Iodine-125–Labeled Estradiol: A Gamma-Emitting Analog of Estradiol That Binds to the Estrogen Receptor

Abstract. *17β-[16α-^{125}I]Iodoestradiol has been synthesized by exchange of 16β-bromoestradiol with Na^{125}I. The iodinated product is readily separated from the bromo reactant by column chromatography. It concentrates in the rat uterus in vivo and binds avidly and specifically to the uterine estrogen receptor in vitro.*

The presence of a steroid receptor in the cytosol of tumors, as in steroid target cells, is a necessary but not sufficient condition for a response to a hormonal stimulus (*1*). Analysis of certain tumors for these binding proteins (receptors) is coming into general use as a means of determining whether a specific cancer might be amenable to hormonal therapy (*2*). These analyses are difficult to perform because they deal with minute amounts of labile proteins that are ultimately measured by detecting the small amount of tritium present in the labeled steroids used to bind to the receptor. Steroids labeled with the gamma-emitting isotopes of iodine would have marked advantages over the presently used tritium-labeled ones.

As emphasized by Katzenellenbogen *et al.* (*3*), there are three criteria for an iodinated steroid to be useful for receptor studies: The linkage of the iodine atom must be sufficiently stable chemically and metabolically, the compound must bind with high affinity to the receptor, and the physiochemical properties of the compound must be such that it does not bind excessively to high-capacity, low-affinity proteins found in target tissues and serum. There are additional constraints on the synthesis of radioiodine-containing steroids for use in receptor assays. Since the half-lives of the various useful radioactive isotopes of iodine are short, a procedure for incorporating them into steroids should not be time-consuming, and the incorporation of the radionuclide should be high enough to provide a specific activity at least as great as that of the available tritiated steroids. Until now, the synthesis of compounds meeting all of these criteria has eluded numerous investigators (*4*).

I have synthesized a steroid, 17β-[16α-^{125}I]iodoestradiol, that meets all of the criteria outlined above. The compound was made by a halogen-exchange procedure that probably will be generally applicable to the synthesis of other radioiodine-labeled organic compounds (Fig. 1). Since halogen exchange reactions usually occur by mechanisms that cause epimerization, as in this case, it is possible to separate the iodinated product from the starting material by chromatography. Thus, carrier-free products may be obtained. I chose to label estradiol at C-16 because it has been shown that estriol, like estradiol, binds to the receptor (*5*), and it seemed reasonable that substitution at this position would not block this interaction. While this work was in progress it was reported in an abstract (*6*) that various C-16 and C-6 iodo derivatives of analogs of estradiol can displace estradiol from the estrogen receptor.

16β-Bromoestradiol. 16α-Bromoestrone was prepared by bromination of the enol acetate of estrone and then epimerized with LiBr (*7*). The resulting mixture of 16α- and 16β-bromoestrone was reduced with $NaBH_4$. All four of the possible bromohydrins were isolated from the reaction mixture by chromatography on silica gel in 5 percent ethyl acetate in benzene. The second compound eluted from the column (60 percent of the reaction mixture) melted at 176° to 178°C, whereupon a new crystalline structure formed (melting point, 255° to 256°C). It was identified as 16β-bromoestradiol by reaction with KOH to form estrone (*cis*-bromohydrin); epimerization with LiBr to form 16α-bromoestradiol (compared to an authentic sample) (*8*); and its mass spectrum. 16α-Iodoestradiol (melting point, 190° to 191°C) was synthesized from 16β-bromoestradiol by exchange with NaI. Its nuclear magnetic resonance (NMR) (*9*) and mass spectra were consistent with the predicted structure.

