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SIGNIFICANCE OF THE 8S COMPLEX IN OESTROGEN RECEPTOR RECOGNITION

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Summary—Previous studies from this laboratory have drawn attention to discrepancies between enzyme-linked immunoassay (EIA) and steroid binding assay (SBA) in the analysis of oestrogen receptors (ER) in breast tumours. In particular, EIA values were at least 3-fold higher than SBA values in tumours which also contained progesterone receptors (PR) when both 4 and 8S isoforms of the ER are present. To test the influence of these isoforms on the two assay systems, the relationships between the oestrogen receptor (ER) values obtained by EIA and SBA were examined in tumour cytosols prepared in the presence of molybdate and protease inhibitors to prevent degradation of the 8S form. Under these conditions, values for ER were the same by EIA and SBA (slope = 1.08, $r = 0.886$, $n = 25$) when EIA was performed using low salt phosphate buffer instead of the high salt-containing Abbott-diluent provided with the kit. However, after disruption of the 8S assembly using high K^+ concentration, the slope of the regression was 6.37, $r = 0.865$, $n = 25$.

Using ER from rat uterus, EIA was also performed on intact 8S oligomers, on 8S ER dissociated by high salt, and on glycerol density gradient-fractionated 4S ER. The identity of the ER oligomers and components was confirmed by glycerol density gradient fractionation, and by isoelectric focussing. For the 4S ER, EIA gave similar values whether using low or high salt phosphate buffer. However EIA values for the 8S form were 2-fold higher when the supplied diluent was used than when the assay was performed in low salt buffer. The amount of oestradiol which could be extracted was affected by the different conditions used. Addition of KCl or trypsin to disrupt the 8S ER caused an increase in the amount of extractable oestradiol compared with control values (control = 52 ± 4.0, high KCl = 91 ± 4.4, trypsin = 152 ± 7.5, pg oestradiol/mg protein). We conclude that further antibody binding sites are revealed from the 8S ER form after its disaggregation by high salt. The steroid extraction data also suggests the possibility that tightly bound steroid is retained within the 8S ER structure, and released by 8S disaggregation. Both of these may contribute to the differences between EIA and SBA values.

INTRODUCTION

The oestrogen receptor (ER) may exist in different forms, and it is thought that the active species which binds to DNA and initiates gene transcription is the dimeric form with a sedimentation coefficient of 5S [1, 2]. In the soluble ER fraction obtained from cytosol, other forms may exist, whose significance have not been fully explained. In cytosols prepared from hypotonic homogenates the ER can be recovered either as a large complex with a sedimentation coefficient of 8S or a smaller monomeric 4S form [3, 4]. The ER can also be shown by isoelectric focussing or HPLC to be present in various molecular forms [5–9] which may at least in part reflect the differences in sedimentation coefficients. Such differences in form may be related to known changes in physiological functions, including the untransformed or, following hormone binding, in the "transformed" state [10, 11], nuclear bound [12], or co-valently modified [13, 14] and in "abnormal" forms [15].

Because of the existence of these variants, it is conceivable that the two radically different systems of measurement of ER in current use, steroid binding assay (SBA) and monoclonal antibody binding (enzyme linked immunoassay, EIA) may estimate the various forms and physiological states of ER with differing

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sensitivity. We have previously reported a discrepancy between EIA and SBA assays performed on the same tumours [16, 17]. In particular, in tumours in which both ER and progesterone receptors (PR) were present, the ER levels obtained by EIA were more than three times higher than those obtained by SBA. The presence of PR in these tumours coincides with the presence of both molecular forms of the soluble ("cytosolic") ER, 4 and 8*S*. On the other hand, in PR-negative tumours, in which the 4*S* form predominates, both EIA and SBA estimate the same amount of ER. Clearly the intact monomeric form of the receptor expresses steroid and antibody binding sites equally. If the 8*S* is formed from an assembly of similar intact monomeric units then the 3:1 antibody–steroid binding stoichiometry suggests that steroid binding sites are hindered by the quaternary structure or that not all of the receptor bound steroid is freely available to exchange with endogenously added labelled steroid.

