RADIOCHEMISTRY AND RADIOPHARMACEUTICALS

Receptor-Binding Radiopharmaceuticals for Imaging Breast Tumors: Estrogen-Receptor Interactions and Selectivity of Tissue Uptake of Halogenated Estrogen Analogs

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Four halogenated estrogen analogs—*o*-fluorohexestrol, and 1-fluoro-, 1-bromo-, and 1-iodohexestrol—have been prepared and tritium-labeled in high specific activity, to investigate their potential as estrogen-receptor-based agents for imaging breast tumors. These compounds bind with high affinity in vitro to the cytoplasmic uterine estrogen receptor from rat and lamb and sediment as 8S receptor complexes on sucrose gradients. After 1 hr in immature rats, these compounds show high uptake into the uterus, but low uptakes (10–25% of the uterine levels) into most nontarget tissues. The uterine uptake is estrogen specific since it is depressed by excess nonradioactive estradiol. Uptake selectivity is greatest for the fluorohexestrols and decreases for the bromo and iodo compounds. In mature rats bearing DMBA-induced mammary tumors, selective uptake by the uterus and tumors is seen with 1-fluoro[$^3H_4$]hexestrol and *o*-fluoro[$^3H_3$]hexestrol. The studies indicate that these four halogenated hexestrols are promising candidates as estrogen-receptor-based agents for the imaging of human breast tumors.

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Estrogen receptors that are found in the majority of human breast tumors have been the focus of great clinical interest, because the receptor content of a breast tumor appears to be the most accurate index of how the cancer will respond to alternative therapies (*1*). Tumors that lack estrogen receptors do not respond to hormonal therapy, whereas those that contain them in significant concentration show a high rate of response to hormonal therapy. Many studies have dealt with the quantitative aspects of estrogen-receptor content and response rate of breast cancer (*2,3*). More recently there has been interest in developing radiopharmaceuticals that will concentrate in breast tumors on the basis of estrogen-receptor binding (*4–10*). Such agents could provide valuable diagnostic information, as they would assist tumor location and would delineate the pharmacodynamics of estrogen-receptor interaction noninvasively and under in vivo conditions. Because the estrogen-receptor content of breast tumors is generally quite low (*2,3*), agents with high specific activity are needed to ensure that the receptor-based uptake of activity by the tumor is adequate for detection. In addition, from the results of preliminary studies (*4,5*), it is apparent that in order to achieve satisfactory contrast (tumor uptake against background), agents need to have both high affinity for the receptor *and* relatively low affinity for nonreceptor sites.

We have recently described the synthesis of two series of halogenated estrogens: estradiol and hexestrol derivatives fluorinated in the aromatic ring (*11*), and hexestrol derivatives bearing a halogen at the terminus of the hexane chain (*12*). In competitive binding assays, most of these compounds display high affinity for the estrogen receptor from lamb or rat uterus. In this report we describe the preparation of four of these compounds in high-specific activity, tritium-labeled form, and we report on their interaction with estrogen receptors in vitro

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and on the selectivity of their tissue uptake in rats under in vivo conditions. The results suggest that some of these compounds (particularly the fluorine-substituted ones) have promise as estrogen-receptor-based agents for imaging breast tumors.

MATERIALS AND METHODS

Materials were obtained from the following sources: tris(hydroxymethyl) aminomethane(Tris),* charcoal Norit A,* Dextran grade C,* *p*-bis[2-(5-phenyl oxazolyl)]benzene; tritiated sodium borohydride,† Nuclear Chicago Solubilizer,† 17β-[6,7-^{3}H]estradiol (40–50 Ci/millimole); Triton X-114‡; (ethylenedinitrilo)-tetraacetic acid (EDTA)‖; sodium azide§; 17β-estradiol¶; pyridinium chlorochromate (98%)**; boron tribromide (99.9%)††; 2,5-diphenyloxazole‡‡; and diethylaminosulfurtrifluoride (DAST)‖‖. (Caution: This last reagent and its hydrolysis product (HF) are corrosive and extremely toxic. It may decompose explosively if heated to about 50°C. Use only with adequate protection and in a well-ventilated hood.) The syntheses of compounds **2a**, **7a**, and **8a** have been described previously (*12*). Marker proteins used in sucrose-gradient experiments* were C-14-labeled by the procedure of Rice and Means (*13*). Radiochemical purity determinations were performed as described previously (*14*). Melting points§§ are corrected.

Except where mentioned otherwise, a standard procedure was used for product isolations; this involved quenching by addition to water, exhaustive extraction with a solvent (washing on occasion to extract with aqueous solutions), drying over an anhydrous salt, and evaporation of solvent under reduced pressure. The particular solvents, aqueous washes (if used), and drying agents are mentioned in parentheses after the phrase "product isolation."

Radioactivity was measured in minivials in a liquid-scintillation counter, using ~5 ml of xylene-based cocktail containing 0.55% 2,5-diphenyloxazole, 0.01% *p*-bis[2-(5-phenyloxazolyl)]benzene, and 25% Triton X-114. Tritium counting efficiency was 25–55%.

***Erythro*-3,4-bis(4-methoxyphenyl-1-hexanal (1a).** Pyridinium chlorochromate (275 mg, 1.275 millimole) was stirred in 2 ml of dichloromethane, and a solution of *erythro*-3,4-bis(4-methoxyphenyl)-1-hexanol (**2a**) (250 mg, 0.769 millimole) in 4 ml of dichloromethane was rapidly added at room temperature. After 1.5 hr, anhydrous ether (20 ml) was added and, after being stirred for 2 min, decanted. The residue was triturated with two further portions of ether, and the combined brown organic extract was filtered through a pad of florisil and evaporated under reduced pressure to give 230 mg (92.5%) of *erythro*-3,4-bis(4-methoxyphenyl)-1-hexanal (**1a**) as white crystals (mp 137–138°C).

