Oestrogen Receptor of Mammary Gland

INHIBITION OF AGGREGATION AND CHARACTERIZATION OF RECEPTOR FROM LACTATING GLAND IN THE PRESENCE OF SODIUM BROMIDE

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1. When NaBr, a chaotropic salt, is added, in concentrations ranging from 0.5 M to 2 M, to low-salt mammary cytosol, (i) age-dependent aggregation of oestrogen receptor is inhibited, (ii) the receptor sediments as a sharp peak at 4.2 S on sucrose-gradient centrifugation, with complete disappearance of heavier forms, and (iii) on gel filtration with Sephadex G-200, the receptor is included in the gel matrix. On a calibrated column, the receptor has a Stokes radius of 3.7 nm ($\pm 6\%$). 2. Because NaBr inhibits interaction of receptor with other components of cytosol, the values of the sedimentation coefficient, measured by sucrose-gradient sedimentation, and of the Stokes radius, measured by gel filtration, can be accepted with confidence. From these values, it can be computed that the oestrogen-receptor form in NaBr has a mol.wt. of 64000, with a frictional ratio of 1.4. 3. Also, inhibition of aggregation by NaBr allows a 30–90-fold purification of oestrogen receptor. Analysis of this partially purified receptor by sucrose-gradient sedimentation and gel filtration in NaBr gives the same results as for receptor in crude cytosol. On electrofocusing on a pH 5–8 gradient, the partially purified oestrogen receptor focuses at pH 6.2. On removal of NaBr, receptor aggregates even in this partially purified state. It seems likely that at the protein and ionic concentrations of cytoplasm *in vivo*, the 64000-mol.wt. receptor form is part of higher states of self- and/or hetero-association with other cytoplasmic components. 4. NaBr up to a concentration of 2 M does not inhibit binding of oestrogen by receptor, nor does it decrease the affinity of the interaction ($K_D \approx 8.9 \times 10^{-10}$ M). The total number of binding sites in cytosol, however, decreases by approx. 10%, but this decrease may actually be the result of elimination of lower-affinity binding by non-receptor components of cytosol. 5. NaSCN, another chaotropic salt, was also tested but gave less satisfactory results with the mammary cytosol than with uterine cytosol. EDTA was omitted from the buffers because it favours aggregation of mammary oestrogen receptor. KCl (0.4 M), sucrose (15%) and ZnSO₄ (3 mM) did not prevent aggregation of receptor.

Since the demonstration that the mammary gland of C3H-strain mice binds oestradiol-17 β *in vivo* with high affinity (Bresciani & Puca, 1965; Puca & Bresciani, 1969), specific receptors for oestrogen have been shown to exist in the mammary gland of mice (Shyamala & Nandi, 1972; Auricchio *et al.*, 1976) and rats (Gardner & Wittliff, 1973). In normal human breast, oestrogen receptors are barely detectable (Hänel & Twaddle, 1973). Oestrogen receptors have also been found in variable concentrations in most breast cancers of experimental animals (Bresciani & Puca, 1965; Mobbs, 1966) and of humans (Korenman & Dukes, 1970; Johanson *et al.*, 1970). The main aim of the tumour studies has been the measurement of concentrations of receptor in an attempt, only partially successful at present, to find a correlation

with tumour responsiveness to endocrine therapy (McGuire *et al.*, 1975).

Progress in the understanding of how the oestrogen receptor is implicated in the mechanism of oestrogen action on mammary tissue is likely to be furthered by characterization and purification of this protein. Once this goal is achieved, it should be possible to clarify the nature of the molecular changes of the cytoplasmic receptor after it has interacted with the hormone, and to understand how these changes confer on the receptor the ability to transfer to the nucleus and interact with chromatin. Also, it will be possible to isolate other components of cytoplasm and nucleus with affinity for the receptor–oestrogen complex, and thus possibly elucidate the chain of molecular events by which the informational content

of the oestrogen molecule is delivered to the nucleus and regulates gene expression responsible for oestrogen-induced cellular changes.