Synthesis of [16α-^{125}I]iodoestradiol. A solution of 1 mCi of Na^{125}I (17 Ci/mg; New England Nuclear) in 3 μl of water, *p*H 8 to 10, was added to 100 μl of acetonitrile in a 300-μl tapered Microflex tube (Kontes) and evaporated to dryness under a stream of nitrogen. To the dried residue was added 10 μg of 16β-bromoestradiol dissolved in 10 μl of freshly distilled 2-butanone. The vial was sealed with a Teflon-lined stopper, placed in an oven at 66° to 68°C, and heated for 4 to 6

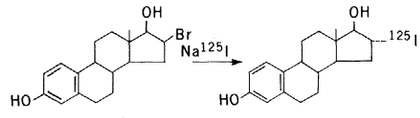

Fig. 1. Synthesis of [16α-^{125}I]iodoestradiol by exchange of 16β-bromoestradiol with Na^{125}I.

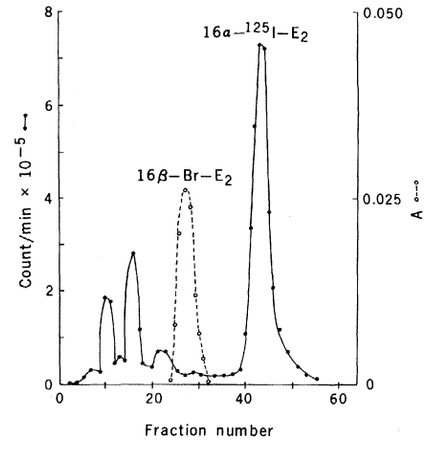

Fig. 2. Purification of ^{125}I-labeled estradiol by silica gel chromatography. The residue of the methylene chloride extract obtained from the reaction of Na^{125}I with 16β-bromoestradiol was dissolved in 0.25 ml of benzene–ethyl acetate (99:1) and chromatographed on a column (8.5 by 1 cm) of thin-layer grade silica gel in the same solvent. The column was eluted under N_2 pressure at a flow rate of approximately 60 ml/hour. Fractions of 4 ml were collected. Unreacted 16β-bromoestradiol was detected by high-pressure liquid chromatography, using an ultraviolet detector at 280 nm.

Table 1. Uptake of ^{125}I-labeled estradiol in vivo. Female Sprague-Dawley rats (200 to 250 g) were castrated 48 hours before subcutaneous injection of ^{125}I-labeled estradiol (1×10^6 count/min) dissolved in 10 μl of ethanol and 0.2 ml of saline. Twenty minutes before the administration of the iodinated estradiol, one group of rats ($N = 6$) were injected with 5 μg of DES dissolved first in 50 μl of ethanol and then in 0.5 ml of saline. Another group of castrated rats ($N = 6$) were injected with 0.5 ml of 10 percent ethanol in saline before receiving the radioactive steroid. The rats were killed 6 hours later and small pieces of the tissues were weighed and counted. All values are means ± standard deviations. To determine ether-extractable radioactivity, the uterus, liver, and thyroid were homogenized in methanol and filtered. The filtrate was evaporated and the resulting residue was partitioned between ether and 0.1*N* $NaHCO_3$. The ethereal solution was evaporated and counted.

Tissue	Radioactivity (count/min per 100 mg of tissue)		Ether-extractable radioactivity (%)
	Without DES	With DES	
Uterus	313 ± 150	73 ± 8	> 90
Liver	446 ± 184	425 ± 105	10–15
Kidney	73 ± 33	75 ± 20	
Serum	69 ± 11	68 ± 6	
Lung	47 ± 23	56 ± 11	
Muscle	33 ± 23	18 ± 12	
Heart	23 ± 5	26 ± 7	
Thyroid*	1839 ± 802	2287 ± 805	0–1

*Radioactivity in the thyroid is for the entire organ.

