Iodohexestrols. I. Synthesis and Photoreactivity of Iodinated Hexestrol Derivatives[†]

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ABSTRACT: The five possible ortho-iodinated hexestrols, containing from 1–4 iodines per molecule, have been synthesized as potential iodine-containing estrogenic ligands. After chromatographic separation and purification, each derivative has been fully characterized by spectroscopic methods. $[^3H]$-3-Iodohexestrol, $[^{131}I]$- and $[^{125}I]$-3-iodohexestrol, and $[^{125}I]$-3,5-diiodohexestrol have also been prepared. Photolysis of 3,5-diiodohexestrol in methanol results in rapid deiodination to 3-iodohexestrol; further reduction to hexestrol is slower. Photolysis of 3-iodohexestrol in benzene gives 3-phenylhexestrol.

Target tissues for estrogens contain binding proteins that have high affinity for estrogenic compounds. These proteins, often termed receptors, are initially localized in the cytoplasm; but after associating with estradiol,[1] they undergo a temperature-dependent transformation and are then translocated into the nucleus (Williams and Gorski, 1973). Considerable evidence has been accumulated which implicates the subcellular dynamics of this estradiol-binding protein complex as important events in the action of estrogen in these tissues (Jensen and DeSombre, 1973).

Several factors led us to consider the synthesis of an estrogenic ligand which contained iodine. It would, in principle, be possible to prepare such a ligand with two radioisotopic labels of high specific activity, that could be differentially counted (e.g., iodine-125 and -131, or tritium and iodine-131). Using such an isotope pair, one could monitor in much greater detail the kinetics of the subcellular movement shown by the estrogen–binding protein complex, in particular, the rate of estrogen movement into the nucleus, the rate of turnover of estrogen in the nuclear and cytoplasmic pools, and the ultimate fate of nuclear-bound estrogen.

Second, our interest in photoaffinity labeling of estrogen binding proteins (Katzenellenbogen et al., 1973a–c, 1974) led us to speculate that the known photohomolytic cleavage of aromatic carbon–iodine bonds (Wolf and Kharasch, 1965) might provide a means for covalently attaching either the estrogenic ligand (tritium labeled) or the radioiodine atom to amino acids residues in the estrogen binding site.

In this report we describe the synthesis and characterization of the five possible ortho-iodinated hexestrols (containing 1–4 iodines, **2–6**) and the radiochemical synthesis of certain of these derivatives (tritium and iodine-125 and iodine-131 labeled). These studies also include an investigation of the photochemical behavior of these compounds in solution and a study of their potential as photosensitive reagents for labeling the estrogen binding sites in rat uterine cytosol.

In the accompanying report (Katzenellenbogen et al., 1975), we describe studies on the binding of these iodinated hexestrol derivatives in rat serum and in uterine and other tissue cytosols, and we characterize the estrogenic activity of these derivatives, in vitro and in vivo.

Experimental Section

Materials and Methods. The following compounds were obtained from the sources indicated: estradiol (Searle; Steraloids); $[6,7\text{-}^3H]$-estradiol (46.5–48 Ci/mmol; New England Nuclear); $[^{131}I]$- and $[^{125}I]$NaI (Amersham-Searle, carrier-free; 10 mCl in *ca.* 0.02 ml of dilute NaOH); *meso*-hexestrol and dienestrol (Mann); *p*-cresol (Eastman); charcoal and Norit A (Sigma); dextran, grade C (Schwarz/Mann); Triton X-114 (Rohm and Haas); chloramine T (Sigma); sodium metabisulfite (Baker); dimethylformamide (Baker); ethylenediaminetetraacetic acid (Eastman); tris(hydroxymethyl)aminomethane (Nutritional Biochemicals). A xylene-based scintillation fluid (Anderson and McClure, 1973) was modified to contain 0.55% 2,5-diphenyloxazole, 0.01% *p*-bis[2-(5-phenyloxazolyl)]benzene, and 25% Triton X-114. Counting was done in minivials (Research Products) containing 5 ml of scintillation fluid in either a Nuclear Chicago Isocap 300 (43–48% tritium and >55% iodine-125 efficiency) or a Packard 314 EX2 (18–22% tritium efficiency) instrument.

Nuclear magnetic resonance (NMR) spectra were obtained on a Varian A60A or HA100 spectrometer and are expressed as parts per million downfield from tetramethylsilane as internal standard (δ scale). Other spectroscopic data were obtained as follows: infrared, Perkin-Elmer 237; mass spectra, Varian MAT CH5, and uv, Cary 15. Melting points were determined on a Fisher-Johns apparatus and, unless noted, are uncorrected.

Thin-layer chromatographic separations were made on Eastman chromatosheets 6060. For the determination of radiochemical purity, the radiolabeled sample was spotted both on top of and parallel to unlabeled carrier on a 15-cm chromatosheet strip. After development and visualization with iodine vapor, 1-cm bands were cut and counted by liquid scintillation techniques. Liquid chromatography was

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[1] Common names and abbreviations used in this paper are: hexestrol **1** (Hex), *meso*-3,4-bis(4′-hydroxyphenyl)hexane; **2** (I-Hex), *erythro*-3-(3′-iodo-4′-hydroxyphenyl)-4-(4′-hydroxyphenyl)hexane; **3** (I_2s-Hex), *meso*-3,4-bis(3′-iodo-4′-hydroxyphenyl)hexane; **4** (I_2u-Hex), *erythro*-3-(3′,5′-diiodo-4′-hydroxyphenyl)-4-(4′-hydroxyphenyl)hexane; **5** (I_3-Hex), *erythro*-3-(3′,5′-diiodo-4′-hydroxyphenyl)-4-(3′-iodo-4′-hydroxyphenyl)hexane; **6** (I_4-Hex), *meso*-3,4-bis(3′,5′-diiodo-4′-hydroxyphenyl)hexane; estradiol (E_2), 1,3,5(10)-estratriene-3,17β-diol.

conducted on a Varian-Aerograph Model 4100 liquid chromatograph, equipped with an ultraviolet detector (254 nm) and a multilinear automatic gradient programmer. Columns were either: 1.3 m × 2.2 mm Corasil II (Waters) or 0.5 m × 2.2 mm MicroPak SI-10 (Varian; packed with 10-μ of silica gel, LiChrosorb SI-60, E. Merck).