Studies on molecular characterization and purification of mammary receptor are still scarce. Mostly, mammary oestrogen receptor has been studied by sucrose-gradient centrifugation. Mammary receptor from both mouse and rat glands sediments at 8–10S at low-salt concentrations (10mM) and at about 4S when 0.4 M-KCl is present (Shyamala & Nandi, 1972; Gardner & Wittliff, 1973; Auricchio *et al.*, 1976). Also, after injection *in vivo* of [³H]oestradiol-17 β , a radioactive receptor–oestradiol complex of approx. 4–5S can be extracted from the nuclear pellet of glands from pregnant and lactating mice (Gardner & Wittliff, 1973). From studies with chelating agents, it has been suggested that the mammary oestrogen receptor is a metalloprotein (Shyamala, 1975).

The limited work with mammary oestrogen receptor is mainly due to technical difficulties when working with the mammary gland. Virgin glands have very little parenchymal tissue. Pregnant or lactating mammary glands, on the other hand, are rich in fat, and although total receptor content is increased in these glands, on a weight basis it is still only about one-third of the uterine content (Auricchio *et al.*, 1976). Also, and perhaps most important, the oestrogen receptor of mammary glands is even more prone than uterine receptor to rapid and virtually irreversible aggregation after tissue homogenization.

The findings described by Sica *et al.* (1976) that NaSCN, a chaotropic salt, can inhibit age-dependent aggregation and dissociates uterine oestrogen receptor suggested to us that this salt could also be utilized in studies of mammary receptor. However, NaSCN was found to be detrimental to mammary receptor. Another chaotropic salt, NaBr, proved instead to be compatible with a stable receptor–oestrogen complex even at concentrations as high as 2M, and to inhibit age-dependent aggregation of receptor. In the presence of NaBr (0.5–2M), the receptor sediments sharply at 4.2S on sucrose gradients and is included in the Sephadex G-200 matrix. Because NaBr inhibits receptor interactions with other components of cytosol, meaningful calculations of molecular parameters of the cytosol receptor can be carried out. Also, a 30–90-fold purification of mammary receptor could be achieved by avoiding aggregation of receptor during the purification procedures by means of NaBr.

Experimental

Materials

Reagent-grade materials were used in all experiments. NaSCN and NaBr were from C. Erba, Milano, Italy. Horse heart cytochrome *c*, horse

muscle myoglobin, ovalbumin, bovine plasma albumin and aldolase were from Serva (Entwicklungslabor), Heidelberg, Germany. Dithiothreitol was from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephadex G-25 (coarse grade) and G-200 and Blue Dextran 2000 were from Pharmacia Fine Chemicals, Uppsala, Sweden. [2,4,6,7-³H]Oestradiol-17 β (85 Ci/mmol) and [³H]leucine (30 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Buffers

The following buffer solutions were used: 10mM-Tris/HCl, pH 7.4 (T-buffer); 10mM-Tris/HCl, pH 7.4, containing 1mM-dithiothreitol (TD-buffer); 10mM-Tris/HCl, pH 7.4, containing 1mM-EDTA and 1mM-dithiothreitol (TED-buffer).

Sucrose-gradient centrifugation

Sucrose gradients (5–20%, w/v; total vol. 4.2ml) were used. Centrifugation was carried out in an L2-65 Beckman–Spinco ultracentrifuge at 2°C for 12h at 55000 rev./min with an SW-60 rotor. The sample was layered on the gradient in a volume of 0.3ml. Bovine plasma albumin (4.45S) was used as a reference for computing the sedimentation coefficients of sample proteins. Preliminary tests on the linearity of gradients were carried out as previously described (Puca *et al.*, 1971).