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hours. At the end of this time, 10 μl of 2-butanone was added to ensure against the complete evaporation of solvent and the reaction was continued overnight. The contents of the tube were transferred to a test tube (15 by 100 mm) with 5 ml of methylene chloride. This solution was washed once with 1 ml of 0.1*N* $Na_2S_2O_3$ and then twice with 1 ml of water, after which the organic solvent was dried over anhydrous Na_2SO_4, filtered, and evaporated under nitrogen. The residue was then chromatographed on a silica gel column, which cleanly separates the ^{125}I-labeled estradiol from the starting material (Fig. 2). In several experiments the yield of ^{125}I-labeled estradiol ranged from 25 to 65 percent of the incubated ^{125}I. The purified radioactive iodinated compound was stored at 0° to 4°C in a benzene-methanol mixture (95:5). Unlike [^{3}H]estradiol at 100 Ci/mmole, the ^{125}I-labeled compound is very stable and showed no signs of decomposition after weeks of storage. To prove the identity of [16α-^{125}I]iodoestradiol, it was analyzed by reverse isotope dilution and crystallization with authentic 16α-iodoestradiol (*10*). Constant specific activity was taken as evidence for the purity and identity of the radioactive material.

The apparent specific activities of various preparations of [16α-^{125}I]iodoestradiol, as determined by analysis of its binding to rat uterine cytosol receptor, ranged from 42 to 150 Ci/mmole (*11*). The specific activities of all preparations made exactly as described above ranged from 95 to 140 Ci/mmole. The reason for the failure to obtain the theoretical specific activity, ~2000 Ci/mmole, is not clear but seems to be related to impurities in the Na^{125}I preparation that cause epimerization of 16β-bromoestradiol to the 16α-bromo isomer. Nevertheless, the iodinated estradiol, made as described, is more than adequate for use in estrogen receptor studies.

Binding of [16α-^{125}I]iodoestradiol to the rat uterine receptor. To determine whether [16α-^{125}I]iodoestradiol would be recognized by estrogen-responsive tissues, it was injected into castrated female rats with and without diethylstilbestrol (DES); after 6 hours (*12*) the concentration of radioactivity in the uterus was compared to that in several other tissues. The results in Table 1 show that the concentration of radioactivity was greater in the uterus than in all other tissues except the liver and thyroid gland. However, the uterus was the only organ in which the uptake of the radioactivity was decreased by DES. This competition for uptake in the uterus by the nonsteroidal estrogen DES demonstrates the specificity of the concentration of the radioactivity by this organ. Furthermore, when the amount of ether-soluble radioactivity was compared, the uterus had a much greater concentration of radioactivity than both the liver and the thyroid (Table 1). Several micrograms of 16α-iodoestradiol were added to the ether extract of the uterus and the mixture was submitted to high-pressure liquid chromatography (*13*). Virtually all the radioactivity migrated with the carrier, demonstrating that the radioactive material in the uterus was unmetabolized ^{125}I-labeled estradiol. The radioactivity in the thyroid gland, approximately 2000 count/min, is probably due to the uptake of free iodide arising from the metabolism of ^{125}I-labeled estradiol in vivo and is equivalent to only 0.2 percent of the injected dose.

Table 2. Binding of ^{125}I-labeled estradiol and [6,7-^{3}H]estradiol to rat uterine cytosol. Uteri obtained from rats (200 to 250 g) ovariectomized 24 hours previously were homogenized in four volumes of TED buffer (0.01*M* tris-HCl, *p*H 7.4, 2.5 m*M* EDTA, and 5 m*M* dithiothreitol) with two to three pulses of 15 seconds each in a Brinkmann Polytron. The homogenate was centrifuged at 4000*g* for 10 minutes and the resulting supernatant for 60 minutes at 150,000*g*. Portions (100 μl) of the supernatant (cytosol) containing 98 μg of protein were added to the radioactive estrogens (^{125}I-labeled estradiol, 112 Ci/mmole; [6,7-^{3}H]estradiol, 45 Ci/mmole) dissolved separately in 150 μl of TED buffer with and without 7×10^{-7}*M* DES. Samples were incubated for 4 hours at 0° to 2°C. Free steroid was removed by addition of 250 μl of a suspension of 0.5 percent dextran-coated charcoal (*14*). The tubes were mixed and then centrifuged at 5000*g* for 10 minutes. The bound radioactivity remaining in solution was then counted.

