J. Steroid Biochem. Molec. Biol. Vol. 38, No. 1, pp. 35–42, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

MULTIPLE ESTROGEN BINDING SITES IN THE UTERUS: STEREOCHEMISTRY OF RECEPTOR AND NON-RECEPTOR BINDING OF DIETHYLSTILBESTROL AND ITS METABOLITES

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(Received 10 July 1990)

Summary—Indenestrol A (IA), an oxidative metabolite of the synthetic estrogen diethylstilbestrol (DES), has high binding affinity for estrogen receptor in mouse uterine cytosol but possesses weak biological activity. Racemic mixture of optically active [^{3}H]indenestrol A (IA-Rac) was separated and purified into individual enantiomers on a semi-preparative scale by HPLC with a Chiralpak OP(+) column. The structure–activity relationship was investigated among the [^{3}H]IA enantiomers (IA-*R* and IA-*S*) and [^{3}H]DES through direct saturation binding assays using mouse uterine cytosol. Specific binding curves and Scatchard plots were obtained for each [^{3}H]ligand; DES, IA-Rac, IA-*R* and IA-*S*. IA-*S* enantiomer ($K_d = 0.67$) binds to the estrogen receptor with the same affinity as DES ($K_d = 0.71$) and four times higher affinity than IA-*R* ($K_d = 2.56$). The number of binding sites for IA-*S* is approximately the same as estradiol, DES and IA-Rac while IA-*R* binds far fewer sites than the other ligands. Saturation binding assays indicated that [^{3}H]DES and [^{3}H]IA enantiomers exhibited a higher level of non-specific binding to the cytosol receptor compared to estradiol which has a low level of non-specific binding. These binding studies led to the detection of an additional binding component for the stilbestrol compounds in estrogen target tissue cytosol preparations. Sucrose density gradient separation assays under low salt conditions showed that both [^{3}H]DES and [^{3}H]IA compounds bound to the 8S form of the receptor, the same as E_2. But, in addition both DES and IA bound to another binding component in 4S region. The binding to the 4S component were partially displaced by the addition of excess unlabeled E_2 and DES. Further characterization of the 4S component is described.

INTRODUCTION

Diethylstilbestrol (DES), a synthetic non-steroidal estrogen, exhibits biological activities similar to the endogenous potent steroidal form, estradiol [1]. In utero exposure of DES has been linked to the development of carcinogenic lesions in the genital tract in experimental animals [2]. DES exposure and associated lesions in humans has recently been reviewed [3]. The exact mechanism by which DES elicits such a biological response is unknown. It is not yet established whether the toxicity is related to its potent estrogenic activity or the interaction of the oxidative metabolites of DES with estrogen receptor (ER). The oxidative metabolites of DES and their respective pathways have been identified [4, 5]. The hormonal and ER binding activities of the metabolites have been extensively studied [5, 6]. These studies indicated that oxidative metabolites of DES retain some hormonal and ER binding activities. It is assumed that the toxic action of DES could be the result of the interaction of one of the DES metabolites with the estrogen receptor. One group of metabolites, indanyl-DES (indenestrol A, IA) has become of particular interest because it has stronger binding affinities with the receptor than the endogeneous estradiol but weaker uterotropic activity [5, 7]. Since the IA compound has two stereoisomers (IA-*R* and IA-*S*), it has been used as a probe to study stereo-selectivity of metabolite interaction with the receptor. The IA enantiomers have been separated and isolated by chiral HPLC [8]. These enantiomers exhibited different biological and receptor binding activities [9]. Earlier we proposed that the difference in biological activity could be result of the

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receptor having a binding preference for one of the enantiomers of IA racemic mixture [7]. The two enantiomers were examined through competitive equilibrium binding assays and hormone responsiveness [10]. It appeared that the ertrogen receptor had a binding affinity twenty times greater for the IA-*S* enantiomer than IA-*R*, and elicited more biological activity. From these results, it was concluded that IA-*S* was the biologically active enantiomer and the receptor demonstrated a chiral preference for the IA enantiomers. The availability of newly synthesized, purified high specific activity ^{3}H-labeled IA and [^{3}H]DES made it possible to study the binding characteristics of these compounds through direct binding analysis. In the present study we investigate the structure–activity relationship among the [^{3}H]IA enantiomers, and [^{3}H]DES through direct binding activity in the mouse uterine cytosol. These binding studies led to the detection of an additional binding component for the stilbene compounds in estrogen target tissue cytosol preparations. The presence of such a component may help explain the hormonal potency and associated toxicity of DES in these tissues.