3-Iodohexestrol (**2**). Hexestrol (4 mmol, 1.08 g) was dissolved in 15 ml of methanol and 5 ml of concentrated ammonium hydroxide. A solution of 1.016 g of iodine (4 mmol) in 5 ml of tetrahydrofuran, prepared previously, was added to the hexestrol solution with stirring. The brown iodine color disappeared within a few minutes. After 30 min, glacial acetic acid was added to neutralize ammonia (pH 5). Water (50 ml) was then added and reaction products were extracted by three 50-ml portions of ethyl acetate. The organic extract, analyzed by thin-layer chromatography (TLC) on silica gel with methylene chloride, showed five components as well as starting hexestrol. The products were separated by silica gel column chromatography (1.8 cm × 50.0 cm) with benzene as eluent. Fractions (6 ml) were collected every 10 min, dried under a stream of nitrogen, and reanalyzed by TLC. The five major peak fractions were tentatively identified as **2–6.**

Material from each peak fraction was recrystallized from hexane–benzene and then from water–methanol; white flake-shaped crystals of **2** were formed: mp 125–126°; uv (EtOH) $\lambda_{\max}$ 285 (ϵ 4600); NMR (THF) δ 6.80 (m, 6 H, aromatic), 7.48 (d, 1 H, J = 1 Hz), 7.96 (s, 1 H, PhOH), 8.63 (s, 1 H, PhOH); mass spectrum (10 eV) m/e (rel intensity) 396 (M^+, 10), 135 (15), 134 (23), 72 (100), 71 (59). Anal. Calcd for $C_{18}H_{21}O_2I$: C, 54.60; H, 5.32; I, 32.05. Found: C, 54.65; H, 5.43; I, 31.87.

In order to ascertain the purity of **2,** the iodinated hexestrols were analyzed by liquid chromatography on a 0.5 m × 2.2 mm MicroPak SI-10 column according to the elution conditions given in Figure 1. Analysis of a sample of purified **2** showed that it contained less than 0.2% (detection limit) of hexestrol.

3,3′-Diiodohexestrol (**3**) *and 3,5-Diiodohexestrol* (**4**). The two diiodohexestrol derivatives (**3** and **4**) have the same R_f (0.19) on silica gel TLC with benzene as developing solvent. However, they can be partially resolved using $CHCl_3/CCl_4$ 1:1 (R_f (**3**) 0.21, R_f (**4**) 0.17) or CH_2Cl_2 (R_f (**3**) 0.27, R_f (**4**) 0.36). Preparative TLC, using two developments with CH_2Cl_2, separated the isomers: mp (**3**) 151–152°, mp (**4**) 113–114°; uv (**3**) $\lambda_{\max}$ (EtOH) 286 (ϵ 6010) and 293 (ϵ 5560); uv (**4**) $\lambda_{\max}$ (EtOH) 280 (ϵ 3740), 297 (ϵ 2660), and 310 (ϵ 2440); mass spectrum (**3,** 70 eV) m/e (rel intensity) 522 (M^+), 261 (89), 260 (22), 135 (76), 42 (100); mass spectrum (**4,** 70 eV) m/e (rel intensity) 522 (M^+), 387 (3.6), 386 (1.6), 359 (3.1), 261 (1.4), 260 (2.3), 135 (100), 134 (8.8), 107 (24.4); NMR (**3,** THF) δ 6.80 (m, 4 H), 7.49 (d, J = 1 Hz, 2 H), 8.68 (s, 2 H); NMR (**4,** THF) δ 6.80 (m, 4 H), 7.51 (s, 2 H), 8.00 (s, 1 H), 8.68 (1 H). Anal. Calcd for $C_{18}H_{20}O_2I_2$: C, 41,37; H, 3.83; I, 48.56. Found **3:** C, 41.41; H, 3.89; I, 48.46. Found **4:** C, 41.04; H, 3.79; I, 48.63.

3,3′,5-Triiodohexestrol (**5**). Derivative **5** was purified by silica gel column chromatography and recrystallized from methanol-H_2O and hexane-benzene: mp 100–103°; uv $\lambda_{\max}$ (EtOH) 286 (ϵ 5400) and 293 (ϵ 4600); mass spectrum (70 eV) m/e (rel intensity) 648 (M^+, 0.43), 387 (15.8), 262 (10.8), 261 (100), 233 (13.9), 135 (3.8), 134 (22.6), 133 (5.7), 57 (24.0), 43 (70.8); NMR (THF) δ 7.80 (m, 2 H), 7.49 (d, J = 1 Hz, 1 H), 7.51 (s, 2 H), 8.52 (2 H). Anal. Calcd for $C_{18}H_{19}O_2I_3$: C, 33.33; H, 2.93; I, 58.64. Found: C, 33.47; H, 2.98; I, 58.21.