***Erythro*-3,4-bis(4-methoxyphenyl)-[1-^{3}H]-1-hexanol (2b).** *erythro*-3,4-bis(4-methoxyphenyl)-1-hexanal (**1a**) (25 mg, 0.08 millimole) was dissolved in a mixture of 8 ml of ethanol and 2 ml of THF and cooled to −10°C in an ice-acetone bath. To this solution was added 100 mCi (0.057 millimole of $^3H^-$; 7 Ci per millimole) of NaB^3H_4, and the reaction mixture was slowly allowed to warm to room temperature. The progress of the reaction was monitored by following the increasing ratio of the ethyl acetate-extractable radioactivity, measured after acidification, as compared to the total radioactivity measured directly; the reaction was complete after 19 hr. The solvent was then evaporated under a stream of nitrogen, and to the solid residue was added 2 ml 0.5 *M* HCl. Product was extracted with 1:1 ether:dichloromethane and was dried (Na_2SO_4). This material was purified by preparative TLC (1:1 hexane:ether, two developments) or column chromatography (silica gel, 1:1 hexane:ether), giving a combined yield of 93.6 mCi of the tritiated alcohol (**2b**). Radiochemical purity was greater than 93%, as measured by analytical TLC in 1:2 hexane:ether, two developments).

***Erythro*-3,4-bis(4-hydroxyphenyl)-[1-^{3}H]-1-hexanol (3b) and *erythro*-3,4-bis(4-hydroxyphenyl)-1-bromo-[1-^{3}H]hexane (4).** A 12 mCi aliquot of the tritiated alcohol **2b** was dissolved in 2 ml of dry dichloromethane (4 Å molecular sieves) and cooled to −78°C. Boron tribromide (0.5 ml of a 1.05 *M* solution in dichloromethane) was slowly added under nitrogen atmosphere, and the reaction mixture was warmed to room temperature over a period of 3 hr. At this point, TLC analysis showed the absence of **2b** and the formation of **3b** (78%) and **4** (22%). The reaction mixture was cooled to −78°C, and the excess boron tribromide destroyed by slow addition of anhydrous methanol. The solvents were evaporated under a stream of nitrogen, and 1 ml of water was added to the residue. Product isolation (EtOAc, Na_2SO_4) and preparative TLC (1:1 CH_2Cl_2:EtOAc, two developments) gave 2 mCi (16%) of the tritiated 1-bromohexestrol (**4**) as the less polar component (radiochemical purity 82% by TLC in 1:1 CH_2Cl_2:EtOAc), and 8 mCi (67%) of tritiated 1-hydroxyhexestrol (**3b**) as the more polar component.

***Erythro*-3,4-bis(4-hydroxyphenyl)-1-fluoro-[1-^{3}H]hexane (5).** *Method 1*. (*From* **3b**). An aliquot of the tritiated phenolic alcohol, **3b**, was dissolved in 1 ml of dry THF and cooled to −10°C with an ice-acetone bath. To this cold solution was added 20 μl of a DAST stock solution (prepared by adding 875 μl THF to 125 μl DAST). The reaction mixture was then slowly warmed to room temperature, and the progress of the reaction monitored by analytical TLC. After 17 hr, an additional 150 μl of the DAST stock solution was added, and stirring was continued for an additional 30 hr. The reaction was quenched with sodium bicarbonate, and product isolation (ethyl acetate, dilute HCl, H_2O, brine, $MgSO_4$) gave the crude product that was purified by column chromatography on silica gel (4:4:2 hexane:CH_2Cl_2:EtOAc); peak fractions totaling 0.8 mCi (10% yield) were combined (radiochemical purity 92%).

Method 2 (**7c**). (*From* **7b**). (This reaction was performed at New England Nuclear Corp, Boston, MA; purification was done in our laboratories.) Tetraiodo-1-fluorohexestrol **7b** (40 mg, 0.05 millimole) and 60 μl of triethylamine (44 mg, 0.44 millimole) were dissolved in 5 ml ethyl acetate; 65 mg of 5% palladium on alumina was added, and the mixture was stirred overnight with 25 Ci of tritium gas. After filtration and removal of labile tritium and solvent *in vacuo*, the residue was dissolved in 10 ml of 10:1 benzene:ethanol for storage and shipping. Two aliquots were chromatographed on 1- × 15-cm silica-gel columns, eluting with a gradient (0–40% ether in 2:1 hexane:pentane) to give 122 mCi of material ranging from 94 to 98% radiochemical purity by TLC.

***Erythro*-3,4-bis(4-hydroxyphenyl)-1-iodo-[1-^{3}H]hexane (6).** The solvent from a solution containing 1.0 mCi of tritiated phenolic bromide, **4**, was evaporated under a stream of nitrogen. A saturated solution of sodium iodide in acetone (5 ml) was added to this residue, and the resulting solution was heated under reflux in the dark under nitrogen atmosphere for 6 hr. After evaporation of the solvent under a stream of nitrogen, product isolation (EtOAc, H_2O, aq. NaCl, $MgSO_4$) gave a residue that was purified on a silica-gel column (1:1 CH_2Cl_2:EtOAc). The desired fraction contained 0.5 mCi (50% yield) of **6** (radiochemical purity 92% by TLC in 1:1 CH_2Cl_2:EtOAc).