EXPERIMENTAL

Materials

[^{3}H]Indenestrol A, 1-ethyl-2-([3,5-^{3}H]4-hydroxyphenyl)-[4,6-^{3}H]3-methyl-5-hydroxyindene (IA) having a sp. act. of 11.5 Ci/mmol with a radiochemical purity of >98%, unlabeled IA and [^{3}H]diethylstilbestrol (DES) with a sp. act. of 46.5 Ci/mmol were prepared by Chemsyn Science Laboratories (Lenexa, Kans.). 17β-[2,4,6,7-^{3}H]Estradiol (E_2), 110 Ci/mmol, with >98% radiochemical purity was obtained from Du Pont-New England Nuclear (Boston, Mass). Unlabeled *E*-DES and 3,3′,5-triiodo-L-thyronine (T_3) were obtained from Sigma (St Louis, Mo.) and tamoxifen was purchased from Amersham (Chicago, Ill.). Anti-Er monoclonal antibody, H-222, was a kind gift from Dr Chris Nolan of Abbott Laboratories (Chicago, Ill.). HPLC-grade solvents were obtained from Fisher (Fairlawn, N.J.).

High-performance liquid chromatography

[^{3}H]Indenestrol A (IA) racemic mixture was separated and purified into individual enantiomers according to the earlier procedure [8]. High-performance liquid chromatography (HPLC) was performed on an IBM Instrument LC/9533 ternary gradient liquid chromatography system (Danbury, Conn.) equipped with a variable-wavelength u.v. detector set at 280 nm, and a Bakerbond Chiralpak OP(+) column (4.6 × 250 mm, J. T. Baker Co. Phillipsburg, N.J.) which was packed with optically active helical polymer of triphenylmethyl methacrylate coated silical gel. Radioactivity was measured using a Radiomatic Flo-One/Beta Radiodetector (Tampa, Fla). For preparative HPLC, flow splitter (8:2 ratio) was used to collect the fractions. A solvent mixture of methanol–water (80:20, v/v) was used at a flow rate of 0.5 ml/min.

Tissue preparations and binding assays

Mouse uterine cytosol was prepared as described previously [10]. Ovariectomized female CD-1 [ICR]BR mice from Charles River Breeding Laboratories (Wilmington, Mass) were killed by cervical dislocation, the uteri were removed and frozen on dry ice. The frozen uteri were placed in ice-cold TEGM buffer (10 mM Tris, 1.5 mM EDTA, 10% glycerol, 3 mM $MgCl_2$, pH 7.6) and homogenized at a ratio of 50:1 (mg wet tissue wt/ml buffer). The 105,000 *g* supernatant was used immediately for cytosol receptor binding assays.

Binding assays were performed according to the previously reported procedure [10] with minor modification. All ligand-binding reactions were carried out at 4°C. Mouse uterine cytosol was incubated with one of the ^{3}H-labeled ligands at a concentration range of 0.2–10 nM either in the presence or absence of 200-fold excess unlabeled DES. After incubation for 16 h at 4°C, the bound ligand was quantified by adsorption to hydroxyapatite (HAP). The resulting HAP pellet was washed with TEGM buffer, suspended in toluene-based Liquifluor and the radioactivity was measured by scintillation counting in an Tri-Carb 4530 counter (Packard Instrument, Sterling, Va). Specific binding was determined as the difference between total (radioactive ligand only) and non-specific binding (radioactive ligand with excess unlabeled ligand) in the preparation.

Sucrose density gradient centrifugation

The assays were performed as previously reported [12]. Cytosols were incubated with 10 nM of labeled ligand in the presence or

absence of 200-fold excess of unlabeled competitor in a total volume of 0.25 ml of TEG buffer (10 mM Tris, 1.5 mM EDTA, 10% glycerol, pH 7.6). After incubating at 4°C for 4 h, the reactions were terminated by the addition of the reaction mixture to pellets of dextran-coated charcoal (DCC, 0.5% Norit, 0.05% dextran in TEG buffer, pH 7.6) to remove the unbound free ligand from the mixture. The 0.2 ml aliquots were applied on 5–20% linear sucrose gradient solution. The gradients were centrifuged for 17 h at 105,000 *g* in a Beckman SW60 rotor. Using a Haake Buchler Auto Dens-Flow IIC, the gradient was removed through the top of each tube and collected in three drop fractions into scintillation vials. Liquifluor was added to each vial and counted. [^{14}C]ovalbumin and [^{14}C]*r*-globulin were used as markers in the gradient tubes.

