokes radius of 3.4 nm measured by gel filtration. 2. A large-scale purification procedure of the calf mammary-gland oestrogen receptor based on the inhibition of receptor aggregation by NaBr and interaction with heparin-Sepharose is reported. The receptor is purified more than 1500-fold over that in the 27000g supernatant of the homogenate, with a 30% yield. In 'low-salt' buffer the purified receptor sediments through sucrose gradients at 4S and the Stokes radius, measured by gel filtration in the presence of heparin, is 3.4 nm. The mol.wt. computed from these values is about 60000, and the frictional ratio is 1.3.

Oestrogen receptors have been found in mammary glands and tumours of various animals (Bresciani & Puca, 1965; Shyamala & Nandi, 1972; Gardner & Wittliff, 1973; Auricchio *et al.*, 1976) and of humans (Korenman & Dukes, 1970). Nevertheless, reports on molecular characterization and purification of mammary oestrogen receptors are rare, whereas uterine oestrogen receptors have been extensively characterized and purified (De Sombre & Gorrell, 1975; Molinari *et al.*, 1977; Bresciani *et al.*, 1978). This difference is, at least in part, due to a stronger tendency towards aggregation and to a lower concentration of receptor in the mammary gland (Auricchio *et al.*, 1978a). Giving adequate consideration to these two facts we have developed a large-scale method of purification of oestrogen receptor from mammary gland.

It has been recently reported that NaBr, a chaotropic salt (Hatefi & Hanstein, 1969), is a powerful inhibitor of the interaction between oestrogen receptor and other components in cytosol of mouse mammary gland (Auricchio *et al.*, 1978a). Furthermore cytosol oestrogen receptor from mouse mammary gland, which is similar to uterine receptor (Molinari *et al.*, 1977), interacts with heparin covalently bound to Sepharose (Auricchio *et al.*, 1978b).

A large amount of tissue for the purification of mammary-gland cytosol oestrogen receptor can be obtained from calf gland. This receptor, with regard

to NaBr and heparin-Sepharose, behaves like the mouse mammary-gland oestrogen receptor (Auricchio *et al.*, 1978a,b). We have characterized and purified the receptor utilizing this behaviour. The results reported here represent a significant progress in isolating large amounts of mammary-gland oestrogen receptor, an important step in the study of the mechanism by which oestradiol-17 β controls normal and neoplastic growth of mammary gland.

Experimental

Materials

Reagent-grade materials were used in all experiments. NaBr was from C. Erba, Milano, Italy. Horse muscle myoglobin, bovine plasma albumin and bovine immunoglobulin G were from Serva, Heidelberg, Germany. Dithiothreitol was from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephadex G-25 (coarse grade) and G-200, Blue Dextran 2000 and Sepharose 4B were from Pharmacia Fine Chemicals, Uppsala, Sweden. [2,4,6,7- 3 H]Oestradiol-17 β (85 Ci/mmol) and [3 H]leucine (30 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Heparin (activity 140 i.u./mg) was from Fluka A.G., Buchs, Switzerland.

Buffer

The following buffer solution was used: 10 mM-

Tris/HCl, pH 7.4, containing 1 mM-dithiothreitol (TD buffer).

Sucrose-density-gradient centrifugation

Sucrose gradients (5–20%, w/v; total volume 4.2 ml) were used. Centrifugation was carried out in the SW-60 rotor of a Beckman Spinco L2-65 ultracentrifuge at 2°C for 14 h at 45 000 rev./min. The sample was layered on the gradient in a volume of 0.3 ml. Bovine plasma albumin (4.45 S) was used as a reference for computing the sedimentation coefficients of the receptor.

Gel filtration

Standard Pharmacia columns K 16–40 and K 50–100 with upward flow adaptor were used. The upward flow was generated by peristaltic pump. The void volumes (V_0) of the Sephadex G-200 columns were measured by using Blue Dextran 2000, and the total volume (V_t) by using [^3H]leucine. Filtrations were carried out at 4°C. The following reference proteins were used [molecular weights and Stokes radii, given in parentheses, are from Andrews (1970)]: myoglobin (17 800, 2.07 nm), plasma albumin (67 000, 3.55 nm), immunoglobulin G (160 000, 5.3 nm).