***Erythro* - 3,4-bis(3,5-diiodo-4-hydroxyphenyl)-1-fluorohexane (7b).** Resublimed iodine (96 mg, 0.38 millimole) was dissolved in 2 ml THF and added dropwise under photographic darkroom lighting to a solution of 29 mg (0.1 millimole) *erythro*-3,4-bis(4-hydroxyphenyl)-1-fluorohexane (**7a**) (*12*) in 3 ml of methanol and 0.5 ml of concentrated aqueous ammonia. After being stirred for 1.5 hr under nitrogen, the mixture was acidified with glacial acetic acid. Product isolation (EtOAc, aq. $Na_2S_2O_3$, H_2O, $MgSO_4$) gave 72 mg (91%) of white crystalline tetraiodofluorohexestrol, **7b**, pure by TLC. After recrystallization from THF:ethanol, it had a

melting point of 230°C.

***Erythro*-3-fluoro-3′,5,5′,-triiodohexestrol (8b).** Resublimed iodine (665 mg, 2.6 millimole) was dissolved in 1.5 ml THF and added dropwise under photographic darkroom lighting to a solution of 250 mg of *o*-fluorohexestrol (*11*) (**8a,** 0.87 millimole) in 3 ml of methanol and 1.5 ml of concentrated aqueous ammonia. After being stirred for a total time of 1.5 hr under nitrogen atmosphere, the mixture was acidified with glacial acetic acid and partitioned between ethyl acetate and water. After the organic layer was washed (H_2O) and dried (Na_2SO_4), and the solvent removed, the crude product was purified by MPLC, (15% ether in hexane) to give 450 mg (78%) of the triiodinated fluorohexestrol, **8b.** The analytical sample was recrystallized from chloroform: cyclohexane and from ethanol: water, mp 160–161°C.

***Erythro*-3-fluoro-[3′,5,5′-$^3H_3$]hexestrol (8c).** (This reaction was performed at New England Nuclear Corp., Boston, MA; purification was done in our laboratories.) 3-Fluoro-3′,5,5′,-tri-iodohexestrol (**8b,** 66 mg, 0.1 millimole) was dissolved in 5 ml of ethyl acetate plus 85 μl of triethylamine, and 100 mg 5% palladium on alumina and 25 Ci of tritium gas were added. After stirring for 5 hr at atmospheric pressure, labile tritium was removed *in vacuo* using ethanol as solvent. The catalyst was removed by filtration, and the crude product was dissolved in 10:1 benzene:ethanol for shipping and storage. A 1000-mCi aliquot was purified in three batches on open silica-gel columns eluted with 6:4:1 hexane:dichloromethane:ethyl acetate; the peak fractions showed >95% radiochemical purity and were pooled and stored in ethanol.

Binding measurements. Rat cytosol was prepared as described previously (*15*) from immature (21–25 day) female Holtzman rats and was stored in liquid nitrogen. Lamb cytosol was prepared and stored as described previously (*16*). All binding experiments were done in TEA buffer (0.01 *M* Tris·HCl, 0.0015 *M* EDTA, 0.02% sodium azide, pH 7.4 at 25°C) as previously reported (*15*), the free estrogen being removed by charcoal-dextran adsorption. The relative binding affinities of unlabeled compounds were determined according to our published method (*17*) and are expressed as the ratio of association constants: $100 \times K_a$ (compound)/K_a (estradiol). The affinity constants of the tritium-labeled compounds were determined directly by Scatchard analysis (*18*).

Density gradient centrifugation. Linear 5–20% sucrose-density gradients (4 ml) were prepared in TEA (low salt) or 0.4 *M* KCl in TEA (high salt). The gradients were centrifuged at 200,000 *g* for 17 hr. All other operations were carried out as previously described (*15*). The receptor preparations for the low-salt gradients were frozen rat cytosol. For the high-salt gradients, the receptor was prepared by extraction of a washed nuclear pellet from immature rat uteri incubated in vitro. The in vitro incubation and extraction was performed as described by Ruh et al. (*19*).

In vivo tissue uptake studies. Animals used in these studies were either immature female Holtzman rats (21–25 day, 50 g) or mature female Sprague–Dawley rats (300 g) bearing mammary tumors induced by dimethylbenzanthracene, as described by Tsai and Katzenellenbogen (*20*). Animals were injected i.v. (tail vein) with physiological saline containing 0.3 μCi of tritiated compound per gram body weight, with or without a 100-fold excess (0.15 μg/g) of unlabeled estradiol. The animals were provided with food and water and were decapitated at the indicated times. Blood was collected and tissues were excised and immediately weighed. The solid tissues were digested in NCS (Nuclear Chicago Solubilizer) at 1 ml NCS per 0.1 g tissue at 50°C until dissolved (usually overnight). The samples were cooled and 1–3 ml were added to 10 ml of scintillation fluid. The samples were neutralized with 100 μl of glacial acetic acid and counted. The blood was either treated as above, or homogenized to break up the clot and then extracted with an equal volume of 100% ethanol at 0°C for 30 min. The ethanol precipitate was pelleted at 800 *g* for 10 min; 99% of the dpm remained in the supernatant.

Metabolism studies. Animals were treated with tritiated compound as described above. Tissues were excised and weighed, then minced and homogenized in two volumes of TEA buffer. A volume of 100% ethanol equal to the total homogenate volume was added, and the sample was allowed to precipitate. Centrifugation (800 *g* for 10 min) gave clear supernatants that were removed and evaporated to dryness under a stream of nitrogen; extraction efficiency was 93%. The residue was resuspended in a minimum volume of ethanol, and an aliquot equivalent to 10,000 cpm was used for chromatographic analysis. Thin layer chromatography was performed on plastic-backed silica-gel plates;[§] and high-pressure liquid chromatography was carried out[¶¶] using gradient elution starting at 60% methanol in water, with the methanol concentration increasing at 2%/min after the first 2 min; the flow rate was 100 ml/hr. Samples of authentic unlabeled compound were added to the extract to permit identification of the eluting radioactivity by ultraviolet absorbance at 254 nm.