In these studies, high salt concentrations, or trypsin were used to disassemble the 8*S* isoform of the ER to examine the relationship between the isoform profile and SBA/EIA stoichiometry. In addition, extractable oestrogen was assayed at different stages of the process to determine whether non-exchangeable oestrogen may be a component of the system. The observations reported here provide further information regarding the structure of the 8*S* ER oligomer.

EXPERIMENTAL

Tissue handling

Human breast tumour tissue was obtained at operation and stored in liquid nitrogen until processed. Uteri were excised from Wistar rats (250–350 g body weight).

All tissue processing was performed at 4°C. The tissue was homogenized, using a polytron homogenizer, in glycerol phosphate buffer (10% glycerol, 10 mM phosphate, 1.5 mM EDTA, 5 mM monothioglycerol, pH 7.4) 1:10 w/v (GPB), or in the same phosphate buffer containing 20 mM Na_2MoO_4 and 1 μg/ml of each of the protease inhibitors aprotinin and soybean trypsin inhibitor (GPBI). The homogenate was centrifuged for 60 min at 100,000 *g*, and the supernatant was used for receptor analysis.

Scatchard analysis

Aliquots (100 μl) of tumour supernatant were incubated with increasing concentrations of [1,2,6,7,-^{3}H]oestradiol (0.08–10 nM, final concentration) in the presence or absence of 100-fold excess of diethylstilboestrol (DES) for 18 h at 4°C. Free and bound steroid were separated by dextran coated charcoal pellet (DCC). Aliquots of the charcoal treated supernatants were counted in a Beckman LS7500 counter. At all concentrations counts for competed tubes were subtracted from counts for non-competed tubes to give values for hormone bound specifically to the receptor. The data were plotted according to the method of Scatchard [18].

Single saturating dose (SSD) method

Aliquots (100 μl) of tumour supernatant were incubated with a single concentration of radioactive oestradiol (10 nM, final concentration) which is sufficient to saturate the receptor, with or without addition of 100-fold excess of DES. Free and bound ligand were separated as described above.

EIA

The EIA was performed according to the instructions provided with the Abbott kit, either using the Abbott diluent supplied, or phosphate buffer alone or phosphate buffer supplemented with 0.8 M KCl. This assay uses two monoclonal antibodies raised against two different epitopes on the ER from MCF-7 breast cancer cells. The first (D547), attached to a glass bead, recognizes an epitope between the DNA and steroid binding domains. The second antibody (H222) is supplied in solution, and recognizes a sequence close to the steroid binding domain.

Isoelectric focussing (IEF)

The IEF gels were cast in slabs of size 125 × 260 mm and separation was conducted along the long axis. Polyacrylamide gels, containing 12% glycerol, 2 mm thick, with high porosity (T = 5%, C = 3%) were used. A pH 3.5–10 gradient was achieved using 0.7% (w/v) LKB ampholine 3.5–10 (LKB, Bromma, Sweden) and 0.3% (w/v) LKB ampholine 5–8. Gels were photopolymerized, at room temperature by means of a TR 26 polymerization light (Hoefer S. I., San Francisco, CA, U.S.A.), using riboflavin (0.004%) for at least 8 h. IEF was performed in a cold room and the temperature of the cooling water was kept constant at 4°C,

using a LKB Multiphor II System. Electrode solutions of 1 M sodium hydroxide (cathode) and 1 M sulphuric acid (anode) were used.

After DCC extraction, aliquots (270 μl) of the radioactive supernatants (3 mg protein/ml) derived from SSD assay were loaded near the cathode. The runs were carried out for 4 h using a 3000xi CC Power supply (Bio-Rad, Hemel Hampstead, Herts., England) at 2500 V/20 mA/20 W, constant power. A mixture of nine natural proteins (Bio-Rad) was used for pH calibration. After the run, the gels were cut into 2.5 mm slices and each slice was incubated with 5 ml of scintillation cocktail (Packard) for 24 h at room temperature and radioactivity assayed.

Steroid extraction and RIA of oestradiol

To study the effects of 8–4*S* transformation on extractability of steroids, rat whole uteri were incubated with 5 nM (final concentration) of cold oestradiol for 1 h in ice.