Oestrogen-binding activity

The assay was performed with separation of bound from free [^3H]oestradiol-17 β by filtration through standard Sephadex G-25 columns (1.4 cm $\times$ 13 cm) (Auricchio *et al.*, 1976).

Radioactivity assay

Radioactivity was assayed as previously described (Auricchio *et al.*, 1976).

Heparin–Sephacrose preparation

Heparin (2 mg/ml of 0.1 M-NaHCO₃, pH 8.3) was coupled to CNBr-activated Sepharose 4B (300 mg of CNBr/ml of packed gel) as described by Cuatrecasas (1970): 1 g of activated Sepharose was used per ml of heparin solution.

Preparation of cytosol oestrogen receptor

Calf mammary glands were taken from the local slaughterhouse, soon after the animals had been killed; they were put on ice and divided into pieces. The fat portions were roughly eliminated, and the pieces either immediately used or frozen at –20°C. The tissue was minced with scissors, mixed with 2 vol. of ice-cold TD buffer and homogenized with an Ultra-Turrax homogenizer in a glass tube kept in crushed ice. Four bursts at 70 V lasting 15 s each were applied at 60 s intervals. The homogenate was filtered through a single layer of cheesecloth and centrifuged for 45 min at 45 000 rev./min at 2°C in the Ti 50 rotor of a Beckman Spinco L2-65 ultracentrifuge.

Cytosol was radioactively labelled by incubation with 2 nM-[^3H]oestradiol-17 β for 12 h at 4°C.

Specific oestradiol binding was carried out in parallel trials by decreasing the specific radioactivity of [^3H]oestradiol by addition of a 300-fold excess of unlabelled oestradiol-17 β . Proteins were assayed as described by Lowry *et al.* (1951), with bovine plasma albumin as standard, unless otherwise specified.

Results

Specificity of oestradiol-17 β binding

Fig. 1 shows the sedimentation pattern of cytosol incubated with [^3H]oestradiol-17 β and centrifuged through 'low-salt' sucrose density gradients. The material with steroid-binding activity sediments heterogeneously, with a major peak in the 4S region, whereas material containing all remaining activity sediments in the lower part of the gradient. When cytosol is labelled in the presence of an excess of unlabelled oestradiol-17 β or diethylstilboestrol, no [^3H]oestradiol-17 β -binding activity is detectable.

Fig. 1. Sucrose-density-gradient centrifugation of [^3H]oestradiol-labelled cytosol in Tris buffer

Cytosol was labelled by 24 h incubation with high-specific-radioactivity (85 Ci/mmol) 2 nM-[^3H]oestradiol-17 β in the absence (●) or presence of a 10-fold excess of either oestradiol-17 β (○) or diethylstilboestrol (Δ) and treated with charcoal before layering on the 5–20% (w/v) sucrose gradients. Centrifugation was in a Beckman L2-65 ultracentrifuge, rotor SW-60, at 45 000 rev./min for 14 h at 2°C; fraction volume was 0.2 ml. The arrow indicates the peak of the reference protein, bovine plasma albumin (4.45 S).

Fig. 2. Measurement of the affinity of the interaction between oestradiol and mammary receptor in Tris buffer

Cytosol samples (0.30 ml) were incubated at 4°C for 24 h with various amounts (5.8–2960 fmol) of [3 H]oestradiol of high (85 Ci/mmol) or low (0.28 Ci/mmol) specific radioactivity. The final volume of each incubation mixture was 0.5 ml. After incubation, separation of bound from free oestradiol was obtained by gel filtration at 4°C on Sephadex G-25 columns. When low-specific-radioactivity [3 H]oestradiol was used, no change in the bound/free ratio was observed (as specific binding). Before plotting the data, the aspecifically bound portion of radioactivity was subtracted from the results obtained by using high-specific-radioactivity [3 H]oestradiol (Scatchard, 1949). K_d value is 0.5 nM. The number of binding sites (abscissa intercept) is 40 fmol/mg of cytosol protein.