RESULTS

Radiochemical synthesis. We have previously described the synthesis of 1-fluoro, 1-bromo, and 1-iodohexestrol from the triol, **3a,** (*12*). Tritium could be introduced into this series of compounds by sodium borotritide reduction of the aldehyde methyl ether, **1a,** a compound that was prepared from the corresponding alcohol, **2a,** whose synthesis we have described (Fig. 1) (*12*). In order to utilize the labeled borohydride efficiently, it was important to use an excess of aldehyde, **1a,** under conditions that would ensure complete consumption of the active hydride. (In a preliminary study, we found the phenolic aldehyde, **1b,** to be unstable and thus unsatisfactory as a precursor for tritium incorporation.) The labeled methoxy alcohol, **2b,** easily separated from the excess starting material by silica-gel chromatography. Ether cleavage with boron tribromide gave a 4:1 mixture of the corresponding labeled alcohol, **3b,** and phenolic bromide, **4,** which were easily separated by preparative thin layer chromatography. The phenolic iodo compound, **6,** was prepared from **4** by iodide displacement of bromine, and the fluoro compound, **5,** was obtained by the treatment of the triol, **3b,** with DAST. The yields are shown in Fig. 1.

The specific activities of both the bromophenol, **4,** and the fluorophenol, **5,** were determined by measurements of receptor-binding capacity (Scatchard analysis) to be 1.6 Ci/millimole, or 91% of that theoretically possible. While this specific activity is adequate for certain of the biochemical experiments, we desired to have the 1-fluoro compound labeled at higher specific activity for tissue distribution studies. Treatment of the fluorophenol, **7a,** with excess iodine in methanolic ammonium hydroxide gave the tetra ortho iodinated analog, **7b,** (Fig. 2). The iodine was replaced by tritium (at New England Nuclear) by exposure to carrier-free tritium gas over 5% palladium on alumina in ethyl acetate: triethylamine. The crude product, **7c,** showed high radiochemical purity and was separated from a small amount of colored impurity by column chromatography. The material purified

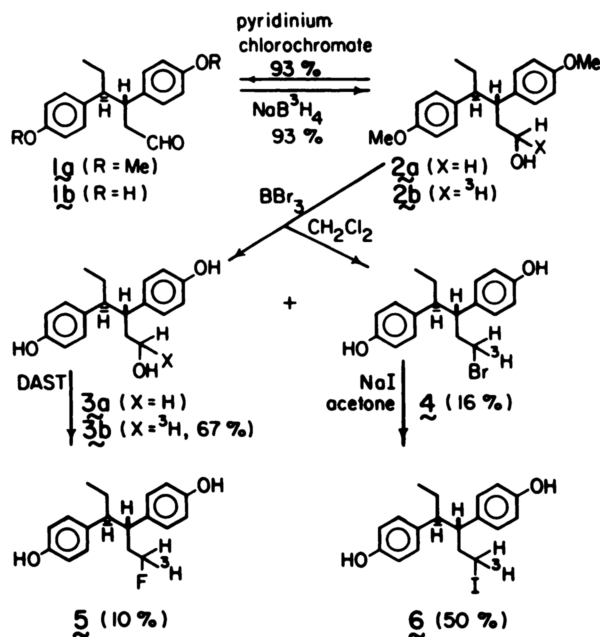

FIG. 1. Synthesis of tritium-labeled side-chain-halogenated hexestrols using sodium borotritide.

by column chromatography had a radiochemical purity of 94–98% and a specific activity of 60 Ci/millimole, determined both by receptor binding and uv analysis.

In a similar fashion, *o*-fluorohexestrol, **8a**, was tritium-labeled by iodination to 3-fluoro-3′,5,5′-triiodohexestrol, **8b**, followed by iodide reduction with carrier-free tritium gas (Fig. 2) to give, after purification, material (**8c**) with a radiochemical purity of >95% and a specific activity of 52 Ci/millimole as determined by uv analysis.

In vitro binding studies with estrogen receptor. The four halogenated hexestrols show high-affinity binding to estrogen receptor in cytosol from rat or lamb uterus. The binding-affinity data for all four compounds are summarized in Table 1, and a representative Scatchard plot for estradiol and 1-fluorohexestrol is shown in Fig. 3. In general, there is good agreement between the estimates of receptor-binding affinity obtained directly using the tritium-labeled compounds and those measured indirectly, by competitive binding assay against [^{3}H]estradiol.

Sedimentation through sucrose gradients is a classical method for characterizing estrogen receptor binding in cytosol preparations (*21*). The sedimentation profiles of [^{3}H]estradiol and the four tritium-labeled halogenated hexestrols bound to rat uterine cytoplasmic receptor under low-salt conditions are shown in Fig. 4. Under these conditions, the estrogen receptor runs as an aggregated species with a sedimentation coefficient of 8S. The specificity of the binding is confirmed by the displacing action of an excess of unlabeled estradiol (dashed lines).

o-fluoro[^{3}H]hexestrol and 1-fluoro[^{3}H]hexestrol have

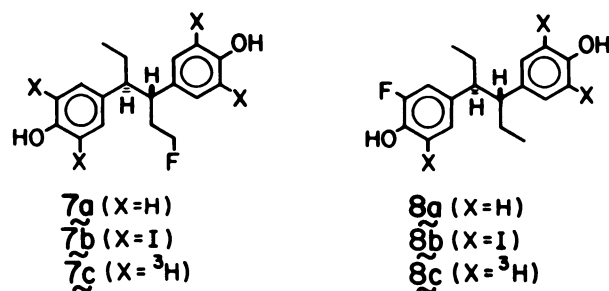

FIG. 2. Tritium labeling of fluorinated hexestrols by iodine reduction with tritium gas.