Steroids were extracted twice with 3 ml of ethyl acetate from a charcoal treated high speed (100,000 *g*) soluble fraction obtained from the whole rat uteri: (a) immediately (control), (b) after leaving the sample for 30 min in the presence of 0.4 M KCl, at 4°C, or (c) after leaving the sample for 20 h at 4°C with and without trypsin (130 μg/mg protein). Extracted steroids were incubated with 200 μl of a specific antiserum raised against oestradiol-6-(*o*-carboxymethyl)-oxime-bovine serum albumin (BSA) in 50 mM Tris buffer (pH 8.0) containing 100 mM NaCl and 0.1% NaN_3, together with 0.6–0.7 nM of [^{3}H]oestradiol at 37°C for 1 h. Free steroid was extracted with a DCC pellet and radioactivity measured in aliquots of the supernatants.

Glycerol density gradient ultracentrifugation (GDG)

To verify the predominant ER form after these treatments, aliquots of radioactive cytosol obtained from whole uteri, prepared as described above, were layered on to 4.8 ml gradients of 5–20% glycerol with or without 0.4 M KCl. The samples were centrifuged at 4°C for 5 h at 50,000 rpm in a Beckman SW 50.1 rotor. Fractions were collected and radioactivity measured. The fraction containing the 8 or 4*S* components were taken from a duplicate gradient and run on an IEF gel as described above.

Protein determination

Proteins were determined according to Lowry *et al.* [19], using BSA, fraction IV, as standard.

RESULTS

ER content was assayed in 89 human breast cancer samples by EIA and SBA. 64 Of these tumours were homogenized in GPB and the remaining 25 were homogenized in the same buffer containing 20 mM Na_2MoO_4 and 1 μg/ml of each of the protease inhibitors aprotinin and soybean trypsin inhibitor (GPBI).

In the group homogenized with GPB, 40 out of 64 tumours (62.5%) were ER-positive (ER > 10 fmol/mg protein) and 14 out of 25 were ER-positive in the group homogenized in GPBI (56%). Comparison between the ER concentration values obtained by EIA and SBA gave a correlation coefficient of $r = 0.8$, slope = 3.0 when using GPB [Fig. 1(A)] and $r = 0.86$, slope = 6.37 using GPBI homogenizing buffer [Fig. 1(B)].

Comparison between EIA values performed in the presence or absence of 0.4 M KCl

To check the possibility that the antibodies require high ionic strength for binding to the receptor, EIA was performed in the presence of the diluent provided with the ER–EIA Abbott kit (phosphate buffer containing 40 mM Na_2MoO_4, oestradiol and progesterone and 0.8 M KCl) or in phosphate buffer containing 40 mM Na_2MoO_4 but without steroids or KCl) on all 25 tumours homogenized in GPBI buffer. A correlation $r = 0.91$ and slope = 4.59 was obtained [Fig. 2(A)]. When ER concentrations were assayed by EIA using buffer without KCl (instead of diluent) and compared with values obtained by SBA using GPBI a correlation coefficient of $r = 0.886$ and slope = 1.08 [Fig. 2(B)] were observed.

The possibility that components of the diluent other than KCl (e.g. steroids) may effect the data was checked on GPBI homogenized tumours by comparison of the data obtained by EIA performed using diluent or our phosphate buffer containing 40 mM Na_2MoO_4 and 0.8 M KCl. In this case we obtained a correlation $r = 0.99$ and slope = 1.06 (Fig. 3).

Discrepancies between EIA and SBA on the ER content were also observed in rat uterus, in which values obtained by EIA performed in presence of the diluent (in fmol/mg

protein ± SEM) were 250 ± 40. The ER concentrations (±SEM) in the same samples, by SBA, were like 116 ± 15 ($n = 6$).

EIA performed on the 4 and 8S forms of ER from rat uterus

Rat uterus cytosol was used as a source of ER and its content was assayed by SBA and EIA; a qualitative GDG and IEF were performed to establish the ER isoform profile.