Gel filtration

Standard Pharmacia columns K16/40 and K26/40 with upward-flow adaptors were used. The upward flows were generated by peristaltic pumps. The void volumes (V_0) of the columns were measured by using Blue Dextran 2000, and the total volumes (V_t) by using [³H]leucine. All operations were carried out at 4°C. Calibration of columns was carried out with the following reference proteins (Stokes radius in parentheses): horse heart cytochrome *c* (1.64 nm), horse muscle myoglobin (2.07 nm), ovalbumin (2.73 nm), bovine plasma albumin (3.55 nm), aldolase (4.50 nm). Stokes radii are from studies by Andrews (1970).

Electrofocusing

The LKB standard (110ml) electrofocusing column, obtained from LKB-Producter, Stockholm, Sweden, and equipped with a double cooling jacket, was used. A pH 5–8 gradient was obtained by using 2% Ampholine (LKB) in a 5–50% (w/v) sucrose gradient. For further details, see Puca *et al.* (1975).

Oestrogen-binding-activity assay

The assay was performed as described previously (Auricchio *et al.*, 1976) with separation of bound from free [^3H]oestradiol-17 β by filtration through standard Sephadex G-25 columns (20m; 1.4cm $\times$ 13cm). The only modification was that in the present paper T-buffer, pH7.4, replaced the TE-buffer, pH7.4, used previously.

Protein assay

This assay was performed by the Eggstein & Kreutz (1955) modification of the procedure of Lowry *et al.* (1951). Bovine plasma albumin was used as reference.

Radioactivity assay

Radioactivity was measured in a Nuclear-Chicago (Des Plaines, IL, U.S.A.) liquid-scintillation counter, by standard procedures. Either a mixture containing 2.66g of 2,5-diphenyloxazole/l, 33mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l and 30% Triton X-100 in toluene, or Instagel (Packard Instrument Co., Downers Grove, IL, U.S.A.) was used, depending on the volume of the sample. Details of the assay were described previously (Auricchio *et al.*, 1976).

Preparation of cytosol

Mammary glands from 7–14-days-lactating Swiss mice were used. The lymph nodes were removed and the glands, after mincing with scissors, were mixed with 2 vol. of ice-cold buffer (TD-buffer, pH7.4, unless otherwise specified in the text) and homogenized, in a glass tube kept in crushed ice, by using an Ultra-Turrax homogenizer (Janke and Kunkel). Four bursts at 70 V, lasting 5 s each, were applied at 60 s intervals. The homogenate was filtered through a single layer of cheesecloth and centrifuged at 45000 rev./min for 30 min in a Beckman-Spinco L2-65 ultracentrifuge at 2°C by using the Ti50 rotor. If not otherwise specified, cytosol was labelled by incubation with 5 nM-[^3H]oestradiol for 30 min at 4°C. Checks of specificity of binding were carried out in parallel trials, by decreasing the specific radioactivity of [^3H]oestradiol by addition of a 150-fold excess of unlabelled oestradiol-17 β . Cytosol prepared as described above contains 6–7 mg of protein/ml.

Results

Sedimentation on sucrose gradients in the presence of NaSCN

Previous work (Sica *et al.*, 1976) has shown that when 0.1 M-NaSCN is added to calf uterus cytosol in low-salt buffers, (i) there is no destruction of the

oestrogen-receptor complex, (ii) age-dependent aggregation of the complex is inhibited, and (iii) instead of sedimenting at 8–9 S, the receptor sediments as a sharp peak at 4.1 S on sucrose gradients, independent of KCl or protein concentration in the cytosol.