Ammonium sulfate fractionation of cytosol

Ammonium sulfate was added to the cytosols in small portions with gentle stirring to obtain a final concentration of 50%. The solution was stirred slowly for 60 min at 0°C, then centrifuged at 30,000 *g* for 30 min, and the precipitate was collected. The second fractionation of 80% precipitate was also collected. The 50 and 80% precipitates were resuspended in TEGM buffer and used for incubation.

RESULTS

HPLC with a Chiralpak OP(+) column permitted separation and purification of optically active [^{3}H]indenestrol A racemic mixture (IA-Rac) into individual enantiomers on a semi-preparative scale. Optimal separation was achieved with an eluting solvent mixture of 20% water in methanol similar to the separation achieved for unlabeled IA [8]. Since the Chiralpak OP(+) column was packed with silica gel coated with optically active polymer of triphenylmethyl methacrylate, this chiral phase slowly leaches out from the column over repeated runs. The Chiralpak OP column lasted longer under this condition than the Chiralpak OT column used in the previous report [13]. Radiopurity of the separated individual enantiomers was ⩾98%.

Saturation and Scatchard binding analysis were used to demonstrate the structure–activity binding relationship of the IA enantiomers. Direct saturation binding curves were generated for each [^{3}H]ligand; E_2, DES, IA-Rac, IA-*R*, IA-*S* from the cytosol binding assay. The

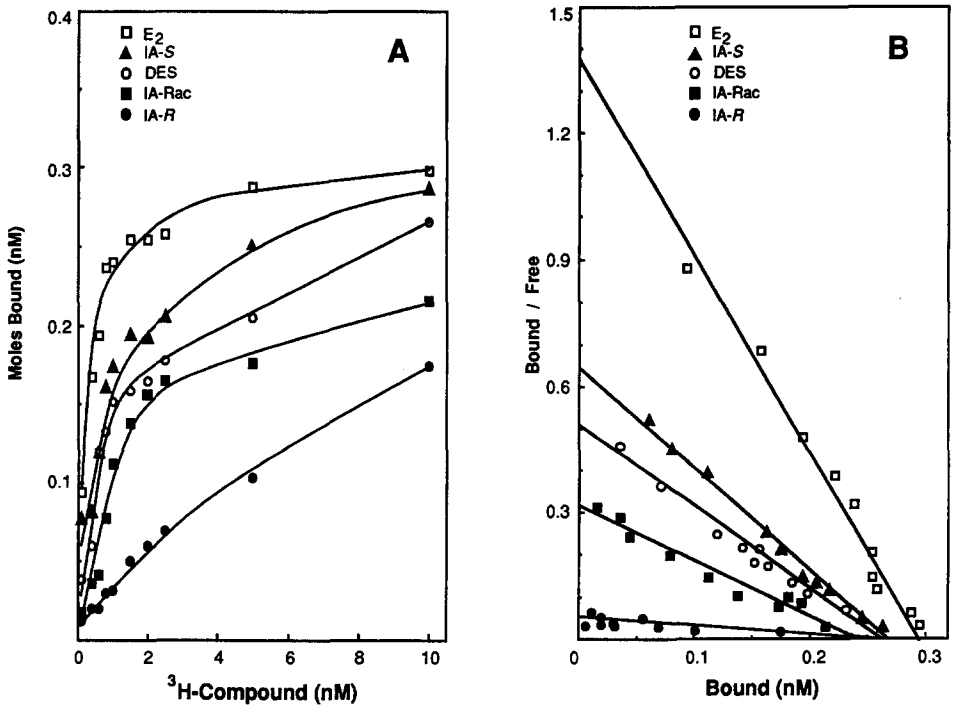

Fig. 1. (A) Specific binding of [^{3}H]ligands in mouse uterine cytosols. The cytosol was incubated with increasing concentrations of [^{3}H]ligands (0.2–10 nM) as described in Experimental. Non-specific binding was obtained at each concentration using a 200-fold excess of unlabeled DES competitor. Saturation binding was expressed in the preparation as specific binding. (B) Scatchard plots of the data presented in (A).