When fresh whole rat uteri were incubated with 5 nM of [^{3}H]oestradiol for 1 h at 4°C, and homogenized in Tris buffer containing Na_2MoO_4 and protease inhibitors, the soluble high speed molybdate-stabilized ER appeared, on GDG, as a homogeneous protein with a sedimentation coefficient of about 8*S*. The same preparation analysed by IEF showed the ER focused as a single band at pI 6.1.

This partially purified ER was collected from the glycerol gradient and assayed by EIA with and without 0.4 M KCl. The EIA performed without KCl recognized < 50% of the amount of ER assayed with KCl [Fig. 4(A)].

The same assay was performed on ER obtained from rat uterus homogenized (without any pre-incubation with oestradiol) in Tris buffer without Na_2MoO_4 and without inhibitors. Following this procedure, the ER appears, by GDG, as two distinct forms with a sedimentation coefficient of approx. 4 and 8*S*.

The partially purified 8 and 4*S* forms of ER were taken from the gradients and assayed by

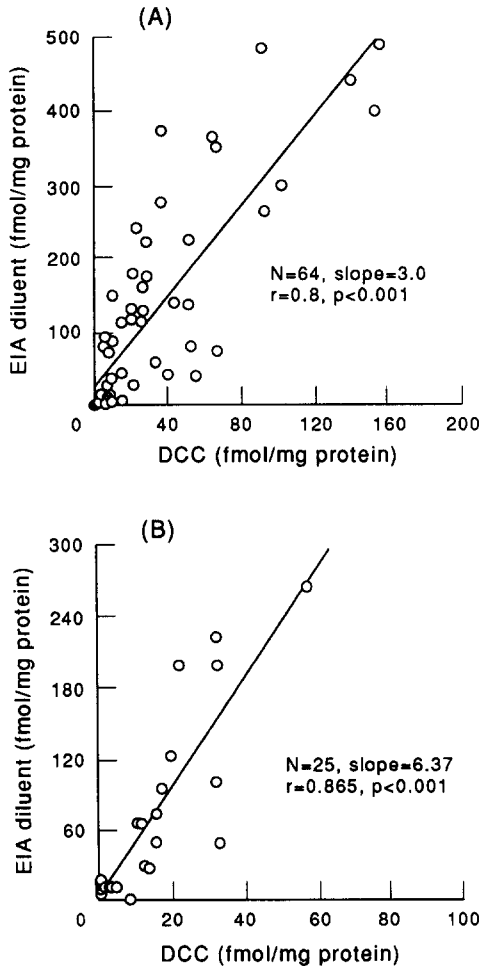

Fig. 1. Correlation between ER concentrations obtained in analysis of supernatants of primary breast cancer homogenates using DCC or EIA methods. (A) Tumours homogenized in glycerol phosphate buffer in the absence of Na_2MoO_4 and protease inhibitors. (B) Tumours homogenized in glycerol phosphate buffer containing 20 mM Na_2MoO_4 and 1 μg/ml each of the protease inhibitors aprotinin and soybean trypsin inhibitor.