However, when 0.1 M-NaSCN was added to mammary cytosol, the results were somewhat different. Fig. 1 shows a comparison of results when [^3H]oestradiol-labelled mammary cytosol was prepared and centrifuged on sucrose gradients in buffer solutions of different composition. In TED-buffer (Fig. 1a) or TD-buffer (Fig. 1b), the receptor sedimented at about 8 S or 7 S, respectively. When 0.1 M-NaSCN was added to the TD-buffer (Fig. 1c) the receptor sedimented more slowly, as a broad band in the 7–5 S region. Dilution of cytosol to lower protein concentration (2–3 mg/ml) or use of TED-buffer, pH7.4, did not modify the effect of NaSCN on the sedimentation pattern of receptor from that illustrated in Fig. 1(c). If, however, the concentration of NaSCN was increased to 0.5 M, the receptor-oestrogen complex was destroyed during centrifugation (results not shown).

The sedimentation patterns in Fig. 1, which are typical of patterns repeated many times, thus clearly show that 0.1 M-NaSCN, although able to favour (in

Fig. 1. Sucrose-gradient centrifugation of [^3H]oestradiol-labelled cytosol in Tris buffers with or without NaSCN. Cytosol was labelled by incubation with high-(85 Ci/mmol, ●) or low-specific-radioactivity (0.57 Ci/mmol, ○) [^3H]oestradiol before layering on the 5–20% (w/v) sucrose gradients in (a) TED-buffer, (b) TD-buffer, (c) TD-buffer containing 0.1 M-NaSCN. Centrifugation was in a Beckman L2-65 ultracentrifuge, rotor SW-60, at 55000 rev./min for 12 h at 2°C. Fractions of volume 0.2 ml were collected. Arrows indicate peak of the reference protein, bovine plasma albumin (4.45 S). To obtain the low-specific-radioactivity [^3H]oestradiol, unlabelled oestradiol was used to dilute the labelled compound 150-fold.

Fig. 2. Sucrose-gradient centrifugation of [^3H]oestradiol-labelled mammary cytosol in Tris buffers with or without NaBr

Cytosol was labelled by incubation with high- (85 Ci/mmol, ●) or low-specific-radioactivity (0.57 Ci/mmol, ○) [^3H]oestradiol before layering on the 5–20% (w/v) sucrose gradients in (a) TD-buffer, (b) TD-buffer containing 0.5M-NaBr, (c) TD-buffer containing 2M-NaBr. Centrifugation was in a Beckman L2-65 ultracentrifuge, rotor SW-60, at 55000 rev./min for 12 h at 2°C. Fractions of volume 0.2 ml were collected. Arrows indicate peak of the reference protein, bovine plasma albumin (4.45S). Low-specific-radioactivity [^3H]oestradiol was prepared as described in the legend to Fig. 1.

part) formation of slower-sedimenting forms of receptor in mammary cytosol, cannot produce a definite, smaller molecular species. The presence of EDTA, however, appears to favour formation of faster-sedimenting forms, a result in agreement with the finding by Shyamala (1975), suggesting that EDTA favours aggregation of mammary receptor. EDTA was therefore omitted from subsequent experiments.

Sedimentation on sucrose gradients in the presence of NaBr

The lack of satisfactory results with NaSCN prompted a search for other chaotropic salts possibly better suited to studies with mammary oestrogen receptor. Among these, NaBr was shown to give the best results. Preliminary trials showed that concentrations of NaBr up to 2M are compatible with a stable receptor-oestrogen complex in mammary cytosol. Fig. 2 shows a comparison of results when [^3H]oestradiol-labelled cytosol was prepared and centrifuged on sucrose gradients in TD-buffer (Fig. 2a) or TD-buffer containing 0.5M- (Fig. 2b) or 2M-

NaBr (Fig. 2c). In the absence of NaBr, the receptor sediments at about 7S. However, in the presence of NaBr sedimentation is at 4.2S. At 2M-NaBr, the peak of receptor is much sharper than at 0.5M and there is virtually no receptor sedimenting at the bottom or in the lower part of the gradient. Considering that the same amount of cytosol was used in all sucrose gradients illustrated in Fig. 2, we conclude that, at 2M-NaBr, virtually all receptor in cytosol is present as a well-defined molecular species sedimenting at 4.2S, with total disappearance of heavier forms. No difference was found in subsequent trials between the effect of 2M- and 1M-NaBr (results not shown).