specific binding of the different compounds to the estrogen receptor is shown in Fig. 1. A comparison of the specific binding curves for each ligand showed that the specific binding of E_2, DES, and IA-*S* were approximately the same. IA-Rac was slightly lower and IA-*R* was about one half the level of the other ligands. Scatchard analysis of the saturation binding curves for each ligand was performed with EMF Software (Baltimore, Md) and the Scatchard plots shown in Fig. 1B. Dissociation constants (K_d) and the number of binding sites were determined for each ligand-receptor interaction (Table 1) from the plots. IA-*S* ($K_d = 0.67$ nM) binds to the receptor with the same affinity as DES ($K_d = 0.71$ nM) and four times higher affinity than IA-*R* ($K_d = 2.56$ nM). The low binding affinity of IA-*R* is also reflected in its low biological activity [7]. From the Scatchard analysis, IA-*S* binds to approximately the same number of binding sites as E_2, DES and IA-Rac while IA-*R* binds to far fewer sites than the other ligands. The K_d value of IA-*S* from the Scatchard plot is comparable to the value reported for IA-Rac ($K_d = 0.7$ nM) from the competition assays [7] indicating that the IA-*S* enantiomer is principally responsible for the binding activity measured in the competition assays. From this direct saturation binding assay with [^{3}H]ligands, DES (Fig. 2A) and the IA-*S* (Fig. 2B) compounds showed a higher level of non-specific binding to the cytosol receptor compared to E_2 (Fig. 2C) which exhibited a very low level of non-specific binding.

Sucrose density gradients were performed to characterize the cytosol sample components to which the [^{3}H]ligands were binding. Estrogen receptors (ER) are known to exist in an 8S form under low salt conditions [11]. As shown in Fig. 3, [^{3}H]E_2 showed (Fig. 3A) virtually only specific binding to the 8S component while [^{3}H]DES (Fig. 3B) and [^{3}H]IA-*S* (Fig. 3C)

Table 1. Comparison of dissociation constants (K_d) from scatchard and competitive binding assays

^{3}H-Labeled ligand	Scatchard K_d (nM)	Competition* K_d (nM)
E_2	0.21	1.0
DES	0.71	0.4
IA-*S*	0.67	—
IA-Rac	2.03	0.7
IA-*R*	2.56	—

The ^{3}H-labeled ligands were incubated with mouse uterine cytosol in the presence or absence of 200-fold excess unlabeled DES. Binding was assayed as described in Experimental.
*From Ref. [7].

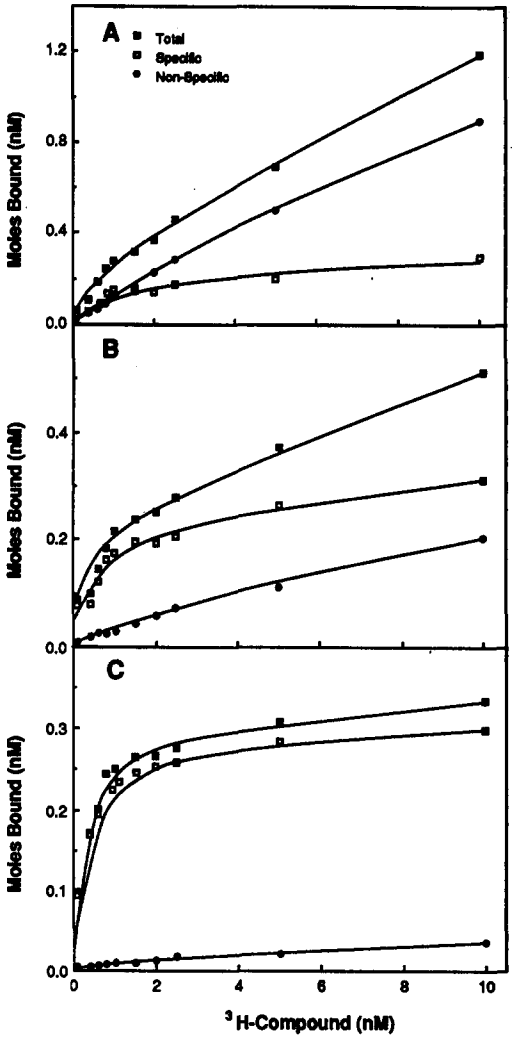

Fig. 2. Saturation analysis of [^{3}H]ligands. Mouse uterine cytosol was incubated with (A) [^{3}H]DES, (B) [^{3}H]IA-*S* and (C) [^{3}H]E_2 in the absence or presence of 200-fold molar excess of DES. Specific binding was determined as difference between total and non-specific binding.

bound to other components in the 4S region of the gradient in addition to the 8S component. Addition of 200-fold excess of unlabeled E_2 partially displaced the [^{3}H]DES or [^{3}H]IA binding in the 4S component. A 200-fold excess of unlabeled DES displaced one-half of the [^{3}H]DES binding to the 4S component.