3,3′,5,5′-Tetraiodohexestrol (**6**). Derivative **6** was purified by silica gel column chromatography and recrystallized from hexane–benzene. It has slightly yellowish color: mp 245–246°; uv $\lambda_{\max}$ (EtOH) 289 (ϵ 5660) and 300 (ϵ 5200); mass spectrum (70 eV) m/e (rel intensity) 773.8 (M^+, 2.3), 387.8 (12.5), 386.8 (100), 385.8 (46.8), 358.8 (29.1), 261 (14.6), 260 (27.4), 133 (18.3); NMR (THF) δ 7.43 (s, 4 H), 7.85 (s, 2 H). Anal. Calcd for $C_{18}H_{18}O_2I_4$: C, 27.95; H, 2.32: I, 65.60. Found: C, 27.93; H, 2.27; I, 65.53.

[^{125}I]-3-Iodohexestrol. Carrier free $Na^{125}I$ was used as supplied. The following solutions were prepared: hexestrol (0.15 mg in 1 ml of Spectrograde methanol), chloramine T (1.5 mg in 1 ml of methanol), and sodium metabisulfite ($Na_2S_2O_5$, 1.5 mg in 1 ml of MeOH–H_2O 1:1 v/v).

Hexestrol (72 μl, 40 nmol) and chloramine T (30.4 μl, 200 nmol) solutions were added via Hamilton syringe to the $Na^{125}I$ solution (10 mCi, 4 nmol in 20 μl of dilute NaOH) which was contained in the original 0.3-ml capacity conical bottomed vial. The vial was recapped with a Teflon-lined cap, agitated briefly, and left at room temperature. After 20 min, 25.3 μl (200 nmol) of the sodium metabisulfite solution was added to quench the reaction. The solvent was removed under a gentle stream of nitrogen, and 20 μl of distilled water was added to redissolve the residue. The reaction products were transferred to a 5-ml test tube with five 0.2-ml portions of ethyl acetate, and the extracts were dried with a few grains of sodium sulfate. The radiochemical purity and recovery were monitored at this stage. The extract was concentrated under a nitrogen stream and transferred to a conical bottomed vial for final evaporation.

The dried product, redissolved in 15 μl of methanol, was injected into a high-pressure liquid chromatography column (0.6 m × 2.2 mm Corasil II, Waters). Elution at 60 ml/hr was controlled by a multilinear gradient programmer: nonpolar solvent A, hexane; polar solvent B, 2-propanol–methylene chloride 1:4; program, 5% B in A for 5 min, increasing B at 1%/min for 10 min, hold at 15% B in A for 5 min, and regenerate at 5% B in A. Fractions (40 drops) were collected in an automatic fraction collector, and the radioactivity of each fraction was monitored by a Geiger counter. The bulk of the radioactivity eluted in two fractions (16 and 17), which were combined in a 5-ml vial, evaporated to dryness under nitrogen, and redissolved in 1 ml of ethanol for storage at −20°. By this procedure a 60% yield of [^{125}I]-3-iodohexestrol with radiochemical purity 93–97% can be obtained within 4 hr.

The radiochemical purity of [^{125}I]-3-iodohexestrol was also ascertained by cocrystallization with 3-iodohexestrol. A 25-μl aliquot of purified [^{125}I]-3-iodohexestrol solution was combined with 20 mg of 3-iodohexestrol and recrystallized three times from methanol–water and once from hexane–benzene. The specific activity (cpm/mg) was: original sample, 4.85×10^3, recrystallized samples in order, 4.65×10^3, 5.06×10^3, 4.48×10^3, and 4.46×10^3.

[^{131}I]-3-Iodohexestrol. Similar yields and radiochemical purities were obtained starting from $Na^{131}I$ and using the preceding procedure.

[^3H]Hexestrol (**1**). [^{3}H]Hexestrol was prepared by catalytic hydrogenation of dienstrol according to the procedure of Williams and Ronzio (1950). The preparation was done by New England Nuclear. Dienstrol (40 mg) was dissolved in 1 ml of ethyl acetate. Palladium–charcoal catalyst (4 mg of 10%) was added, and reduction was conducted with 25

Ci of carrier-free tritium gas for 1 hr at 25°. After filtration, labile tritium was removed in vacuo using methanol. The yield was 13 Ci.

The material obtained from this reduction is a mixture of meso and dl diastereomers. These are readily separable by TLC (2% EtOH in CHCl$_3$; R_f (meso) 0.19, R_f (dl) 0.08) or liquid chromatography on Corasil II column (solvent A, hexane; solvent B, 2-propanol–methylene chloride 1:4; gradient of 5–25% B in A at 0.8%/min, with total flow rate 40 ml/hr). Preparative separations were performed by liquid chromatography under the above conditions; 50% recoveries of material that is >97% radiochemically pure were routinely obtained.

As only limited amounts of [^{3}H]hexestrol could be handled, its specific activity was determined relative to that of a sample of [^{3}H]estradiol of known specific activity (45 Ci/mmol). Competitive binding assays were run according to the procedures described previously (Katzenellenbogen et al., 1973b), using [^{3}H]E$_2$ and [^{3}H]Hex as tracers and unlabeled E$_2$ as competitor. Comparison of the extent of estradiol competition established the specific activity of [^{3}H]Hex as 86 Ci/mmol.