Formation and affinity of oestradiol-17β-receptor complex in the presence of NaBr

NaBr (1M) was added to mammary cytosol prepared as described in the Experimental section. The cytosol was thereafter divided into portions to which different amounts of [^3H]oestradiol were added. After incubation for 60 min at 4°C, bound hormone was separated from free hormone by filtration on Sephadex G-25 columns. A parallel, control experiment (no NaBr) was also carried out.

The results are plotted by the method of Scatchard (1949) in Fig. 3. Straight lines were fitted to the points by the least-squares method, and the dissociation constants could thus be calculated. In the presence of 1M-NaBr the K_D is $8.9 \times 10^{-10}\text{M}$, and in the absence of the salt $8.8 \times 10^{-10}\text{M}$. Therefore, 1M-NaBr does not significantly affect the affinity of the oestrogen-receptor interaction. It does have a slight effect on the number of binding sites, which decrease to about 90% of those available in the absence of the chaotropic salt.

Gel filtration in the presence of NaBr

When [^3H]oestradiol-labelled cytosol in TD-buffer, pH 7.4, is filtered through a Sephadex G-200 column (50 ml) equilibrated with the same buffer (Fig. 4a), the radioactivity is eluted as a single peak corresponding to the excluded volume of the column (V_0), with a shoulder on the trailing edge. The [^3H]oestradiol eluted at V_0 is specifically bound, since the peak disappears when the high specific radioactivity of [^3H]oestradiol used for labelling the cytosol is decreased by addition of a 150-fold excess of unlabelled oestradiol-17β.

The above elution pattern is not modified by the following changes in the composition of the cytosol and of the TD-buffer used for the filtration through the Sephadex column: (i) addition of 3 mM- ZnSO_4 , (ii) addition of 50 mM-NaSCN, (iii) addition of 15% (w/v) sucrose, (iv) addition of 0.4M-KCl (results not shown). However, when 1M-NaBr (or 0.5M; results not shown) is added, the elution profile from the

Fig. 3. Measurement of the affinity of the interaction between oestradiol and mammary cytosol receptor in Tris buffers with or without NaBr

Cytosol samples (0.3ml) were incubated (60min; 4°C) with various amounts $[(0.8-12) \times 10^{-13} \text{ mol}]$ of $[^3\text{H}]$ oestradiol of high (85 Ci/mmol) or low (0.57 Ci/mmol) specific radioactivity. The final volume of each incubation mixture was 0.5ml. At the end of incubation, separation of bound from free oestradiol was obtained by gel filtration at 4°C on Sephadex G-25 columns. When low-specific-radioactivity $[^3\text{H}]$ oestradiol was used, no change of the bound/free ratio was observed (aspecific binding). The aspecifically bound portion of radioactivity was subtracted from the results obtained by using high-specific-radioactivity $[^3\text{H}]$ oestradiol before plotting the data (Scatchard, 1949). $\circ$, TD-buffer; $\bullet$, TD-buffer containing 1M-NaBr. Straight lines were fitted to experimental results by a computer by using the least-squares method. The coefficients of determination (r^2) are: 0.961 (TD-buffer) and 0.996 (TD-buffer + NaBr). K_D values are $0.88 \times 10^{-10} \text{ M}$ (TD-buffer) and $0.89 \times 10^{-10} \text{ M}$ (TD-buffer + NaBr). The number of binding sites (abscissa intercept) is about 10% lower in TD-buffer + NaBr.