To further characterize the ER and non-receptor binding components, the mouse uterine cytosol was incubated with [^{3}H]E_2 and [^{3}H]DES in the presence of monoclonal antibody, H-222. The antibody has been used for specific detection of ER in mouse tissues [14]. The H-222 antibody shifted only the 8S component in both [^{3}H]E_2 (Fig. 4A) and [^{3}H]DES (Fig. 4B), and the [^{3}H]DES binding 4S component was not affected by the addition of the antibody. The

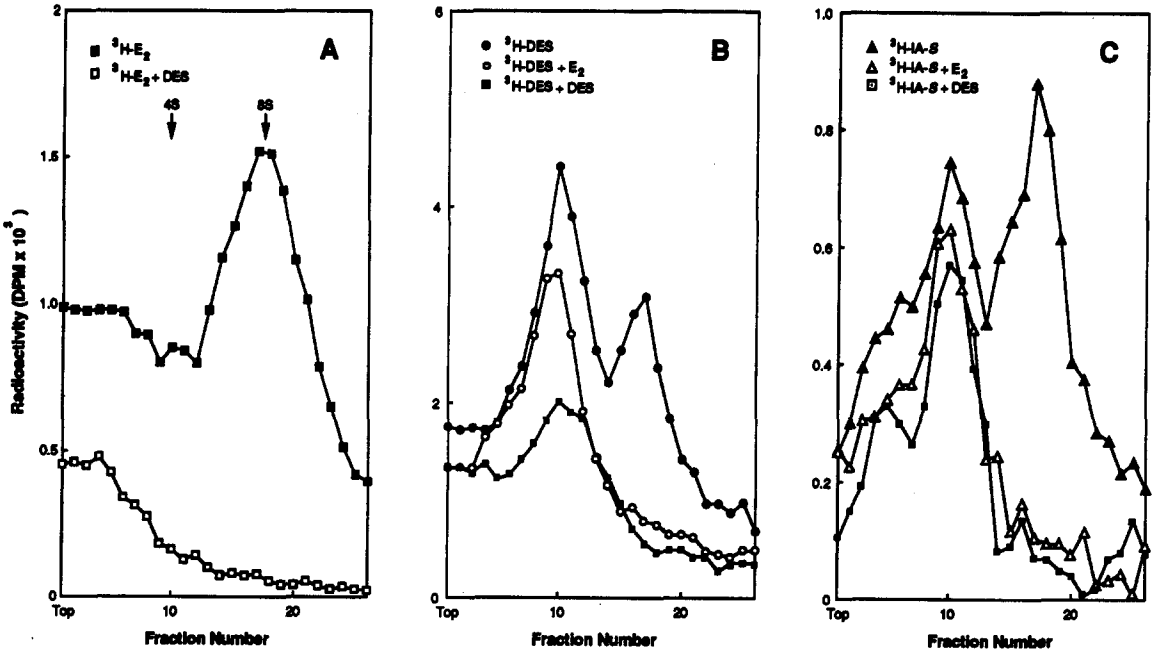

Fig. 3. Sucrose density gradient analysis of [^{3}H]ligands binding in mouse uterine cytosol. The cytosol was incubated with (A) [^{3}H]E_2, (B) [^{3}H]DES and (C) [^{3}H]IA-*S* in the presence of 200-fold excess of unlabeled E_2 or DES as described in Experimental.

alkylaromatic ring structure of the stilbestrol compounds is similar to the thyroid hormones. In order to determine whether the 4S component could be a thyroxine type receptor, the mouse uterine cytosol binding was incubated with unlabeled T_3. The T_3 did not compete for the 8S nor 4S components with [^{3}H]E_2 or [^{3}H]DES (data not presented).