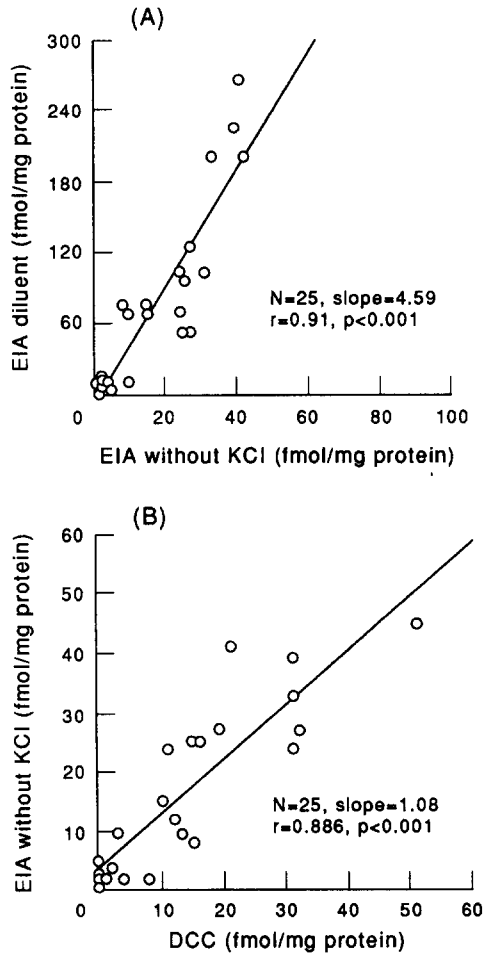

Fig. 2. (A) Correlation of ER concentrations obtained in supernatants of primary breast cancer homogenates by EIA using either the Abbott diluent or GPB. Tumours were homogenized in GPB containing 20 mM Na_2MoO_4 and 1 μg/ml each of the protease inhibitors aprotinin and soybean trypsin inhibitor. (B) Correlation between ER concentrations obtained by EIA, performed using GPB (without KCl), and DCC. Tumours were homogenized in GPB containing 20 mM Na_2MoO_4 and 1 μg/ml each of the protease inhibitors aprotinin and soybean trypsin inhibitor.

EIA in the presence or in the absence of KCl. The EIA on the 8S form performed without KCl was unable to recognize all the receptor present, but no significant difference was found in the amount of 4S form assayed by EIA in the presence or absence of KCl [Fig. 4(B)].

The GDG-separated 4 and 8S forms were subjected to IEF, and the 8S form was shown to be equivalent to the isoform with a pI of 6.1 while the 4S is a composite of 3 separate isoforms separating at about pI 6.6 (Fig. 5).

Steroid extraction from different forms of the ER

Oestradiol was extracted from charcoal-treated rat uterine cytosol containing the various ER forms and assayed by a specific RIA for oestradiol. The amount of extractable oestradiol was different in each of the conditions used. In particular, addition of trypsin caused a large, highly significant increase in the amount of extractable oestrogen.

The values obtained by RIA (in pg of oestradiol/mg of cytosolic protein ± SEM) were: control = 52 ± 4.0; +0.4 M KCl = 91 ± 4.4; +trypsin = 152 ± 7.5 ($n = 7$ throughout) [Fig. 6(A)].

Cytosol loaded on an IEF gel immediately after centrifugation had only one predominant form of the receptor with a pI of about 6.1 (8S form). This form shifted to pI 6.6 after an overnight incubation at 4°C, in the presence of trypsin [Fig. 6(B)].

DISCUSSION

The results show that the presence of the 8S oligomer in breast tumours or rat uterus cytosols is readily assayed by the EIA method when performed with the KCl-containing diluent provided with the Abbott-kit (Figs 1 and 4). It is known, in fact, that the presence of 0.4 M KCl disaggregates the 8S form to a smaller, monomeric steroid binding form of the receptor [20, 21]. This means that the ER is never entirely present in 8S form during the EIA procedure. The effect of the presence of the 8S is appreciated when the assay is performed in the absence of KCl [Fig. 2(B)]. The presence of molybdate and protease inhibitors in the homogenization buffer serves to stabilize the 8S form of the receptor [22, 23], if present, and results in EIA producing values 4.59 times higher when performed in the presence of KCl [Fig. 2(A)].

SBA and EIA, performed in the absence of KCl, measure the same amount of ER when the receptor is present as an oligomeric 8S form [see Fig. 2(B)]. In fact, EIA does not recognize the 8S structure in the absence of KCl as effectively as in its presence [Fig. 4(A) and (B)], but does recognize the 4S, monomeric form, [Fig. 4(B)]. This strongly suggests that the epitopes are "masked", i.e. not accessible to the antibodies, when the ER is present in the 8S complex form.