Sephadex G-200 column changes considerably (Fig. 4b); radioactivity is eluted in two peaks, the first and smaller one emerging with V_0 and the second emerging included in the gel matrix, with an elution volume (V_e) of 27 ml (range of several experiments, 25–29 ml). When the oestradiol-receptor complex is salted out of cytosol by $(\text{NH}_4)_2\text{SO}_4$ at 25% saturation in the presence of 1M-NaBr, and then redissolved and filtered through the Sephadex G-200 column in TD-buffer containing 1M-NaBr, the elution pattern con-

Fig. 4. Gel filtration on Sephadex G-200 columns of $[^3\text{H}]$ oestradiol-labelled mammary cytosol, or $(\text{NH}_4)_2\text{SO}_4$ precipitate of cytosol, in Tris buffers with or without NaBr

Cytosol was labelled with high-specific-radioactivity (85 Ci/mmol, $\bullet$) or low-specific-radioactivity (0.57 Ci/mmol, $\blacktriangle$) $[^3\text{H}]$ oestradiol, and subsequently (a) filtered through a Sephadex G-200 column in TD-buffer, (b) filtered through a Sephadex G-200 column in TD-buffer containing 1M-NaBr, (c) treated with $(\text{NH}_4)_2\text{SO}_4$ (25% saturation) and the resulting precipitate redissolved in TD-buffer containing 1M-NaBr and filtered through a Sephadex G-200 column equilibrated in the same buffer. The total volume of the columns was 50ml. The effluents were monitored for radioactivity ($\bullet$, $\blacktriangle$) and absorbance ($\circ$).

sists of only the included peak emerging at $V_e = 27 \text{ ml}$ (Fig. 4c). These results thus show that by the combined use of $(\text{NH}_4)_2\text{SO}_4$ and NaBr, aggregation of mammary receptor during gel filtration can be virtually eliminated.

Assessment of Stokes radius of receptor in the presence of NaBr

A Sephadex G-200 column (50ml) equilibrated in TD-buffer containing 1M-NaBr was calibrated by using several reference proteins (see the Experimental section). The measured K_D^3 values of the reference proteins gave a straight line when plotted against their Stokes radii (Porath, 1963). With this calibrated column, the Stokes radius of the receptor-oestrogen complex of cytosol was found to be $3.7 \text{ nm} \pm 6\%$ (averaged from five separate measurements). Both crude cytosol or receptor partially purified by the procedure described below show the same Stokes radius on filtration on the above calibrated column.

Partial purification of mammary receptor

From the above results, the following purification procedure was applied to the receptor from the mouse lactating gland (all operations at 0–4°C): (i) cytosol

Fig. 5. Isoelectrofocusing on a pH 5–8 gradient of partially purified mammary oestrogen receptor

[^3H]Oestradiol-labelled (85 Ci/mmol) receptor was partially purified as described in the Results section, and a sample selectively positioned in the pH 7 zone of a prefocused Ampholine pH-gradient. The LKB electrofocusing column (110 ml) was used. The temperature of the cooling water was 3 °C. After application of the sample, the gradient was focused until the current fell to a constant 0.8 mA at 1000 V. Fractions (3 ml) were collected, and pH ($\blacktriangle$), absorbance ($\circ$) and radioactivity ($\bullet$) were measured in each fraction. The two radioactive peaks found were analysed by gel filtration and sucrose-gradient centrifugation. The peak focused at pH 6.2 is [^3H]oestradiol bound to receptor. The broader peak in the neutral zone of the gradient is free [^3H]oestradiol, which clearly did not move from the zone where the sample initially positioned.

was prepared and labelled with [^3H]oestradiol as described in the Experimental section, (ii) NaBr up to 1 M was added to the gently stirred cytosol and then $(\text{NH}_4)_2\text{SO}_4$ up to 25% saturation; (iii) after 30 min, the precipitate was collected by centrifugation in a refrigerated centrifuge at 20000g for 10 min, redissolved in TD-buffer, pH 7.4, containing 1 M-NaBr and filtered through a Sephadex G-200 column in the same buffer; (iv) the fractions corresponding to the included peak were collected, $(\text{NH}_4)_2\text{SO}_4$ was added up to 25% saturation and the precipitate redissolved in a small volume of TD-buffer, pH 7.4, containing 0.2 M-NaBr.