Ligand	Radioactivity (count/min)			Radioactivity specifically bound (count/min)
	Added	Bound without DES	Bound with DES	
[16α-^{125}I]Iodoestradiol	69,600	42,600	3,800	38,800 (56 percent)
[6,7-^{3}H]Estradiol	63,600	28,900	5,800	23,100 (36 percent)

The most probable explanation for the concentration of the iodinated estrogen in the uterus is that the radioactive steroid is bound to the estrogen receptor. This presumption was tested by in vitro experiments designed to determine whether ^{125}I-labeled estradiol binds to the estrogen receptor. Both ^{3}H-labeled and ^{125}I-labeled estradiol were incubated with cytoplasm from the uteri of rats and the amount of bound steroid was determined (*14*). The data in Table 2 show that a larger percentage of the ^{125}I-labeled estradiol than of the [^{3}H]estradiol is bound and that there is less nonspecific binding with the iodinated estradiol. Slightly more [^{3}H]estradiol is bound when the

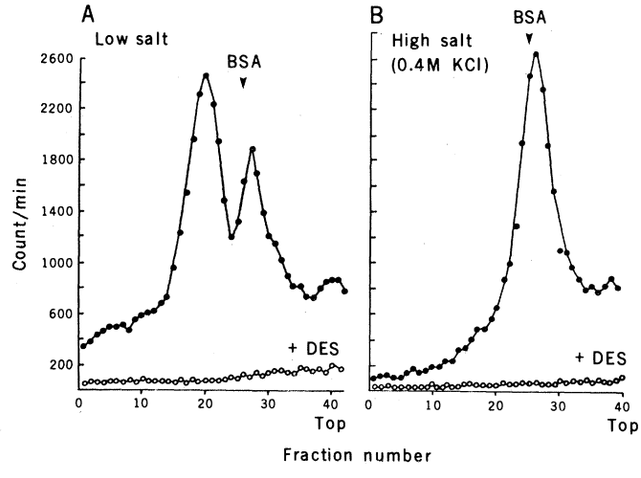

Fig. 3. Sedimentation pattern in linear glycerol gradients of ^{125}I-labeled estradiol bound to the estrogen receptor of rat uterine cytosol. Rat uterine cytosol was prepared as described in the legend to Table 2. Cytosol was diluted 1:1 with TED buffer and 0.5 ml of the mixture was incubated for 3 hours at 0°C with ^{125}I-labeled estradiol (500,000 count/min; 112 Ci/mmole) with and without DES (7×10^{-7}*M*). Separate tubes also contained 0.4*M* KCl. At the end of the incubation, free steroid was removed by adding the incubation mixture to a tube containing a 5-mg pellet of dextran-coated charcoal. After shaking for 5 to 10 minutes at 0°C, the charcoal was removed by centrifugation. A 200-μl portion of each charcoal-treated cytosol was layered on 3.8 ml of a 15 to 40 percent linear gradient of glycerol prepared in (A) TED buffer or (B) TED buffer containing 0.4*M* KCl and centrifuged at 50,000 rev/min for 18 hours in a Beckman SE-60 rotor. Fractions of four drops were collected from the bottom of the tube. Control tubes containing cytosol mixed with ^{14}C-labeled bovine serum albumin (*BSA*) as a marker were also analyzed.

difference in specific activities is taken into account.

In a separate set of experiments the cytoplasmic extracts, incubated with the iodinated and tritiated tracer, were centrifuged on glycerol gradients (*15*) in the presence and absence of 0.4*M* KCl. The low-salt 8*S* form (Fig. 3a) is totally transformed into the 4*S* form by 0.4*M* KCl (Fig. 3b) (*16*). The radioactivity bound to both the 8*S* and 4*S* forms is almost completely displaced by DES. The glycerol density gradients of the cytoplasmic extracts incubated with [^{3}H]estradiol (not shown) were identical to those incubated with the iodinated tracer with two exceptions: there was less bound radioactivity and more nonspecific binding of [^{3}H]estradiol than of ^{125}I-labeled estradiol.