[^{3}H]-3-Iodohexestrol. [^{3}H]Hex (2 mCi, 86 Ci/mmol) was dissolved in 1.5 ml of methanol, and 2 equiv of aqueous potassium iodide (0.88 mg/ml, 9.2 μl) and 10 equiv of chloramine T (4.6 mg/ml, 13 μl) solution were added. The reaction was stirred in an ice bath for 8 min. (Small scale test reactions had established this as the optimum reaction time.) The excess chloramine T was consumed by the addition of 10 equiv of sodium metabisulfite (3.8 mg/ml, 17.3 μl), and the reaction mixture was then dried in a conical bottom vial (1 ml) under a gentle stream of nitrogen. The dried residue was redissolved in 10 μl of THF and purified by liquid chromatography under the conditions given in Figure 1. Fractions of 30 drops were collected, and reanalysis (TLC) of the combined peak fractions of the [^{3}H]I-Hex peak showed it to be >97% pure. The overall yield was 15%.

The identity and radiochemical purity of [^{3}H]I-Hex were confirmed by cocrystallization with samples of unlabeled I-Hex and I$_2$s-Hex in methanol–water. In each case the original specific activity was 4000 cpm/mg. Specific activity (cpm/mg) after successive recrystallization with I-Hex was: 3800 and 3986, but recrystallization with I$_2$s-Hex gave 2502.

Hexestrol Monobenzoate (**7**). Hexestrol (540 mg, 2 mmol) was reacted for 2 hr with 1 equiv of benzoyl chloride (141 mg, 116 μl, 2 mmol) in 10 ml of pyridine. The reaction mixture was poured into 50 ml of deionized water, and organic products were extracted with three 50-ml portions of ethyl acetate. Preparative TLC purification (benzene) gave 294 mg (51%) of the monobenzoate.

3-Iodohexestrol Monobenzoate (**8**). Hexestrol monobenzoate (100 mg, 0.27 mmol) was treated with 53 mg (0.32 mmol) of potassium iodide and 175 mg (1.34 mmol) of chloramine T in MeOH/THF (2:1) for 30 min. Excess chloramine T was consumed by the addition of 127 mg (0.67 mmol) of sodium metabisulfite. The reaction products were extracted with ethyl acetate, and the monoiodo substituted derivatives were purified by preparative TLC on silica gel with benzene or dichloromethane: yield 54%, mp 86–88°; NMR (CDCl$_3$) δ 0.56 (t, 3 H), 0.58 (t, 3 H), 1.1–1.6 (m, 4 H), 2.48 (m, 2 H), 6.95 (m, 2 H), 7.16 (s, 4 H), 7.3–7.6 (m, 5 H), 8.2 (m, 2 H), mass spectrum (70 eV) *m/e* (rel intensity) 500 (M$^+$, 1.8), 240 (11), 239 (45), 238 (11), 105 (100), 77 (16), 31 (11). Anal. Calcd for $C_{25}H_{25}O_3I$: C, 60.01; H, 5.04; I, 25.36. Found: C, 60.08; H, 5.37; I, 24.80.

[^{125}I]-3,5-Diiodohexestrol (**10**). 3-Iodohexestrol monobenzoate (0.1 mg/ml, 10 μl) was treated with 0.5 mCi of carrier free sodium iodide-125 (Amersham/Searle, Batch 51A/390) and 10 μl of a methanolic chloramine T solution (10 μg, 3.5×10^{-5} mmol) for 20 min. The reaction was then quenched with sodium metabisulfite (3.5×10^{-5} mmol) and then hydrolyzed for 5 hr with 1 ml of 2% KOH in ethanol. The ethanol was evaporated under nitrogen; 1 ml of distilled water added, and the organic products were extracted with five 2-ml portions of ethyl acetate. The extracts were combined and stored in a 25-ml erlenmeyer flask over anhydrous sodium sulfate.

[^{125}I]-3,5-Diiodohexestrol was purified by liquid chromatography under the conditions given in Figure 1. The purified product had a radiochemical purity in excess of 93%.

3-Phenylhexestrol Dibenzoate (**9**). 3-Iodohexestrol (250 mg), dissolved in 15 ml of benzene, was transferred to four quartz annular photolysis tubes. The solution was photolyzed at 254 nm for 16 hr in a Rayonet reactor (RPR 1000) using 12 GE (G8T5) lamps (Katzenellenbogen et al., 1974b). After photolysis, the iodine released was reduced with sodium metabisulfite, and the organic products were extracted with ethyl acetate. Separation by preparative TLC (methylene chloride) gave a major band corresponding in mobility to I-Hex. Spectroscopic analysis (NMR, mass spectrum) showed that this material was a mixture of I-Hex and 3-phenylhexestrol. Recrystallization failed to separate these two components.

Reduction of this mixture over 30 mg of 5% palladium on charcoal in 5 ml of ethanol for 16 hr converted it into a mixture of hexestrol and 3-phenylhexestrol. Preparative TLC (methylene chloride) allowed separation of these components and afforded a homogeneous sample of 3-phenylhexestrol. As this material was only poorly crystalline, it was converted to the dibenzoate (benzoyl chloride in pyridine), which was purified by preparative TLC (methylene chloride) and recrystallization from ethanol: 30% yield overall from I-Hex: mp 162–163°; NMR (CDCl$_3$) δ 0.62 (t, 3 H), 0.64 (t, 3 H), 1.5 (m, 4 H), 2.68 (t, 2 H), 7.2 (s, 4 H), 7.24 (s, 2 H), 8.04 (d, d, J = 4 and 1 Hz, 2 H), 8.22 (d, d, J = 4 and 1 Hz, 2 H), 7.2–7.6 (m, 12 H); mass spectrum (70 eV) *m/e* (rel intensity) 554 (M$^+$, 0.17), 315 (30), 239 (14), 105 (100), 77 (15). Anal. Calcd for $C_{38}H_{34}O_4$: C, 82.30; H, 6.13. Found: C, 82.01; H, 6.38.

p-Cresol, 2-Iodo-p-cresol, and 2,6-Diiodo-p-cresol. *p*-Cresol was recrystallized from hexane at 0°. The mono and diiodo derivatives were prepared by reaction with 1 equiv or excess iodine, respectively, in ammonium hydroxide methanol according to the procedure of Burger et al. (1945).