The above purification procedure gives a purification of 30–90-fold over the original homogenate, with a recovery of 30–80% of the original receptor-binding activity. Variations in purification and recovery depend especially on losses during Sephadex G-200 chromatography, which are due mainly to irreversible aggregation. These losses may vary con-

siderably from preparation to preparation, for reasons that are still unknown.

Study of molecular properties of partially purified receptor

Both on sucrose gradients and on Sephadex G-200 chromatography in the presence of NaBr, no difference could be detected between partially purified receptor and receptor in crude cytosol. When NaBr was removed, the partially purified receptor, like receptor in crude cytosol, rapidly formed large aggregates. When analysed by electrofocusing on a pH 5–8 gradient, the partially purified receptor–oestrogen complex focused as a sharp peak at pH 6.2 (Fig. 5). The radioactivity at about pH 7 in Fig. 5 is free oestradiol. Indeed, to avoid exposure of oestrogen receptor to extremes of pH, the technique described by Puca *et al.* (1975) was used, which prescribes pre-forming of the pH gradient and selective positioning of the sample in the zone of the gradient corresponding to about neutral pH. The same pI of 6.2 was found in four separate preparations and runs of mammary receptor.

Discussion

The results in the present paper show that addition of 0.5–2 M-NaBr, a chaotropic salt (Hatefi & Hanstein, 1969), to low-salt mammary-gland cytosol inhibits age-dependent aggregation of receptor. Also, the receptor sediments on sucrose gradients as a sharp peak at 4.2 S instead of at about 7 S and is included in the Sephadex G-200 matrix, instead of being excluded. Gel filtration on a calibrated Sephadex G-200 column shows that the receptor species in NaBr has a Stokes radius of 3.7 nm.

Furthermore, NaBr at the concentration used is compatible with a stable oestrogen–receptor complex. In fact, NaBr does not even interfere with formation of the oestrogen–receptor complex, nor does it decrease the affinity of the association. It does, however, decrease the number of total binding sites of cytosol by about 10%. Considering that the affinity of the oestrogen–receptor interaction is not affected, it seems likely that this 10% decrease actually represents elimination by NaBr of low-affinity oestrogen binding by non-receptor components of cytosol.

The first indication that chaotropic salts are useful for blocking age-dependent aggregation of oestrogen receptor in cytosol, and during purification procedures, was given by Sica *et al.* (1976), who showed that 0.1 M-NaSCN can block aggregation *in vitro* and dissociates the approx. 8 S form of oestrogen receptor in calf uterus cytosol into a form sedimenting at 4.1 S and with a Stokes radius of 3.6 nm. Initially, we tried to apply NaSCN to oestrogen receptor of mammary-

gland cytosol, but with only partial success. NaSCN (0.1M), which is effective for calf uterus oestrogen receptor, does not avoid aggregation of mammary receptor during Sephadex chromatography, and produces a broad band sedimenting in the 7–5S region on sucrose gradients. Whether the reasons for this difference are organ- or species-specific remains to be established. Results showing that NaBr (0.5–2M) is also effective in inhibiting aggregation and in dissociating calf uterus oestrogen receptor (Sica *et al.*, 1977) suggest that mammary cytosol must have some peculiar property that requires NaBr specifically.

Several mechanisms are probably involved in protein dissociation by chaotropic salts (Sawyer & Puckridge, 1973): (i) preferential binding of salt to the lower-molecular-weight species, (ii) disruption of hydrogen-bonded structure of water, with increased solubility of non-polar areas of the protein surface, (iii) direct disruption of inter-subunit hydrogen bonds. In protein associations in which hydrogen bonds predominate, the chaotropic salt effectiveness parallels the Hofmeister series. In associations where, on the contrary, hydrophobic interactions are the main contributor to the interaction energy, there are notable exceptions to the Hofmeister series. For instance, NaBr is much more effective in dissociating β -casein, in which hydrophobic interactions predominate, than the Hofmeister series would predict (Sawyer & Puckridge, 1973). Considering that lactating-mammary-gland cytosol is richer in fat than is uterine cytosol, this property of NaBr could explain the particular effectiveness of this chaotropic salt in mammary cytosol, where lipid may participate strongly in intermolecular interactions.