The charcoal assay and glycerol gradient analysis in concert with the competition by the nonsteroidal estrogen DES show that [16α-^{125}I]iodoestradiol binds to the estrogen receptor. Unlike the results obtained by Katzenellenbogen *et al.* (*3*) with ^{125}I-labeled hexestrol, there was no evidence for increased nonspecific binding sites in the uterus when compared to [^{3}H]estradiol. The specific binding of the ^{125}I-labeled estradiol compares favorably with that of [^{3}H]estradiol, indicating that more sensitive assays than that obtainable with [^{3}H]estradiol could be performed with this high-specific-activity tracer. In addition, ^{125}I-labeled estradiol does not bind to the testosterone-estradiol binding globulin present in human plasma (*11*). This protein (*17*) contaminates most human tissue preparations, complicating the detection of sex steroid receptors. The use of the ^{125}I-labeled estradiol as a ligand in such assays will eliminate the "nonspecific" binding caused by this protein. Kinetic experiments and Scatchard analyses (*18*) indicate that in rat uterine cytosol the binding constant, K_d, of ^{125}I-labeled estradiol, is very close ($K_d = 2.7 \times 10^{-10}$) to that of [^{3}H]estradiol ($K_d = 1.6 \times 10^{-10}$) (*11*).

Its binding characteristics, stability, and ease of preparation, as well as the economic and technical advantages in determining radiation from a gamma emitter as compared to a beta emitter, make [16α-^{125}I]iodoestradiol the compound of choice for experimentation with the estrogen receptor. The studies in the rat in vivo demonstrating that ^{125}I-labeled estradiol is concentrated in the uterus, a tissue containing the estrogen receptor, provides an impetus for experiments to determine whether this steroid labeled with ^{131}I could be used in vivo for the radioimaging of estrogen receptor-containing breast tumors. It has also been found that ^{125}I-labeled estradiol binds to antibodies to estradiol conjugated at positions 3, 6, and 17 of the steroid nucleus and thus can be used as a ligand for the radioimmunoassay of estradiol (*11*).

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19. I gratefully acknowledge M. Lippman, who encouraged me to perform these studies. I thank J. Fishman for the NMR analyses, W. Rosner for his advice and support, and W. Douglas for his excellent technical assistance. This work was supported in part by NIH grants RO1-CA24512, KO4-CA00174, and T32-AM07328.

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Molecular Cloning of Polyoma Virus DNA in *Escherichia coli:* Oncogenicity Testing in Hamsters

Abstract. *Inoculation of suckling hamsters with 2 × 10^8 live cells of* Escherichia coli *K12 strain χ1776, carrying the complete genome of polyoma virus in a recombinant plasmid, failed to induce tumors in any of 32 recipients. Also, lambda phage DNA and particles with a monomeric insert of polyoma DNA did not induce tumors. Purified recombinant plasmid DNA, as well as phage particles and DNA containing a head-to-tail dimer of polyoma DNA, showed a low degree of oncogenicity, comparable to that of polyoma DNA prepared from mouse cells. These findings support the previous conclusions, based on infectivity assays in mice, that propagation of polyoma virus DNA as a component of recombinant DNA molecules in* E. coli *K12 reduces its biologic activity many orders of magnitude relative to the virus itself.*

We have recently reported the results of a series of risk assessment experiments involving derivatives of *Escherichia coli* K12 bearing recombinant DNA that contains the complete genome of polyoma virus (*1, 2*). In those experiments, the circular polyoma viral DNA was converted to the linear form by cleavage with single-cut restriction enzymes, ligated to plasmid or lambda phage vectors, and propagated in *E. coli* K12. The *E. coli* that contained recombinant DNA, as well as the purified recombinant DNA, were tested for their ability to produce polyoma infection. Although the recombinant molecules contained complete polyoma genomes which were infectious when enzymatically excised from the recombinant molecules, *E. coli* carrying these molecules consistently failed to induce polyoma infection, even when massive numbers were fed or injected into mice, a highly sensitive indicator system for productive polyoma infection. Similar results were recently reported by Fried *et al.* (*3*).

As a further step in evaluating the biologic activity of the polyoma-plasmid and polyoma-lambda recombinant DNA host-vector systems, we have tested their ability to induce tumors in suckling

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