The sedimentation similarity of the 4S component to that of the type II estrogen

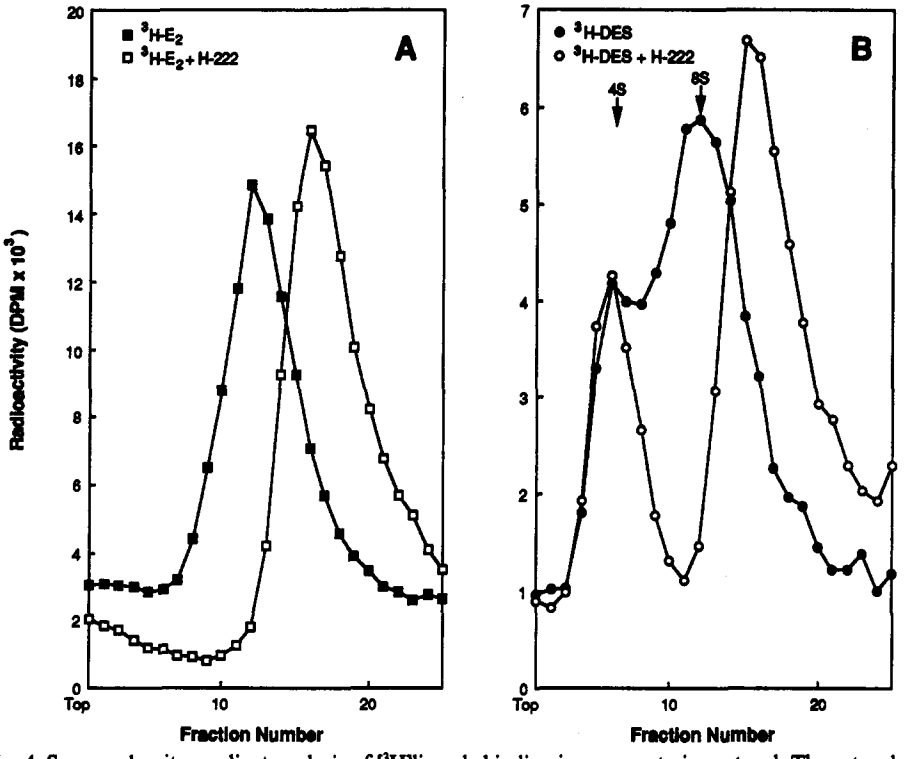

Fig. 4. Sucrose density gradient analysis of [^{3}H]ligands binding in mouse uterine cytosol. The cytosol was incubated with (A) [^{3}H]E_2 and (B) [^{3}H]DES in the absence or presence of ER monoclonal antibody, H-222.

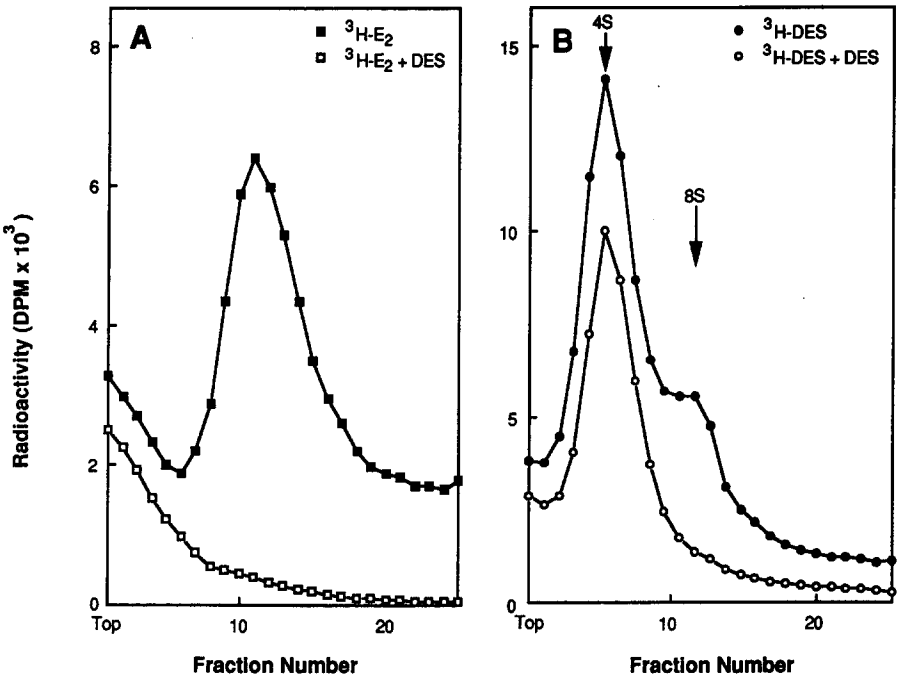

Fig. 5. Sucrose density gradient analysis of [^{3}H]ligands binding in mouse uterine cytosol. The cytosol was incubated with (A) [^{3}H]E_2 and (B) [^{3}H]DES in the absence or presence 200-fold excess of unlabeled DES with 1 mM of dithiothreitol in the preparation.

binder [9] was investigated. It has been reported that the type II estrogen binding sites are sensitive to reducing agents such as dithiothreitol (DTT) [15]. When 1 mM of DTT was added to the TEG buffer, it did not affect the binding or sedimentation characteristics of either the 4S or 8S components in the uterine cytosol (Fig. 5A and B).