Fig. 2 illustrates an experiment in which equal amounts of cytosol were incubated with different amounts of [3 H]oestradiol-17 β in the absence and presence of an excess of the unlabelled steroid. Bound hormone was separated from free hormone by Sephadex G-25 filtration, and the results were plotted by the method of Scatchard (1949). There is a single class of specific binding sites for oestradiol-17 β . Computation of dissociation constant (K_d) gives $K_d = 0.5$ nM, and computation of the number of binding sites gives 40 fmol/mg of cytosol protein. These values are similar to those of cytosol from mouse mammary gland (Auricchio *et al.*, 1976). The

specificity of [3 H]oestradiol binding was studied in greater detail and results are reported in Table 1. Of all the hormones tested in addition to oestradiol-17 β and diethylstilboestrol, whose effect on [3 H]oestradiol binding was previously followed by sucrose-density-gradient centrifugation, only oestrone and oestriol displace [3 H]oestradiol.

Sedimentation and gel filtration of oestradiol-17 β receptor in the presence of NaBr

Fig. 3 compares the results of experiments where equal amounts of cytosol after labelling with [3 H]oestradiol were either centrifuged through sucrose gradients or, after the addition of 1 M-NaBr, were centrifuged through gradients containing 1 M-NaBr. The specific binding activity heterogeneously sedimenting in the absence of NaBr migrates in the presence of the chaotropic salt as a very sharp single peak in the 4S region. The slight increase in binding activity observed in the presence of NaBr could be due to better solubilization of the receptor by the salt. The receptor is eluted, from a calibrated 108 ml Sephadex G-200 column equilibrated with TD buffer plus 1 M-NaBr, as a single protein with a Stokes radius of 3.4 nm, measured as described by Porath (1963) (not shown).

Purification of the receptor

About 2 kg of calf mammary gland was put on ice immediately after the animals were killed, and processed 1 h later. After elimination of fat portions, the tissue was minced, added to 2 vol. of ice-cold TD buffer and homogenized in the cold with an Ultra-Turrax homogenizer (10 bursts at 120 V lasting 15 s each were applied at 1 min intervals). The homogen-

Table 1. Specific oestradiol-17 β -binding activity of cytosol from calf mammary gland in the presence of other steroid hormones

Compounds were in excess of the [3 H]oestradiol by (a) 10-fold and (b) 100-fold. The specific binding activity of the control is computed from the difference between binding of [3 H]oestradiol alone and in the presence of a 300-fold excess of unlabelled oestradiol-17 β .

	Residual binding (% of control)	
	(a)	(b)
Oestradiol-17 β	26	0
Oestrone	35	15
Oestriol	25	5
Diethylstilboestrol	20	5
Deoxycorticosterone	96	93
Progesterone	97	97
Testosterone	93	100
Dihydrotestosterone	90	95

Fig. 3. Sucrose-density-gradient centrifugation of [^3H]oestradiol-labelled mammary-gland cytosol in TD buffer with and without NaBr

Cytosol was labelled by incubation with high- (85 Ci/mmol, ●) or low-specific-radioactivity (0.85 Ci/mmol, ○) [^3H]oestradiol and treated with charcoal before being layered on the 5–20% (w/v) sucrose gradients in (a) TD buffer and (b) TD buffer containing 1 M-NaBr. Centrifugation was in a Beckman L2-65 ultracentrifuge (rotor SW-60) at 45000 rev./min at 2°C for 14 h; fraction volume was 0.2 ml. Arrows indicate the peak of the reference protein, bovine plasma albumin (4.45 S).

ate was centrifuged for 10 min at 16300g at 2°C in the G.S.A. rotor of a Sorvall RC2-B centrifuge. The supernatant was centrifuged at 27000g for 60 min in the SS 34 rotor of the same centrifuge. This supernatant was labelled with 2 nM [^3H]oestradiol, and 30 min later 1 M-NaBr was added. The supernatant was left overnight at 4°C. $(\text{NH}_4)_2\text{SO}_4$ was then added to 40% saturation; the pellet collected by centrifugation at 27000g for 15 min was dissolved in 60 ml of TD buffer containing 1 M-NaBr and filtered through a 2000 ml Sephadex G-200 column equilibrated with TD buffer containing 1 M-NaBr (Fig. 4). The eluate was pooled between 1078 and 1408 ml, $(\text{NH}_4)_2\text{SO}_4$ was added to 40% saturation and centrifuged as described above. The pellet was dissolved in 35 ml of buffer, 1 nmol of [^3H]oestradiol-17 β and 40 ml of packed heparin-Sepharose were added and the mixture was dialysed overnight against 2 litres of buffer containing 5 mM-MgCl₂. When conductivity measurement showed that NaBr concentration was