By inhibiting interactions of receptor with other components of cytosol, NaBr furnishes ideal conditions for the study of molecular properties of receptor in cytosol by methods that can be applied to characterization of protein in mixtures, i.e. sucrose-gradient centrifugation and exclusion chromatography on gels. Therefore, the values of the sedimentation coefficient and of the Stokes radius of cytosol receptor, as measured in the present study, can be accepted with confidence. As suggested by Siegel & Monty (1966), from the Stokes radius and sedimentation coefficient, an unbiased estimate of molecular weight (M) and frictional ratio (f/f_0) can be obtained from the classical equations

$$M = 6\pi\eta Ns / (1 - \bar{v}\rho) \quad (1)$$

$$f/f_0 = a / (3\bar{v}M / 4\pi N)^{1/2} \quad (2)$$

where η = viscosity of the medium, N = Avogadro's number, a = Stokes radius, s = sedimentation coefficient, $\bar{v}$ = partial specific volume, ρ = density of the medium. By using the Stokes radius (3.7nm) and the sedimentation coefficient (4.2S), obtained as

described in the Results section, and assuming $\bar{v} = 0.725$, the following values were calculated: $M = 64000$; $f/f_0 = 1.4$.

It is noteworthy that the molecular properties of the mammary oestrogen receptor do not change when it is analysed after purification by approx. 90-fold by the procedure described in the Results section, a fact that reinforces the conclusion that the molecular parameters assessed are realistic. Also, there is a close molecular resemblance between the mouse mammary oestrogen-receptor species in NaBr and the calf uterus oestrogen receptor in NaSCN (Sica *et al.*, 1976); both have virtually the same sedimentation coefficient (4.2S compared with 4.1S), Stokes radius (3.7nm, 3.6nm), molecular weight (64000, 61000), frictional ratio (1.40, 1.25) and isoelectric point (6.2, 6.0). An axial ratio (prolate) of 5.5 was found for the mouse mammary oestrogen-receptor species in NaBr, derived from f/f_0 (Oncley, 1941) by assuming a hydration value of 0.275 g of water/g of protein.

The use of NaBr for preventing aggregation during purification procedures, such as $(\text{NH}_4)_2\text{SO}_4$ precipitation and gel filtration, has allowed for the first time a partial purification of oestrogen receptor from mammary gland. However, despite the up to 90-fold purification, elimination of NaBr from the medium still results in aggregation of receptor. This result, as well as the presence of forms sedimenting quite sharply at 7S, or faster on sucrose gradients in low-salt buffers, suggests that the 64000-molecular-weight species has a high tendency to form larger molecular structures. In fact, as discussed previously for oestrogen receptor of calf uterus (Puca *et al.*, 1972; Sica *et al.*, 1976), at the ionic and protein concentrations in cytoplasm *in vivo* the 64000-molecular-weight form is most probably part of higher molecular structures resulting from self- and/or hetero-association. The physiological meaning of these higher structures *in vivo*, if any, is still unknown. Certainly, however, these putative physiological associations of the 64000-molecular-weight form *in vivo* should be kept distinct from the large aggregates that form in an age-dependent fashion in the cytosol *in vitro*, a phenomenon that is obviously the consequence of the artificial conditions after cell disruption. It is hoped that the nature and the significance of the molecular interactions involving the cytosol receptor will be elucidated once the 64000-molecular-weight form has been completely purified. It will then be possible to study self-association, as well as hetero-associations, of oestrogen receptor under simplified and rigorous experimental conditions.

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