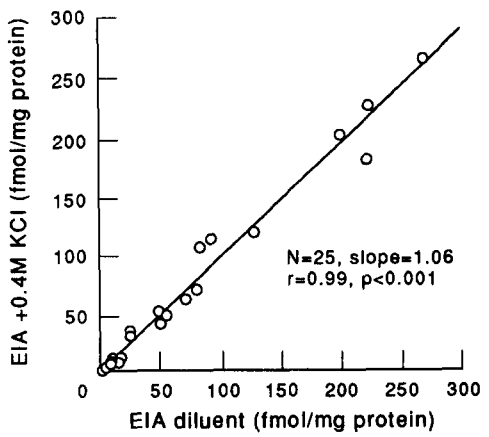

Fig. 3. Correlation between ER concentrations obtained by EIA, performed using GPB in the presence of 0.4 M KCl (final concentration), and Abbott-diluent.

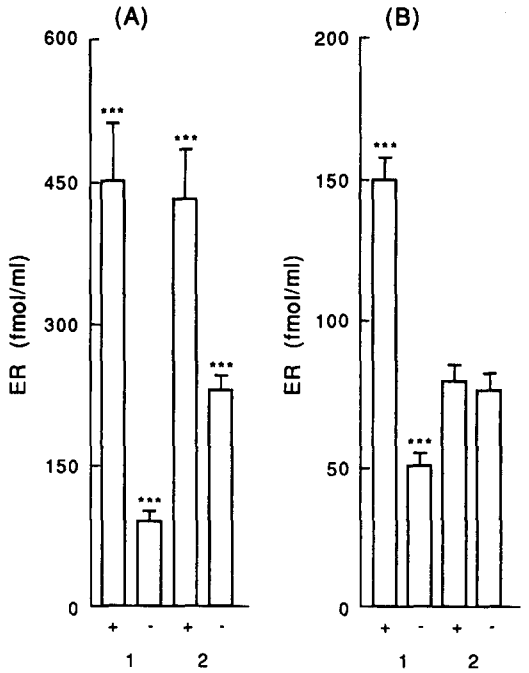

Fig. 4. Assay of the different molecular forms of rat uterus ER using EIA with GPB, either in the presence (+) or absence (−) of 0.4 M KCl. (A) EIA analysis of non-fractionated cytosol (1) and of the partially purified 8S form obtained from GDG (2). (B) EIA of the partially purified 8S (1) and 4S (2) forms collected by glycerol density fractionation. While a ER loss of about 60% is seen in the EIA recognition of the 8S form in absence of KCl, no appreciable difference is seen in the 4S EIA recognition. Values are means ± SEM; $n = 4$ throughout. ***$P < 0.001$.