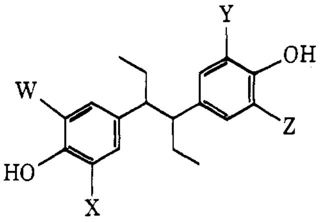
2-Iodo-*p*-cresol was isolated by column chromatography on silica gel (methylene chloride). The brown liquid was subjected to molecular distillation (condensate crystallized) and was recrystallized from hexane at 0°: mp corr. 36–37° (lit. 37–38°, Morgan and Burgstall (1928)).

2,6-*p*-Diiodocresol was recrystallized from methanol–water: mp corr. 61.5–62.5° (lit. mp 61°; Windaus and Schiele (1923)).

Results

Synthesis, Separation, and Characterization of the Five Iodohexestrols. The four positions ortho to the phenolic hydroxyls in hexestrol are susceptible to electrophilic iodination. Even though the rate of conversion of a phenol to an

SCHEME I



	W	X	Y	Z	
1	H	H	H	H	(Hex)
2	I	H	H	H	(I-Hex)
3	I	H	I	H	(I_2s-Hex)
4	I	I	H	H	(I_2u-Hex)
5	I	I	I	H	(I_3-Hex)
6	I	I	I	I	(I_4-Hex)

o-iodophenol is reported to be ca. five times faster than conversion of the *o*-iodophenol to a *o,o*′-diiodophenol (Mayberry, 1967), we have found that the successive iodination reactions of hexestrol (**1**) are sufficiently similar in rate that treatment of hexestrol with 1 equiv of iodine in aqueous ammonium hydroxide-methanol (Albert et al., 1949) produces a mixture of starting material and the five possible ortho-iodinated derivatives **2-6** (Scheme I) in the molar ratio 10: 3:2.5:2:1. Iodination with iodine monochloride in acetic acid (Wallingford and Krueger, 1949) tended to give more highly substituted products and was less convenient. All five iodo compounds can be resolved by silica gel thin-layer chromatography, the most difficult separation being between the two diiodinated derivatives **3** and **4.** Separation by gradient elution, liquid-solid adsorption chromatography on silica gel (10 μ of MicroPak Lichrosorb Si 60 or Corasil II), cleanly resolves hexestrol and the five iodinated compounds on an analytical scale. A typical chromatogram is shown in Figure 1.

The derivatives were separated on a preparative scale by silica gel column chromatography. The fractions containing predominately single isomers were pooled and recrystallized to analytical purity, and the identity of each derivative was established by its spectroscopic properties (NMR and mass spectroscopy) and iodine content, as determined by microanalysis. Distinction between the symmetrical and unsymmetrical diiodo isomer (**3** and **4**) was evident from the fragmentation pattern in the mass spectra, the symmetrical isomer (**3**) giving an intense ion at *m/e* 261 (very weak in **4**) and the unsymmetrical isomer (**4**) giving an ion at *m/e* 387 (absent in **3**).

Purity of the separated isomers could be quantitated by liquid chromatography. In particular, 3-iodohexestrol (**2**), which most resembles the parent estrogen hexestrol, was shown to contain less than 0.2% hexestrol.

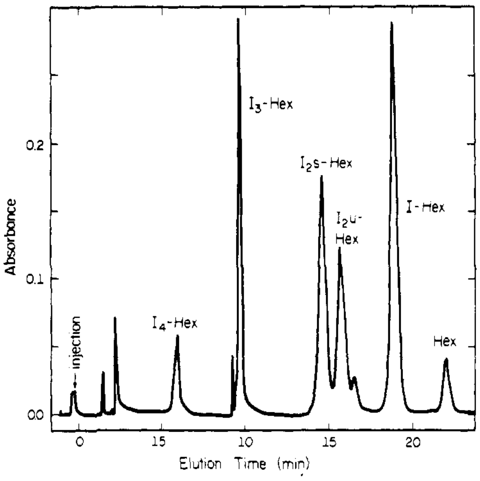


FIGURE 1: Chromatographic separation of the iodinated hexestrols. A mixture of hexestrol (**1**) and the five iodohexestrol derivatives (**2-6**) was subjected to chromatography on silica gel. (1.3 m × 2.2 mm Corasil II column; solvent A, hexane; solvent B, 2-propanol-methylene chloride, 1:4; gradient of 2-30% B in A, 2%/min, after a delay of 10 min; total flow rate 60 ml/hr.)