Cytosols from ER non-responsive tissues were tested to evaluate the tissue specificity of the binding components on [^{3}H]DES. When [^{3}H]DES was incubated with cytosols from mouse lung (Fig. 6A) and liver (Fig. 6B), it only

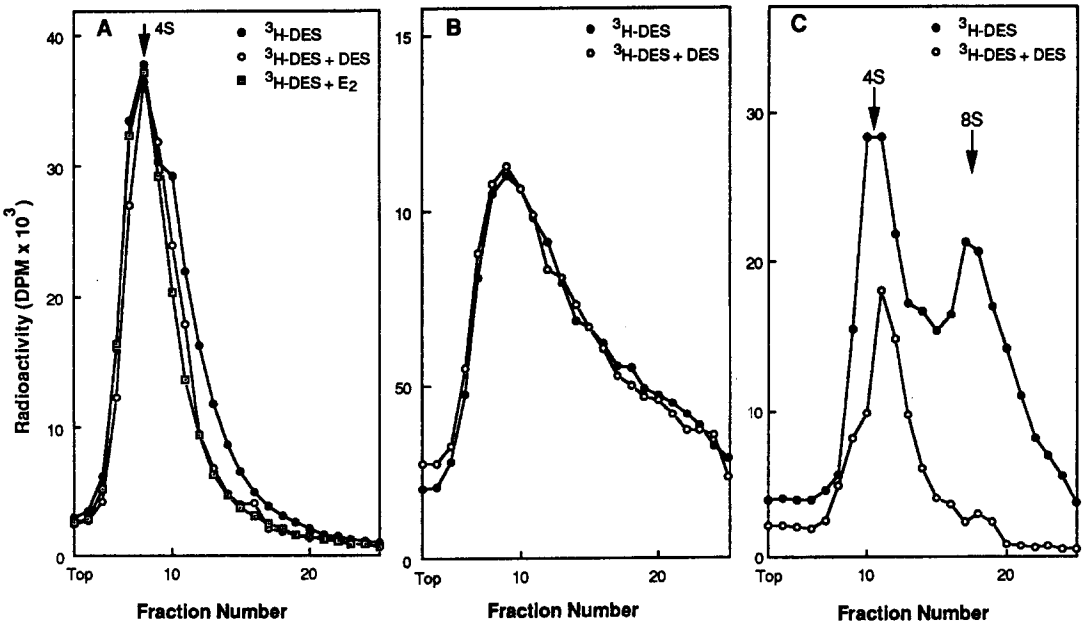

Fig. 6. Sucrose density gradient analysis of [^{3}H]DES binding in mouse (A) lung, (B) liver and (C) pituitary cytosols. The cytosol was incubated with [^{3}H]DES in the absence or presence 200-fold excess of unlabeled DES.

bound to a 4S component which was not displaced by excess unlabeled DES nor E_2. However, other ER-responsive tissues, such as cytosol from mouse pituitary, have both the 4S and 8S components (Fig. 6C).

Ammonium sulfate purification methodology was used to further characterize these 4S and 8S binding components in the cytosol preparation. The mouse uterine cytosols were saturated with 50% ammonium sulfate, followed by 80% saturation. The resulting precipitates were resuspended in TEG buffer, incubated with [^{3}H]E_2 and [^{3}H]DES, and analyzed by sucrose density gradient centrifugation. The specifically bound 8S component is located in the 50% ammonium sulfate precipitate fraction for both [^{3}H]E_2 and [^{3}H]DES, while the 4S [^{3}H]DES binding component is located in the 80% precipitation fraction (data not presented).

DISCUSSION

Purified high specific activity ^{3}H-labeled IA enantiomers and DES were used in this study to determine the receptor binding activities and characteristics through direct binding analysis. Various steroids and synthetic compounds have been used to explore structure–activity relationships between estrogenic compounds and the ER [7, 9]. The Scatchard and saturation binding analyses confirmed the earlier studies with competitive binding assays [7, 10] that mouse uterine cytosolic ER exhibited stereochemical preference for the IA-*S* enantiomer. The X-ray crystal structure [10] revealed that IA-*S* enantiomer has an *S*-configuration at carbon-3 position. The fact that the ER exhibits chiral selectivity emphasizes the usefulness of chiral HPLC in the separation and purification of enantiomeric forms of chiral estrogenic non-steroid compounds. The K_d for the IA-Rac mixture from the competitive and Scatchard analyses are different because in Scatchard analysis the IA-*R* enantiomer of the mixture is measured directly as it interacts with the receptor. In Scatchard analysis, the free ligand is determined by subtracting the amount of ligand bound from the total amount of ligand added. The actual concentration of active ligand (IA-*S*) added to the IA-Rac incubation is less than the calculated concentration because IA-*R* enantiomer in IA-Rac mixture is biologically inactive [9]. The higher resulting concentration of free ligand in the ratios resulted in the different K_d for IA-Rac between these analysis.