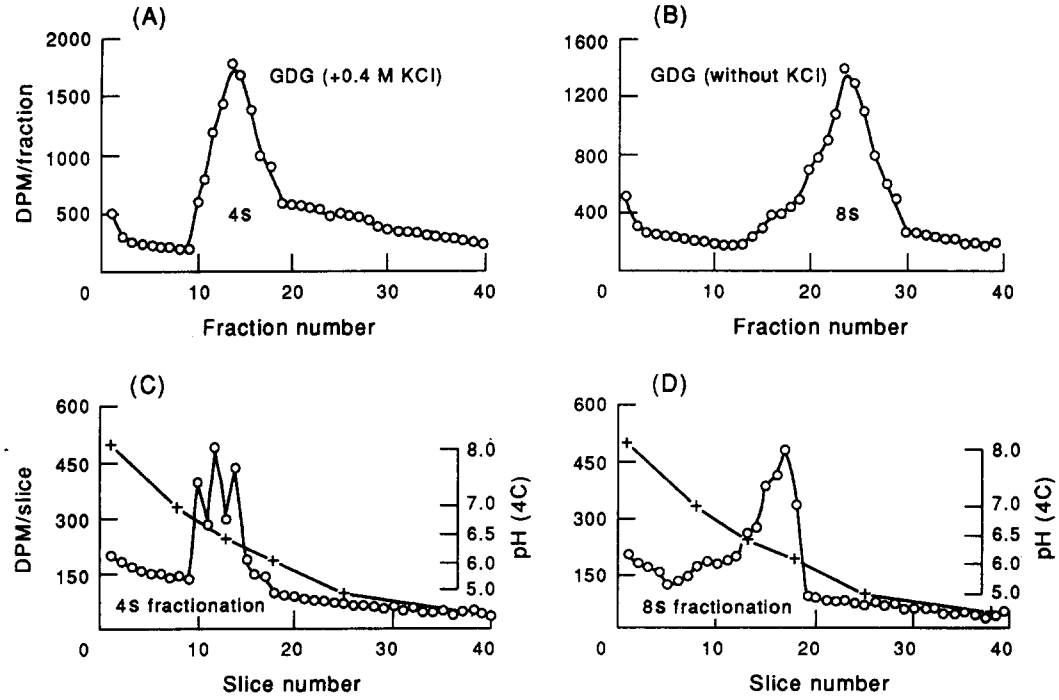

Fig. 5. Comparison between GDG and IEF fractionation of rat uterine ER. The two forms present in the GDG (A and B), with sedimentation coefficients 4 and 8*S*, fractionate as three components (C) distributed around pI 6.6 and as a single component (D) at pI 6.1, respectively.

Several other reports support this hypothesis. Antibodies raised against steroid receptors have been shown to detect the "activated" or "non-activated" forms with differing sensitivity [24, 25] and an antibody raised to the avian PR bound to the transformed 4*S* but not to the 8*S* non-transformed receptor [26]. Moreover, an assay based on antibody recognition of steroid bound to the ER failed to react with the 8*S* form as readily as to the 4*S* [27].

One of the antibodies present in the ER–EIA kit, H222, is also used in the Abbott ER–immunocytochemical assay (ER–ICA) kit. Using this assay it has been shown that both occupied and unoccupied ER are uniquely located in the nucleus of the cell [28, 29]. This antibody has been shown to recognize the nuclear, dimeric 5*S* form of the receptor [30, 31]. Immunohistochemical evidence has shown that unliganded PR [32, 33] and GR is also predominantly located within the nucleus (despite the proven cytosolic location of GR [34]). There is, so far, no evidence that these antibodies bind to steroid receptors in oligomeric form.

The presence of an abundance of the 8*S* complex in the molybdate-stabilized preparations would explain the difference observed between antibody and ligand binding assays. It may also explain the effect of trypsin on steroid extraction. If epitopes for the monoclonal antibody are masked it may also be possible that in the complex 8*S* structure steroid is held in such a way that it is inaccessible to solvents and can only be released when the oligomer has been disassembled or disaggregated.

There have been numerous studies on the action of trypsin concerning the release of steroids from different systems. For instance, Raven *et al.* [36] showed that trypsin increases the production of free extractable steroid (aldosterone and 18-hydroxy-corticosterone) in whole rat adrenal capsules, while Anderson *et al.* [35] showed that addition of trypsin in large excess enhances yields of oestrogen in ER + ve but not in ER − ve tumours. Furthermore, a small amount of trypsin added to ER + ve human breast cancer cytosols leads to a substantial increase in ligand binding capacity [37]. In rat, it has been shown that the mild trypsinization of cytoplasmic ER causes a disaggregation of the receptors, giving a form with sedimentation coefficient of 4.01*S* [38].

Interestingly enough, we found that the amount of extractable steroid is also increased by exposure of the charcoal-treated cytosol to 0.4 M KCl [Fig. 7(A)]. In the presence of high salt, the amount of extractable steroid is almost twice that of the control values. The

perturbation provoked by KCl is not as powerful as trypsin, and therefore does not result in disaggregation to the smallest fragment. However, it is undeniable that in high salt, the oligomeric 8S form is disaggregated to a mixture of 8 and 4S forms. Hence these KCl-extracted steroids further favour the idea that the additional steroid arises from the oligomeric complex.