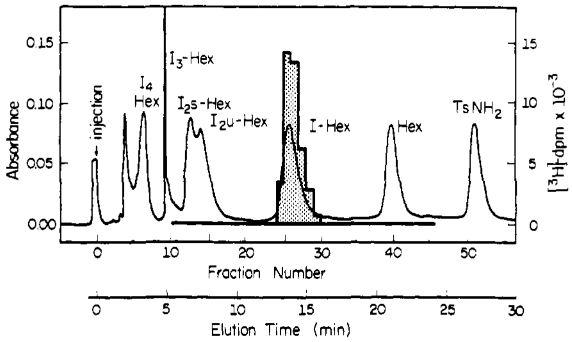


FIGURE 2: Chromatography of [^{125}I]-3-iodohexestrol with unlabeled standards. A sample of [^{125}I]-3-iodohexestrol, purified by liquid chromatography, was mixed with the five iodohexestrols (**2-6**) and *p*-toluenesulfonamide ($TsNH_2$) and subjected to liquid chromatography on silica gel (0.5 m × 2.2 mm MicroPak Si 10 column; see Figure 1 legend for elution conditions). Collected fractions were dried under a gentle stream of nitrogen and then dissolved in 0.5 ml of tetrahydrofuran for radioactivity determination. Counts in fractions other than 24-30 were insignificant.

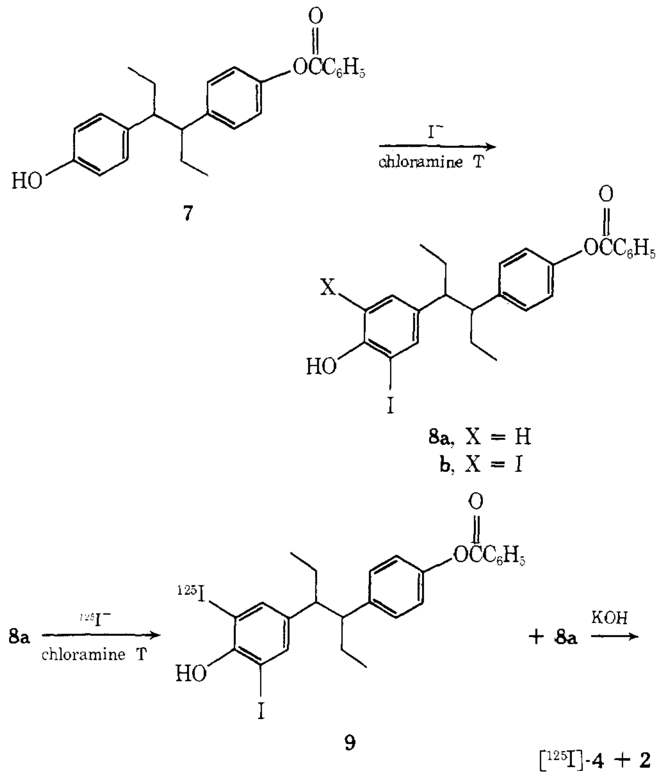
[^{125}I]- and [^{131}I]-3-Iodohexestrol and [^{125}I]-3,5-Diiodohexestrol. Hexestrol could be labeled with ^{125}I or ^{131}I using the iodide-chloramine T procedure normally used to label tyrosine residues in proteins (Greenwood, 1971). Use of a large excess of hexestrol ensured the efficient utilization of the labeled iodine (95%) and the production of only the monoiodo derivative **2.** Purification of [^{125}I]- or [^{131}I]-**2** was accomplished by liquid chromatography on a Corasil II column, which gave excellent separation of the monoiodo derivative from both hexestrol and *p*-toluenesulfonamide, a by-product formed by reduction of chloramine T. This analysis also confirmed the absence of other labeled materials. A chromatographic trace showing the coincidence of radioactivity of the purified material with monoiodohexestrol is shown in Figure 2. The radiochemical purity of the material purified by this chromatographic procedure was also established by its cocrystallization with an authentic sample of unlabeled 3-iodohexestrol in hexane-benzene and methanol-water.

[^{125}I]-3,5-Diiodohexestrol ([^{125}-I]-**4**) was needed for photolytic studies (vide infra). As direct synthesis from hexestrol would produce a mixture of all the iodinated derivatives, a more efficient synthesis was devised, as outlined in Scheme II.

Iodination of hexestrol monobenzoate (**7**) is confined to the free phenolic ring, and the monoiodo derivative (**8a**) is readily obtained by chromatographic separation from **7** and **8b.** Iodination of an excess of **8a** by the [^{125}I]iodine-chloramine T procedure gave a mixture of **8a** and **9,** which was hydrolyzed to a mixture of **2** and [^{125}I]-**4,** from which [^{125}I]-**4** could be readily obtained by liquid chromatography.

[^{3}H]-3-Iodohexestrol ([^{3}*H*]-**2**). Iodination of [^{3}H]hex-

SCHEME II



estrol by the iodide-chloramine T method gave the usual mixture of iodinated derivatives. Up to 40% of [^{3}H]monoiodohexestrol ([^{3}H]-**2**) with radiochemical purity in excess of 95% and smaller amounts of the ^{3}H-labeled derivatives **3–6** could be obtained from this reaction by liquid chromatography. Radiochemical purity of [^{3}H]-**2** was again established by cocrystallization and TLC.

Ultraviolet Spectra of Iodinated p-Cresol and Hexestrol Derivatives. The ultraviolet spectra of hexestrol and the iodohexestrol derivatives **2–6**, showing the B-band region, are given in Figure 3. The relationship between structure and spectral appearance can be appreciated by considering first the spectra of *p*-cresol (A), 2-iodo-*p*-cresol (B), and 2,6-diiodo-*p*-cresol (C), which illustrate the absorbance of the parent chromophores. Successive iodine substitution causes changes consistent with the introduction of electron donating substituents onto the *p*-cresol chromophore (Pasto and Johnson, 1969). The λ_{max} of the B band shifts to longer wavelengths, and a shoulder on the long wavelength side becomes more prominent, developing into a second maxima in C; intensification and bathochromic shifts in the E-band region (ca. 200 nm) causes a bathochromic shift and an elevation of the trough with region 240–260 nm. Furthermore, a long wavelength tail develops, especially in C, where it extends to 340–350 nm. The spectra of the compounds **1–6** can be considered simply as combinations of the chromophore types A, B, and C.