8S peaks that appear comparable in size to that seen with [^{3}H]estradiol, whereas the 8S peaks seen with 1-bromo[^{3}H]hexestrol and 1-iodo[^{3}H]hexestrol appear to be much smaller. This is due to the lower specific activity of the latter two compounds (1.7 Ci per millimole) relative to the others (40–60 Ci per millimole); so the quantity of 8S binding in the last two gradients is, in fact, similar to that in the first three.

The major difference among these gradients is the size of the 4S peak. Binding in the 4S region of low-salt sucrose gradients is generally associated with nonspecific binding; this is confirmed by its persistence even in the presence of excess, unlabeled estradiol. The size of the 4S peak relative to the 8S peak is a direct measure of the binding selectivity of these agents. By this criterion, it is apparent that the two fluorohexestrols have binding selectivities that are quite comparable to that of estradiol,

TABLE 1. ESTROGEN-RECEPTOR-BINDING AFFINITY OF HALOGENATED HEXESTROLS

			Affinity relative to estradiol*	
X	Y	Compound No.	by competitive binding†	by direct binding‡
F	H	(5,7)	126	165
Br	H	(4)	65	35
I	H	(6)	60	64
H	F	(8)	234	200

* Expressed as 100 times ratio of association constants: $100 \times K_a^{compound}/K_a^{estradiol}$. Under these conditions, the K_a for estradiol is 4.5×10^9 M^{-1} (see Fig. 3).

† Average of at least two determinations; measured in lamb uterine cytosol. Data are from Ref. *12* and method is from Ref. *17*.

‡ Binding incubations for Scatchard analysis included 7% dimethylformamide, as did the competitive binding experiments.

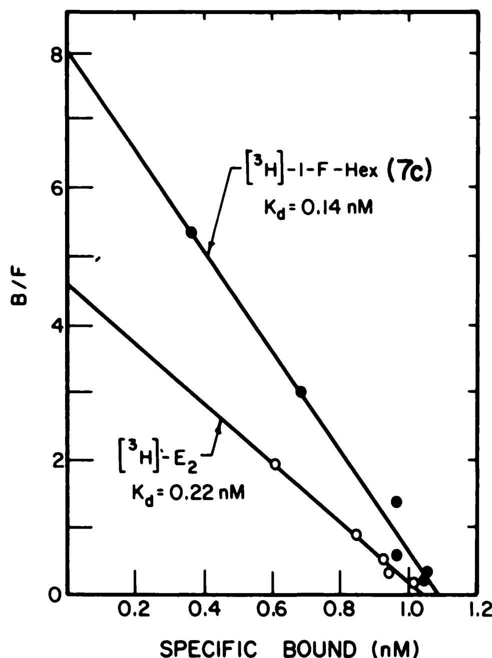

FIG. 3. Binding of [^{3}H]estradiol ([^{3}H]E_2) and [^{3}H] 1-fluorohexestrol ([^{3}H] 1-F-Hex, 7c) to lamb uterine cytosol, plotted according to Scatchard. Concentrations of compounds varied from 0.5–10 n*M*, and incubations contained a final concentration of 7% dimethylformamide.

whereas the bromo- and iodohexestrols have progressively lower selectivities.

In intact cells the binding of estrogens to the cytoplasmic estrogen receptor causes receptor activation, which is then followed by translocation of the hormone-receptor complex from the cytoplasm to the nucleus. This activation and translocation sequence is temperature-dependent and occurs both in vivo and in uterine organ culture in vitro. The hormone-nuclear-receptor complex can be extracted from the nuclear fraction with buffers containing 0.4 *M* KCl, and the complex will sediment as a 5S species on high-salt sucrose gradients.

We have have performed in vitro translocation experiments with [^{3}H]estradiol and *o*-fluoro[^{3}H]hexestrol in immature rat uteri. After incubation at 0°C for 1 hr, the majority of the activity for [^{3}H]estradiol or *o*-fluoro[^{3}H]hexestrol is found in the cytoplasmic compartment, but after 1 hr at 37°C, nearly all the activity is found in the nuclear fraction (data not shown). Extraction of the nuclear fraction with 0.4 *M* KCl removes a major portion of the receptor-bound activity, which sediments as prominent 5S peaks on high-salt sucrose gradients (Fig. 5).

In vivo studies: selectivity of tissue uptake in immature rats. We have repeated the classical experiment of Jensen and Jacobson (*22*) in order to investigate the tissue uptake selectivity of these compounds in vivo. Figure 6 shows the concentrations of radioactivity in various tissues 1 hr after i.v. injection into immature female rats of small amounts of [^{3}H]estradiol or one of the four tritium-labeled halogenated hexestrols (vertical bars). The data (starting as dpm per gram tissue) are normalized so that the uptake by the uterus is 100%. The absolute uptake can be ascertained from the data given in the figure's legend.

There are a number of striking features about these data. First of all, the uptake by the uterus, a classical estrogen target tissue, is much more pronounced than that of the nontarget tissues—esophagus, diaphragm, lung, spleen, and stomach. Not surprisingly, some of the

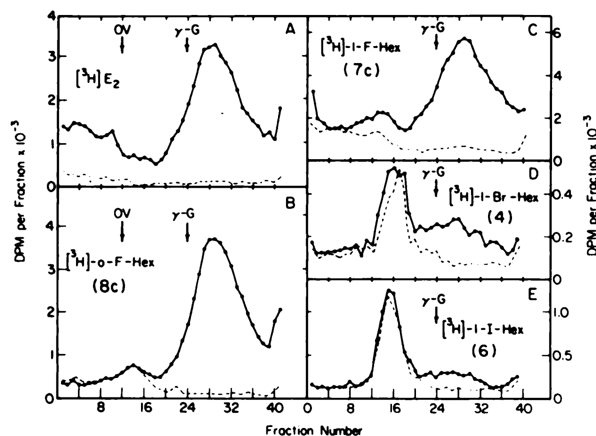

FIG. 4. Low-salt sucrose-gradient analysis of binding of estradiol (E_2, panel A) and four halogenated hexestrol derivatives (panels B–E) in rat cytosol. Sedimentation is from left to right. Gradients with tritium-labeled compounds are shown as solid lines; dashed lines show gradients from samples incubated in presence of 100-fold excess of unlabeled estradiol, to determine nonspecific binding. Carbon-14-labeled ovalbumin (OV, 3.5 S) and γ-globulin (γ-G, 7.0 S) were used as sedimentation standards. In each case, quantity of specific binding (represented by area of 8S peak minus nonspecific binding) corresponds to 0.61–0.84 pmoles of receptor per uterine equivalent.