In order to characterize the receptor form to which the [^{3}H]IA and [^{3}H]DES were binding, sucrose density gradient centrifugation was used. Previous studies [11] showed that the cytosolic form of the ER with [^{3}H]E_2 on sucrose gradients in TE buffer is an 8S form. Both unlabeled DES and IA have been shown to be specific for this receptor through sucrose density gradient and rate inhibition reactions in cytosols [6]. The binding of [^{3}H]DES and [^{3}H]IA to the 8S component and the resulting peak shift by the addition of H-222 antibody verifies that these compounds are binding to the ER. The existence of the 4S binding component for these [^{3}H]ligands raised questions concerning its identity. An additional binding component (4S form) has been observed in rat uterine cytosol with labeled tamoxifen and ER [16]. It has been postulated that such a 4S component may result from weak affinity ligands dissociating from the ligand–receptor complex and binding to non-receptor proteins which sediment in the 4S region as a result of lengthy preparation [16]. However, DES and IA are high affinity ligands for ER, and the fact that excess unlabeled tamoxifen did not displace the ligand binding of this 4S component (data not shown) discounted this hypothesis as a possible explanation. Previous studies have shown multiple binding components to exist in rat uterine cytosol [17]. It is plausible that the component may be the type II receptor since all stilbene compounds were found to interact with both the type II sites in the proper concentration range and the type II site sediments at 4S form [9]. However, our experiment utilized 10 nM ligand concentrations in the incubations mixture while all previous observations of interaction with the type II receptor have been at ligand concentration ranges of 10–40 nM [9]. In addition, the 4S component was not sensitive to sulfhydryl reducing agents such as 1 mM DTT indicating by previous criteria that this is not the type II site.

The presence of this 4S component explains other observations made in our study. First, since the 4S component is partially displaceable by the addition of excess unlabeled DES, the high degree of non-specific binding observed in the saturation analysis most likely is the result of ligand binding to the 4S component. Second, the existence of such a component may explain the difference in K_d values determined from the competitive binding and the Scatchard binding analysis (Table 1).

A most interesting characteristic of these sites is their apparent exclusive presence in estrogen target tissues such as uterus and anterior pituitary. Such a localization would suggest a relationship of the 4S binder and the estrogen receptor protein indicating a possible modified or proteolyzed form of the ER. If these are modified forms of the ER then the modifications have altered the receptor's ligand binding specificity. On the other hand, the 4S binder could also be the expression of a mutant form of the ER in target tissues which has gone undetected in the past due to the lack of highly purified and high specific activity stilbestrol ligands. These possibilities would appear unlikely, since the 4S binder was not detected by the ER monoclonal antibody H-222 which detects all the major *in vivo* forms of the ER [18] and has general reactivity in all species [14]. Therefore, the identity of the [^{3}H]DES and [^{3}H]IA binding 4S component is not conclusive at present. The possibility of it being an enzyme or contaminating microsomal binder could not be ruled out at the present time. However, it would be surprising that the enzymes are specific to estrogen target tissues and would not be present in other tissues. DES is a potent ER ligand and agonist which is retained to high levels by most estrogen target tissues. In the past, this tissue localization has been related to the ER binding for the DES ligand. Another possibility shown by our new data is that the significant tissue localization of DES is also, in part, due to the presence in target tissues such as the uterus of this 4S binder. The binder acts to localize DES to the tissue where it could facilitate binding by the ER. This is particularly related to the comparison between estradiol and DES where the higher DES localization and occupancy had been suggested to be due to a higher receptor binding affinity for DES compared to estradiol. It is plausible that the high tissue concentrations of DES are due, in part, to the 4S binder. Thus DES potency, toxicity or carcinogenecity in these tissues may result, in part, from the presence of such a non-ER binding component.

Acknowledgements—The authors wish to thank Dr Louis A. Levy for helpful discussions and Ella Snead for technical aid.

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