Since we have been using cytosol derived from high speed centrifugation, it seems unlikely that we are dealing with membrane-associated steroids. Furthermore, it is unlikely that this additional extractable oestradiol is released from the type II receptor because the steroid binds with such low affinity [39, 40], that it should be extremely easy to extract with solvents. We feel that the KCl and trypsin data supports the possibility of a pool of unextractable, non-exchangeable steroid present within the 8S oligomeric form of the oestrogen

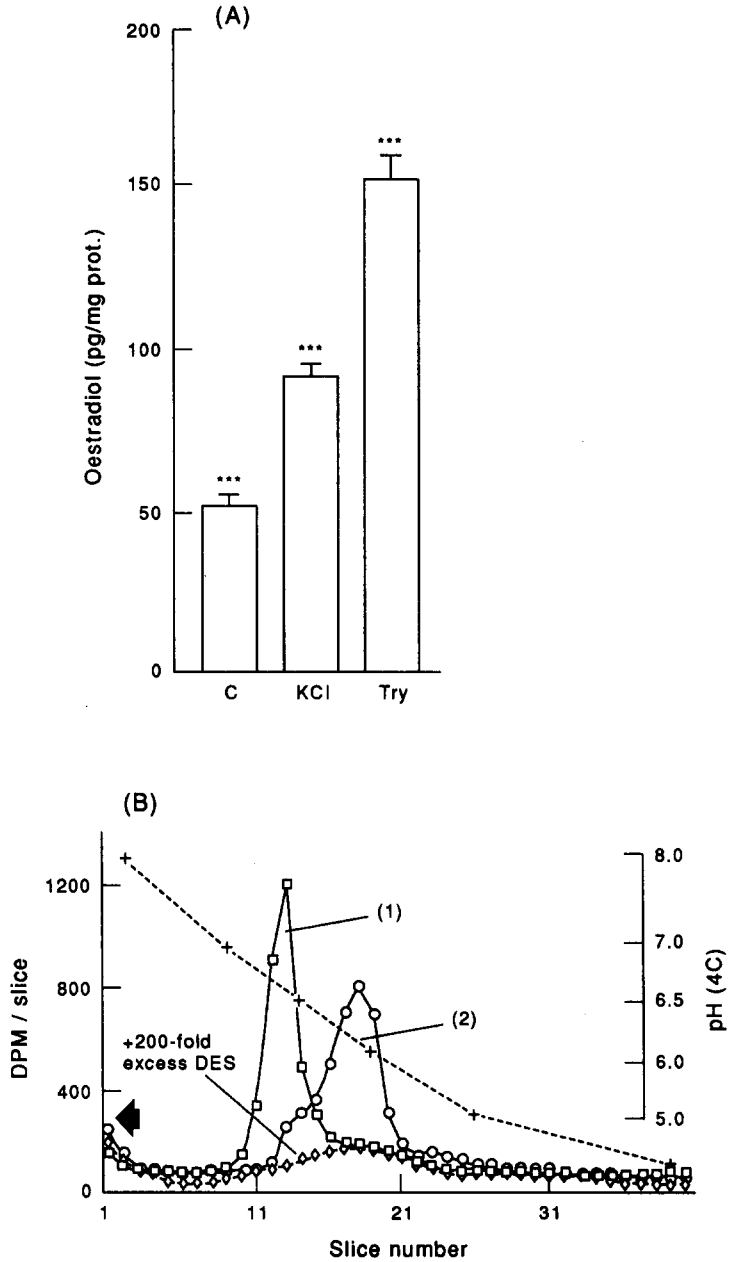

Fig. 6. (A) Effect of 0.4 M KCl and trypsin on the extractable oestradiol from high speed supernatants obtained from homogenates of rat uterus. C = control; KCl = + 0.4 M KCl; Try = + trypsin. Values are means ± SEM; ***$P < 0.001$. $n = 7$ throughout. (B) IEF analysis of ER from whole rat uterus preincubated with labelled oestradiol for 1 h at 4°C, loaded immediately (1), or (2) after incubation at 4°C for 20 h in the presence of 5 μg of trypsin per mg of cytosolic protein.

receptor. The role of this tightly bound steroid is unclear but it may be important in maintaining and stabilizing the complex architecture of the 8*S* receptor and, therefore, ensuring that the oestrogen receptor remains functional. This is particularly important since the presence of the 8*S* assembly has been shown to be a predictive index of an endocrine responsive breast cancer [8].

The data presented here clearly supports the existence of an oligomeric form of the receptor. However, further work must be done to elucidate the structural components of the 8*S* receptor if we are to understand the essential factors required for the normal function of this complex regulatory protein.

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