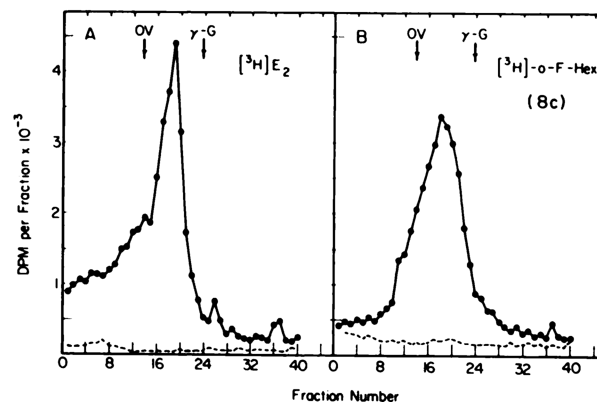

FIG. 5. High-salt (0.4 *M* KCl) sucrose-gradient analysis of nuclear estrogen receptor after in vitro incubation of rat uteri with [^{3}H]-estradiol ([^{3}H]E_2, panel A) or *o*-fluoro[^{3}H]hexestrol ([^{3}H] *o*-F-Hex, panel B). Sedimentation is from left to right. Solid lines represent total binding; dashed lines give nonspecific binding. Under high-salt conditions, sedimentation velocity of carbon-14-labeled marker proteins is 4.1 *S* for ovalbumin and 7.0 S for γ-globulin.

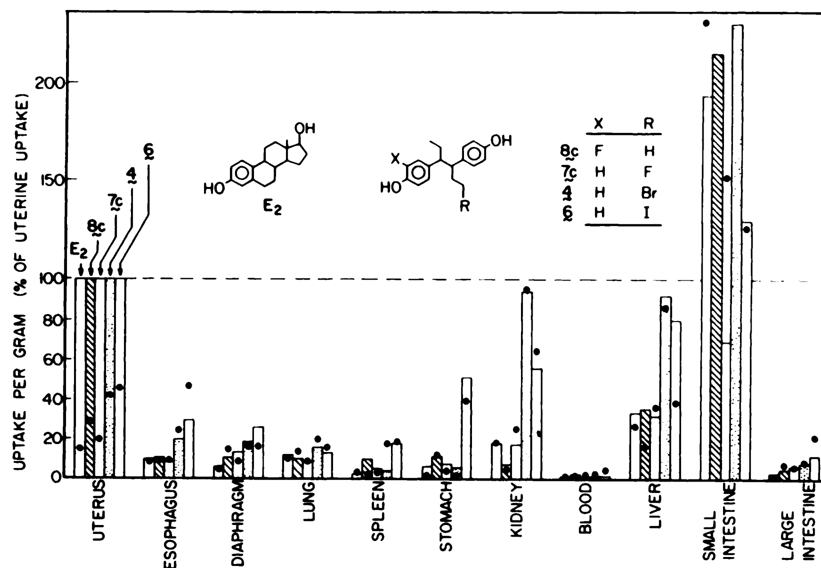

FIG. 6. Uptakes of estradiol (E_2) and four halogenated hexestrols into tissues of immature female rats. Rats were injected with the tritium-labeled compounds in absence (vertical bars) and presence (dots) of an excess (13 μg) of unlabeled estradiol (to determine nonspecific uptake). Uptake levels after 1 hr (dpm/g tissue) are expressed relative to uterine uptake (100%). Absolute uptakes by uterus (% dose/g tissue) were: for E_2 4.2; for o-F-Hex 2.9; 1-F-Hex 4.2; 1-Br-Hex 1.0; 1-I-Hex 1.43. In experiments where multiple (seven to eight) animals were used (o-F-Hex and 1-F-Hex), levels of both total and specific were reproducible within 30%.

compounds show substantial levels in tissues involved in hormone metabolism (liver) and excretion (kidney and intestine).

While the pattern of uptake of total radioactivity appears to be selective, it is crucial to establish that the enhanced uptake noted in target tissue is due to receptor interaction, rather than to nonspecific processes. In parallel experiments, the tissue uptake of these tritium-labeled compounds was determined in rats that were simultaneously treated with excess unlabeled estradiol (13 μg), sufficient to occupy estrogen receptors fully. In this case, the uptake into nontarget tissues (levels indicated by the dots) was essentially the same as before, whereas the uptake by the uterus was depressed nearly to the level seen in the nontarget tissues.

Finally, the trend in the uptake selectivity of the different compounds appears related to their lipophilicity. The more lipophilic bromo- and iodohexestrols show a higher level of uptake into nontarget tissues, relative to their uterine uptake, than do the fluorohexestrols.

We have examined the time dependence of tissue uptake and retention for one compound, *o*-fluoro[^{3}H]-hexestrol (Fig. 7). Uptake by the uterus appears to peak at early times (0.5–1 hr); thereafter, dissociation is relatively slow. Uptake by the nontarget tissues is low, with some showing a small peak at 1 hr. This temporal profile of uptake is very similar to that of [^{3}H]estradiol reported by Jensen and Jacobson (*22*) (small dashes).