less than 0.025 M, heparin-Sepharose was withdrawn from the dialysis bag, poured into a 50 ml syringe and extensively washed with buffer containing 150 mM-KCl and 5 mM-MgCl₂ until neither radioactivity nor protein were measurable in the elution buffer. The receptor was eluted from heparin-Sepharose with buffer containing 3 mg of heparin/ml (Fig. 5a) and stored at 4°C. Table 2 summarizes the results of the entire purification procedure. The final purification is more than 1500-fold over the 27000g supernatant; since the amounts of receptor in the homogenate and in the supernatant are equal, the purification over the homogenate is 20000-fold. The final yield is 30%. The purified receptor sediments in 'low-salt' buffer at 4S and is eluted from the calibrated 108 ml Sephadex G-200 column in the presence of 3 mg of heparin/ml as a single protein with a Stokes radius of 3.4 nm, measured as described by Porath (1963), and a mol.wt. of 60000 measured as described by Andrews (1964) (Figs. 5b and 5c).

Fig. 4. Gel filtration on Sephadex G-200 columns in TD buffer of $(\text{NH}_4)_2\text{SO}_4$ precipitate of $[^3\text{H}]$ oestradiol-labelled 27000g supernatant

27000g supernatant was labelled with $[^3\text{H}]$ oestradiol (85 Ci/mmol), 1 M-NaBr was added, and precipitation was performed with 40%-satd. $(\text{NH}_4)_2\text{SO}_4$. The pellet was dissolved in TD buffer containing 1 M-NaBr and filtered through a 2000 ml Sephadex G-200 column equilibrated with TD buffer containing 1 M-NaBr. Arrow indicates the peak of Blue Dextran (V_0). The effluent was monitored for radioactivity (●) and for A_{280} (○).

Table 2. Summary of the purification of oestrogen receptor from calf mammary gland

Step	Volume (ml)	Total protein (mg)	$10^{-6} \times$ Total receptor (d.p.m.)	$10^{-3} \times$ Specific radioactivity (d.p.m./mg of protein)	Purification factor	Yield
Homogenate	1400	37100	—	—	—	—
16300g supernatant	670	3350	—	—	—	—
27000g supernatant	610	2867	21.18	7.4	1	100
First $(\text{NH}_4)_2\text{SO}_4$ fraction	59	1180	21.18	18.0	2.4	100
Sephadex G-200	330	181	14.18	78.3	10.6	67
Second $(\text{NH}_4)_2\text{SO}_4$ fraction	35	124*	12.48	100.7	13.6	59
Heparin-Sephadex	30	0.54*	6.26	11592	1566	30

* Protein was determined by measurement of A_{280} and A_{260} .

Discussion

The specific $[^3\text{H}]$ oestradiol-17 β -binding activity of calf mammary-gland cytosol sediments through a 'low-salt' sucrose density gradient with a very heterogeneous pattern, quite different from that of mouse mammary-gland oestradiol receptor, which sediments as a single sharp peak at 7S (Auricchio *et al.*, 1978a,b). However, it is unlikely that the observed heterogeneity is partially or completely due to the presence of molecules binding oestradiol-17 β as

distinct from oestradiol receptor. The complete disappearance of $[^3\text{H}]$ oestradiol-17 β -binding activity in the presence of a low concentration of diethylstilboestrol shown by both centrifugation through sucrose gradients (Fig. 1) and specific binding assay (Table 1) does not support the presence of α -foetoprotein (Soloff *et al.*, 1972; Raynaud *et al.*, 1971; Radanyi *et al.*, 1977). Furthermore, since the binding is unaffected by testosterone and dihydrotestosterone (Table 1), the presence of a sex-steroid-binding globulin should also be excluded. The possibility that