The Solution Photochemistry of I-Hex and I₂u-Hex. A series of studies by Wolf and Kharash (1965) established that the photolysis of *o*-iodophenols at 254 nm resulted in deiodination, as evidenced by the production of free iodine and the formation of products resulting from reaction with solvent, reduction in alcohols, and phenylation in benzene. These reactions are presumed to be initiated by a photohomolytic scission of the carbon–iodine bond.

We have investigated the photochemistry of I-Hex (**2**) and I_2u-Hex (**4**), being illustrative of the reaction of chromophores B and C, in methanol and benzene at 254 and >315 nm (Scheme III). Irradiation of I_2u-Hex at 254 nm in methanol causes rapid reduction to I-Hex; further reduction to Hex proceeded more slowly, as expected on the basis of the relative molar absorptivities of I_2u-Hex and I-Hex at 254 nm (cf. Figure 3). Irradiation at >315 nm permitted selective conversion of I_2u-Hex into I-Hex, without significant further reduction. Up to 30% of phenylhexestrol (**10**) could be isolated (as the dibenzoate) upon irradiation of I-Hex in benzene at 254 nm.

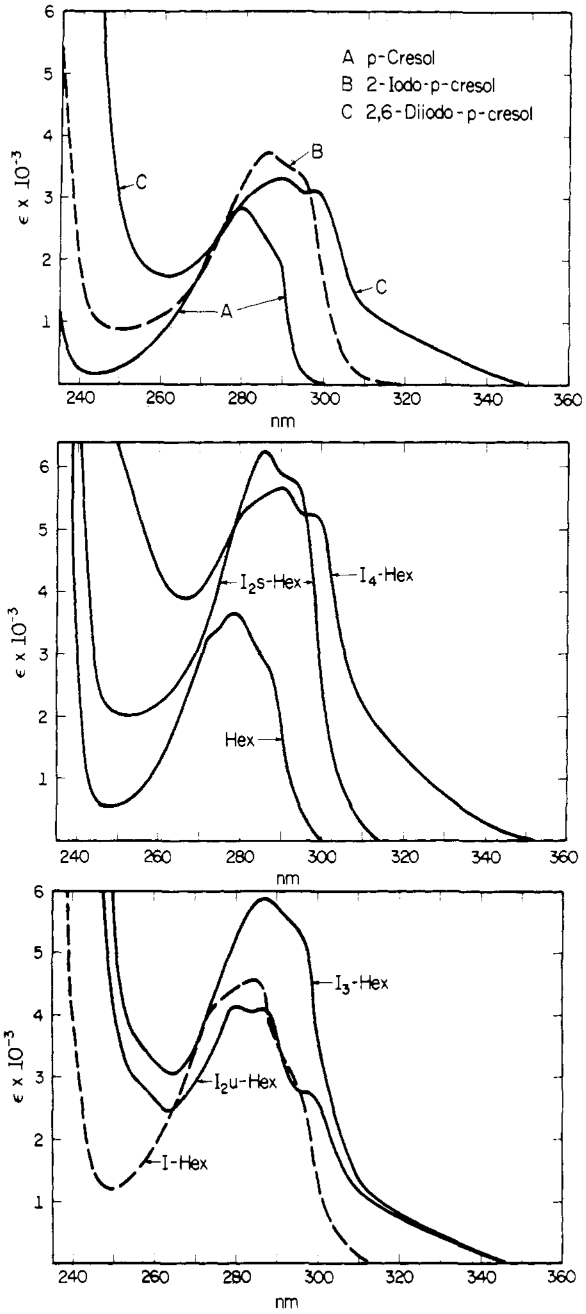
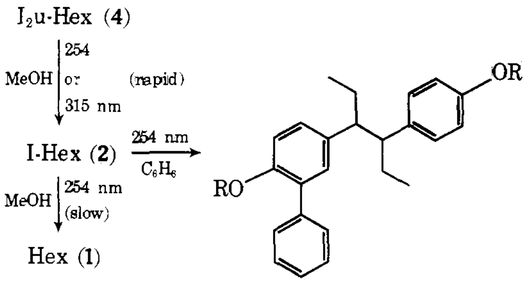


FIGURE 3: Ultraviolet spectra of iodinated hexestrols and *p*-cresols. The ultraviolet spectra of *p*-cresol (A), 2-iodo-*p*-cresol (B), 2,6-diiodo-*p*-cresol (C), and the hexestrols (**1–6**) were measured in 95% ethanol at 50–200 μM.

The Photoreactivity of Iodinated Hexestrols with the Estrogen Binding Protein from Rat Uterus. We have recently described a general method for detecting photolytic reactions of chromophoric ligands bound to the estrogen

SCHEME III



10, R = H; R = COC$_6$H$_5$

binding protein from rat uterus (Katzenellenbogen et al., 1974). According to this assay, a photosensitive ligand, bound at the estrogen binding site, is irradiated; reaction with the protein is evidenced by a decrease in the specific estradiol binding capacity, as monitored in a subsequent exchange reaction with [^{3}H]estradiol (Katzenellenbogen et al., 1973a). We investigated the photoreactivity of these iodinated hexestrol derivatives, using this assay.