The extent of metabolism of the fluorinated hexestrols (**7c** and **8c**) was investigated by thin layer chromatography of radioactivity extracted from tissues 1 hr after in vivo injection. The radioactivity extracted from the uterus is mostly unmetabolized compound (75–93%). In contrast, only 35–50% of the radioactivity extracted from nontarget, nonexcreting tissues (esophagus, diaphragm, lung, spleen, stomach, and blood) co-chromatographs with the unmetabolized compound, and for the organs involved in metabolism, conjugation, and excretion (kidney, liver, intestine) less than 2% of the extracted activity corresponds to unmetabolized material . These results indicate that the receptor-mediated uptake process utilizes unmetabolized compound. Further metabolism studies have been carried out on extracts from mammary tumors (see Fig. 9).

Tissue distribution in mature rats bearing DMBA-induced mammary tumors. A high yield of mammary tumors can be induced in Sprague-Dawley rats by administration of the carcinogen dimethylbenzanthracene (DMBA) during certain phases of mammary gland development (47–50 days of age). Nearly all these tumors are ovary-dependent, and they contain substantial levels of estrogen receptor (*20*). The selectivity of tissue uptake of *o*-fluoro[^{3}H]hexestrol and 1-fluoro[^{3}H]hexestrol in

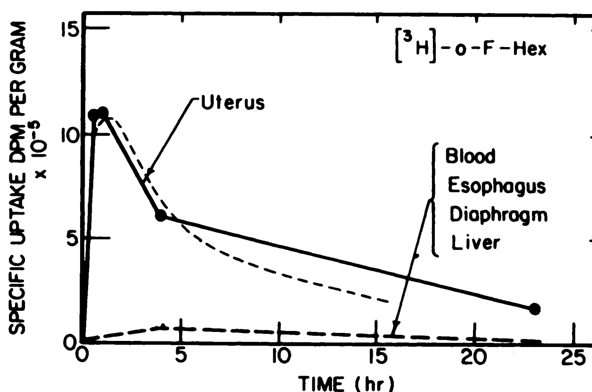

FIG. 7. Radioactivity content of uterus and nontarget tissues at various times after injection of immature female rat with *o*-fluoro-[^{3}H]hexestrol (23 μCi, 0.45 μg). Each point represents specific uptake (total uptake average of two animals) minus uptake seen with simultaneous injection of 13 μg of unlabeled estradiol. Fine dashed line represents time course of uptake of [^{3}H]estradiol in uterus (adapted from Jensen and Jacobson, *22*).

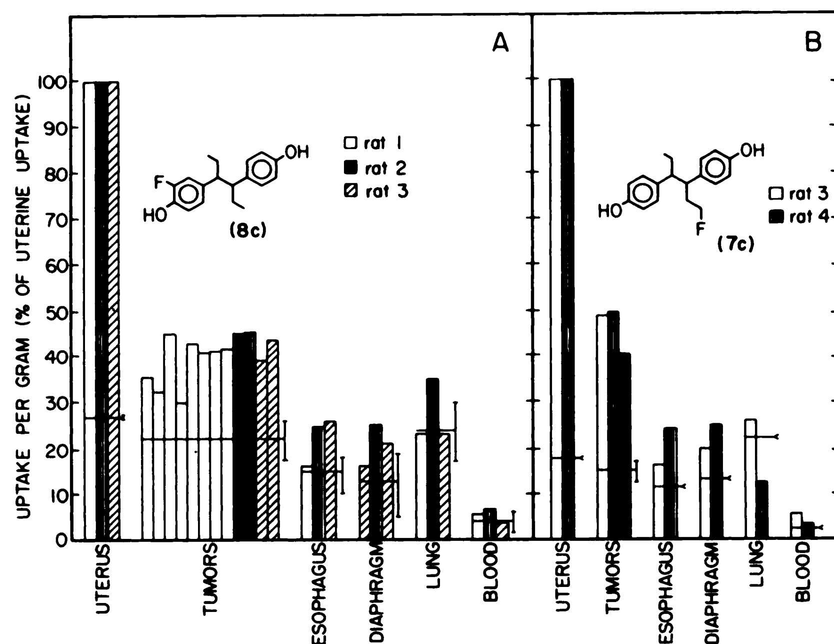

FIG. 8. Uptake of o-fluorohexestrol (panel A) and 1-fluorohexestrol (panel B) in adult rats bearing dimethylbenzanthracene-induced mammary tumors. At 1 hr absolute uptake by uterus (% dose/g tissue) was 0.07–0.15 for o-F-Hex; 0.33–0.38 for 1-F-Hex. Each rat had from one to eight tumors, ranging in size from 0.05–8.7 g. Ranges of nonspecific uptake are shown by —I; levels of nonspecific uptake by —(.

90- to 100-day rats bearing DMBA-induced mammary tumors was investigated by the same protocol used with the immature animals. The data are summarized in Fig. 8.

The tissue uptake of these two compounds in adult rats parallels that seen in the immature animals: the nontarget tissues (esophagus, diaphragm, and lung) show uptake levels that are around 20% of that of the uterus. This appears to be somewhat higher than was seen with these compounds in the immature animals (~10%), but since mature rat uterus contains less estrogen receptor than immature uterus (~10 against 30 pmoles per gram tissue, *20*), higher nontarget uptake values are expected when the data are expressed relative to the uterine uptake. Again, administration of an excess of unlabeled estradiol together with the labeled fluorohexestrol had little effect on the uptake in the nontarget tissues, but it depressed the uterine uptake to the nontarget tissue level.