Fig. 5. Interaction of cytosol oestrogen receptor with heparin-Sepharose

The receptor eluted from the Sephadex G-200 column, see Fig. 4, was precipitated with 40%-satd. $(\text{NH}_4)_2\text{SO}_4$, suspended in buffer and dialysed against TD buffer containing 5 mM-MgCl₂ in a bag containing heparin-Sepharose. When the NaBr concentration in the bag decreased to 0.025 M, the heparin-Sepharose was removed from the bag, poured into a 50 ml column and extensively washed with TD buffer containing 5 mM-MgCl₂ and 150 mM-KCl until neither radioactivity nor A_{280} was measurable in the washing buffer. At this point, binding activity was eluted from the gel with TD buffer containing 3 mg of heparin/ml and 10 ml fractions were collected (a). Then 0.3 ml of the eluted sample containing the binding activity was centrifuged through 'low-salt' sucrose gradients (b). Centrifugation of 5–20% (w/v) sucrose gradients was in a Beckman L2-65 ultracentrifuge (rotor SW-60) at 45000 rev./min at 2°C for 14 h; fraction volume was 0.2 ml. The arrow indicates the peak of the reference protein, bovine plasma albumin (4.45S). (c) Gel filtration of the receptor eluted from heparin-Sepharose through a 108 ml Sephadex G-200 column in TD buffer containing 3 mg of heparin/ml. The void volume, measured by elution of Blue Dextran, is at 35 ml. The effluent was monitored for radioactivity (●).

the heterogeneity of sedimentation is due to the presence of several oestradiol receptors is not, in our opinion, convincing, as the steep linear slope of the Scatchard plot (Fig. 2) shows that there is a single class of binding sites with high affinity for oestradiol, and also in the presence of NaBr all the receptor sediments as a sharp peak at 4S (Fig. 3) and is eluted from a Sephadex column as a single protein. Therefore we think that the sedimentation heterogeneity is due to auto- and/or hetero-association, of varying complexity, of a 4S receptor with other cytosol components. Since NaBr is able to inhibit the interaction of the oestrogen receptor with other cytosol components (Auricchio *et al.*, 1978a), it seems, from the sedimentation coefficient value and from the Stokes radius measured by gel filtration, realistic to calculate the mol. wt. to be about 60000 and the frictional ratio of the NaBr-dissociated calf receptor to be f/f_0 1.3 (Siegel & Monty, 1966). These values are similar to those of mouse oestrogen mammary-gland receptor in NaBr (Auricchio *et al.*, 1978a), showing the similarity of mammary-gland cytosol oestrogen receptors from different animals.

Mouse mammary-gland oestrogen receptor directly interacts with heparin as shown by the tight binding

of this receptor to heparin-Sepharose (Auricchio *et al.*, 1978b). Also calf mammary-gland oestrogen receptor binds to heparin-Sepharose. Therefore this affinity chromatography is a powerful and general tool for cytosol oestrogen receptor purification.

Our final purification procedure initially utilizes the prevention of receptor aggregation by NaBr. In fact, in the presence of NaBr the receptor precipitated by $(\text{NH}_4)_2\text{SO}_4$ still sediments through NaBr/sucrose at 4S (not shown), and a major part of it is largely included through a Sephadex G-200 column equilibrated with TD buffer containing 1 M-NaBr (Fig. 4). Before heparin-Sepharose chromatography the NaBr concentration should be decreased, since the receptor does not interact with heparin-Sepharose in the presence of high NaCl concentration (Molinari *et al.*, 1977). It is also known that at this stage of purification the receptor greatly aggregates shortly after complete removal of the chaotropic salt (Auricchio *et al.*, 1978a). Therefore, after gel filtration in NaBr, the receptor is dialysed against buffer, keeping heparin-Sepharose inside the dialysis bag; when the NaBr concentration decreases, the receptor binds to heparin-Sepharose. Such an interaction and the following elution with heparin prevent wide aggrega-

tion of the receptor; the purified receptor sediments as an homogeneous peak of radioactivity at 4S in a 'low-salt' gradient and is eluted from a Sephadex G-200 column as a single sharp peak, corresponding to a protein with a Stokes radius of 3.4nm (Porath, 1963).

These values are identical with those for the crude cytosol receptor in the presence of NaBr, suggesting that the purification procedure eliminates most of the components that interact in the cytosol with the receptor, components responsible to some extent for the heterogeneity observed in the experiment presented in Fig. 1. From combination of these values the mol.-wt. can be computed to be 60000, and the frictional ratio is 1.3 (Siegel & Monty, 1966). Comparison of these data with those on the isolated calf uterine receptor (Molinari *et al.*, 1977) shows the striking similarity of mammary and uterine oestrogen receptors.

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