The estrogen binding protein was saturated with the I-Hex (70 n*M*), I$_2$s-Hex (480 n*M*), and I$_2$u-Hex (1600 n*M*) derivatives (see Katzenellenbogen et al., 1975 for determination of the affinity of these derivatives for the estrogen binding protein) and irradiated at 254 nm for up to 10 min or at >315 nm (I$_2$u-Hex only) for up to 2 hr. Although these conditions are sufficient to cause substantial deiodination when these compounds are photolyzed in methanol, in no case did the rate of binding site consumption of the iodohexestrol-filled sites exceed that of a control irradiation in which the sites were filled with estradiol. Therefore, either the extent of photoreaction that occurs with these derivatives is minimal, or the reaction is such that the binding activity is left unimpaired.

Additional studies were done to determine whether these derivatives are capable of undergoing photocovalent attachment to the proteins in rat uterine cytosol. Irradiation of ^{3}H- and ^{125}I-labeled I-Hex, I$_2$s-Hex, and I$_2$u-Hex under the conditions of the preceding assay resulted in the attachment of no more than a few percent of the added label; furthermore, protection of the estrogen binding site, by prior incubation with estradiol, failed to reduce the extent of covalent attachment. This indicates that the attachment of these compounds is neither efficient nor selective for the estrogen binding site.

Discussion

Direct iodination is a convenient means for the preparation of the ortho-iodinated hexestrol derivatives; even though it produces a mixture of the five derivatives, chromatographic separation can readily be accomplished. The tritium labeled derivatives can be similarly prepared, although the yield of any one particular derivative is quite low.

For the preparation of the iodine-125 or -131 labeled derivatives, direct iodination is favorable only for the monoiodinated derivative. In this case efficient utilization of iodine can be assured by using an excess of hexestrol, which is later removed chromatographically. Preparation of the more highly iodinated derivatives, on the other hand, would require reaction of near stoichiometric amounts of hexestrol and iodine, which at tracer concentrations would result in both a low yield of the derivatives and an inefficient utilization of the labeled iodine. Therefore, for the preparation of [^{125}I]-I$_2$u-Hex, we resorted to a more involved, but much more efficient synthesis via I-Hex monobenzoate (**8a**).

The ultraviolet spectra of the monoiodocresol and, in particular, the diiodocresol chromophores in the iodinated hexestrol derivatives are attractive in terms of selective photolysis; there is sufficient molar absorptivity in the regions 254 nm and beyond 315 nm to permit the photofragmentation of the *o*-iodophenol unit without substantial destruction to protein. In solution, the reactivity of the radical species generated by photoscission of the carbon-iodine bond is considerable, as evidenced by its ready photoreduction in methanol and photophenylation in benzene. Despite this behavior in solution, there is no evidence that these derivatives are capable of undergoing reaction or covalent attachment with constituents in the iodohexestrol binding sites present in rat uterine cytosol proteins.

A study of the binding activity and the estrogenic character of the iodinated hexestrols is presented in the accompanying paper (Katzenellenbogen et al., 1975).

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References

Albert, S., Heard, R. D. H., Leblond, C. P., and Saffran, J. (1949), *J. Biol. Chem. 177,* 247.

Anderson, L. E., and McClure, W. O. (1973), *Anal. Biochem. 51,* 173.

Burger, A., Wilson, E. L., Brindey, C. O., and Bernheim, F. (1945), *J. Am. Chem. Soc. 67,* 1416.

Greenwood, F. C. (1971), in Principles of Competitive Protein-Binding Assays, Odell, W. D., and Daughaday, W. H., Ed., Philadelphia, Pa. Lippincott, p 288.

Jensen, E. V., and DeSombre, E. R. (1973), *Science 182,* 126.

Katzenellenbogen, J. A., Hsiung, H. M., Carlson, K. E., McGuire, W. L., Kraay, R. J., and Katzenellenbogen, B. S. (1975), *Biochemistry,* following paper.

Katzenellenbogen, J. A., Johnson, H. J., Jr., and Carlson, K. E. (1973a), *Biochemistry 12,* 4092.

Katzenellenbogen, J. A. Johnson, H. J., Jr., Carlson, K. E., and Myers, H. N. (1974), *Biochemistry 13,* 2986.

Katzenellenbogen, J. A. Johnson, H. J., Jr., and Myers, H. N. (1973b), *Biochemistry 12,* 4085.

Katzenellenbogen, J. A., Myers, H. N., and Johnson, H. J. Jr. (1973c), *J. Org. Chem. 38,* 3525.

Mayberry, W. E. (1967), *Biochemistry 6,* 1320.

Morgan, G. T., and Burgstall, F. H. (1928), *J. Chem. Soc.,* 3260.

Pasto, D. J., and Johnson, C. R. (1969), Organic Structure Determination, Englewood Cliffs, N.J., Prentice-Hall, p 99.

Wallingford, V. H., and Krueger, P. A. (1949), Organic Syntheses, Collect. Vol. II, New York, N.Y., Wiley, p 349.

Williams, D. L., and Gorski, J. (1973), *Biochemistry 12,* 297.

Williams, D. L., and Ronzio, A. A. (1950), *J. Am. Chem. Soc. 72,* 5787.

Windaus, A., and Schiele, H. (1923), *Chem. Ber. 56,* 846.

Wolf, W., and Kharasch, N. (1965), *J. Org. Chem. 30,* 2493.