Uptake by the mammary tumors is also shown in Fig. 8. In nearly every tumor, uptake of the tritiated fluorohexestrol is greater than that in the nontarget tissues; this is particularly true with 1-fluoro[^{3}H]hexestrol (Fig. 8B). Excess unlabeled estradiol also depresses tumor uptake to the nontarget level. The receptor-specific uptake in the tumors (i.e., the difference between total uptake and uptake in the presence of excess estradiol) is 25–30% that of the receptor-specific uptake of the mature uterus. Again, this is consistent with the relative estrogen receptor content of the DMBA-induced rat mammary tumors (~3 pmoles per gram tissue, *20*).

Extracts from the mammary tumors of these animals were analyzed by high-pressure liquid chromatography on a reverse-phase system. As is seen in Fig. 9, nearly all

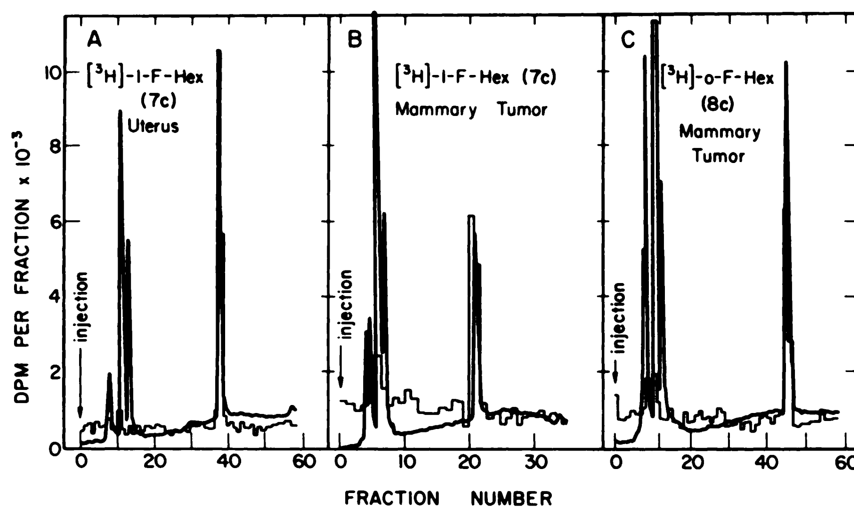

FIG. 9. High-pressure liquid chromatographic analysis of extracts from immature uterus and DMBA-induced mammary tumors. Adult rats bearing DMBA-induced mammary tumors, or immature rats were injected with 1-fluoro[^{3}H]hexestrol ([^{3}H]1-F-Hex, panels A and B) or o-fluoro[^{3}H]hexestrol ([^{3}H]o-F-Hex, panel C); ethanol extracts of tissue or tumor homogenates were prepared after 1 hr. Heavy lines represent uv profile of authentic samples of unlabeled compound measured by uv absorbance at 254 nm; narrow line (rectangular plot) indicates dpm per fraction.

of the radioactivity in these extracts co-chromatographs with authentic samples of the compound, indicating that the unmetabolized compound is involved in the receptor-mediated uptake.

DISCUSSION

We have prepared four halogen-containing estrogen analogs in tritium-labeled form with sufficiently high specific activity to permit study of their interaction with estrogen receptors under in vitro and in vivo conditions. High-affinity binding to estrogen-receptor sites in uterine cytosol can be demonstrated in vitro with all four compounds by direct binding measurement and by sedimentation on sucrose gradients. In general, the degree to which these compounds interact with nonreceptor binding species (low-affinity, nonspecific binding proteins) parallels their lipophilicity (increasing with the increasing size of the halogen).

In in vivo experiments with immature rats, all four compounds show estrogen-receptor-mediated uptake that is highly selective for target tissues such as the uterus; the activity in target tissues is largely unmetabolized and is retained for considerable time periods. Organs involved in hormone metabolism and excretion accumulate considerable activity (mostly metabolites), but not by a receptor-specific mechanism. The studies with *o*-fluoro[^{3}H]hexestrol and 1-fluoro[^{3}H]hexestrol in mature rats bearing DMBA-induced mammary tumors show that receptor-specific uptake is occurring in uterus and tumor tissue, but that the selectivity of uptake (target to nontarget tissue) is lower, reflecting the lower concentration of estrogen receptors in tumor tissues and in the target tissues of mature animals.

As the range of concentration of estrogen receptors in the DMBA-induced rat mammary tumors and human breast tumors are approximately the same, it appears that these two compounds have promise as receptor-based agents for imaging human breast tumors. While in most experiments, *o*-fluorohexestrol appeared to be nearly as selective as 1-fluorohexestrol, the latter appears a better choice as an imaging agent since it can be prepared by direct displacement reactions using fluoride ions (D. F. Heiman and J. A. Katzenellenbogen, in preparation). On the other hand, currently available methods for the introduction of fluorine-18 at aromatic positions adjacent to oxygen substituents are not capable of giving *o*-fluoro[^{18}F]hexestrol with sufficiently high specific activity for tumor detection based on receptor-mediated uptake (*23–25*). The more lipophilic bromo- and iodohexestrols appear to have somewhat lower selectivity in terms of estrogen-receptor interaction and tissue uptake, but these compounds may still have potential as imaging agents for human breast tumors. In addition to suggesting which compounds are suitable for further investigation, these studies reaffirm the importance of the binding selectivity of compounds to be used as breast tumor imaging agents.

FOOTNOTES

* Sigma Chemical Corp., St. Louis, MO.
† Amersham
‡ Central Solvents and Chemical Co.
‖ Baker
§ Eastman
¶ Searle
** Aldrich
†† Apache Chemicals
‡‡ Research Products International Corp.
‖‖ PCR Inc.
§§ Determined on a Fisher–Johns Melting Point Apparatus.
¶¶ Varian Associates 4100 instrument with an Alltech 35-cm × 4.6-mm column packed with 5 μm Spherisorb S-ODS.

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