Interaction of 16α-[125I]iodo-estradiol with estrogen receptor and other steroid-binding proteins

(gamma-emitting estrogen/testosterone-estradiol-binding globulin/estradiol antibodies/estrogen bioassay/breast cancer)

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ABSTRACT   This communication describes the synthesis of 16α-[125I]iodo-estradiol (^{125}I-E$_2$) with specific activities >1000 Ci/mmol (1 Ci = 3.7 × 10^{10} becquerels). We show that it binds to the same specific estrogen receptor sites as does [^{3}H]estradiol and that it does so with an affinity that is indistinguishable from that for the latter steroid. This is true for receptor obtained from calf uterus and from a pool of human mammary carcinomas. There is no significant binding of ^{125}I-E$_2$ to the testosterone-estradiol-binding globulin of human plasma. ^{125}I-E$_2$ also binds tightly to anti-estradiol antibodies raised against estradiol derivatized at carbons 3, 6, and 17. Finally, we show that unlabeled I-E$_2$ is an active estrogen in vivo as demonstrated by its ability to increase uterine weight in ovariectomized rats.

The demonstration of receptors for steroid hormones necessitated the synthesis of such hormones with high degrees of specific radioactivity. Jensen and Jacobson (1) accomplished this task by synthesizing ^{3}H-labeled estradiol (E$_2$) with a sufficiently high specific activity and then showing that estrogen target tissues retained the ^{3}H-labeled hormone even when the hormone concentration in blood was lower. These observations, together with the subsequent elucidation of soluble estrogen receptors (2) in the cytoplasm of estrogen target tissues, formed the foundation for a new area of both basic and applied research in steroid hormone action.

Subsequent to these original experiments it has become clear that important studies could be made possible or greatly simplified if an estrogen containing a γ-emitting isotope were available. The specific activity of ^{125}I, ≈2200 Ci/mmol (1 Ci = 3.7 × 10^{10} becquerels), is much higher than that of ^{3}H, ≈29 Ci/mmol (incorporation of multiple ^{3}H atoms has allowed the synthesis of steroids with a maximal specific activity of ≈100 Ci/mmol) and would allow receptors to be measured with greater sensitivity. Radioautographic studies of tissues containing receptors, which now take months, could be accomplished in days. Through the use of isotope pairs that can be counted simultaneously because of their different energy spectra—e.g., ^{125}I and ^{131}I—many experiments that can now be done only indirectly could be done simply and directly. By using the appropriate isotope of iodine along with currently available scanning equipment, it might be possible to perform external imaging of tissues containing estrogen receptors, such as certain breast cancers. Finally, γ-emitting isotopes are detected and quantitated more cheaply, more rapidly, and with equipment more generally available in clinical laboratories than are β-emitting isotopes.

These advantages of γ-emitting isotopes are well known and the search for an estrogen containing such an isotope (3), which could bind specifically to estrogen receptors, has not been neglected (4–8). Until recently, attempts to synthesize appropriate

compounds were unsuccessful. The synthesis of 16α-iodo-17β-estradiol (I-E$_2$) has been described and preliminary studies on some of its properties have been reported (9).

This communication describes in some detail the binding in vitro of I-E$_2$ to estrogen receptors from various sources, demonstrates that it is estrogenic in vivo, and gives examples of its high binding affinity to several anti-E$_2$ antisera currently in use as reagents in radioimmunoassays for estrogens.

MATERIALS AND METHODS

General. [2,4,6,7-^{3}H]Estradiol (92 Ci/mmol), [6,7-^{3}H]estradiol (42 Ci/mmol), 5α-[1,2-^{3}H]dihydrotestosterone (55 Ci/mmol), and Na^{125}I (in low-pH high-concentration solution) were obtained from New England Nuclear. Unlabeled steroids were purchased from Steraloids, (Wilton, NH). Antisteroid antisera were produced in rabbits immunized with steroid–bovine serum albumin conjugates. Antibodies against estradiol 17-hemisuccinate–albumin were raised in this laboratory. Antibodies against estradiol-6-carboxymethyloxime-albumin were kindly furnished by John O'Connor and David Fukishima of the Steroid Institute, Montefiore Hospital. Antibodies against the 3-carboxymethyl ether of E$_2$ conjugated to albumin were a gift from R. Perrin of Radioassay Systems Laboratories, Inc.

Preparation of 16α-[125I]Iodo-17β-estradiol (^{125}I-E$_2$). In essence, ^{125}I-E$_2$ was prepared by halogen exchange using 16β-bromo-17β-estradiol as the starting material. The entire procedure was described previously (9) but we have made some modifications. After synthesis of the brominated steroid (9), 5 mg of it was dissolved in 500 μl of CH$_2$Cl$_2$ and subjected to high-pressure liquid chromatography on a column of Lichrosorb Diol (250 × 4.6 mm, Merck) which was eluted at 1 ml/min with CH$_2$Cl$_2$. The brominated steroid eluted in a sharp peak in 8 min. This purified Br-E$_2$ served as the substrate for a modified halogen exchange procedure.

One to 2 mCi of carrier-free Na^{125}I in 5–10 μl of aqueous medium was added to 100 μl of freshly distilled acetonitrile in a 100-μl microflex tube (Kontes) and was taken to dryness under a stream of nitrogen. Because some ^{125}I is entrained in the vapor, the entire evaporation process was carried out in a hood in an enclosed vessel that had an exit port connected to a charcoal-filled filter. To the dried residue we added 10 μg of bromo-estradiol and 30 μl of freshly distilled 2-butanone. The mixture was stoppered with a Teflon lined cap and heated overnight at 78°C in a heating block; the next morning the products were extracted and chromatographed on a silica gel column (9). Those fractions containing ^{125}I-E$_2$ were combined, evaporated, dissolved in 250 μl of CH$_2$Cl$_2$, and subjected to high-pressure liquid chromatography as above. The ^{125}I-E$_2$,

Abbreviations: I-E$_2$, 16α-iodo17β-estradiol; E$_2$, estradiol; DES, diethylstilbestrol; TE$_2$BG, testosterone-E$_2$-binding globulin.

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which eluted in about 12 ml, was separated cleanly from the Br-E_2, which was eluted in 8 ml (Fig. 1). The mass associated with the tracer was detected at 280 nm and quantified by comparing the area of the UV-absorbing peak with a standard curve produced with known amounts of I-E_2. Although the UV peak was small, it allowed a preliminary estimate of the specific activity of the ^{125}I-E_2.

Analyses of Estrogen Receptors. Fresh calf uteri obtained from a slaughterhouse were brought to the laboratory on ice, immediately trimmed of fat, and cut into small pieces; the pieces were frozen in liquid nitrogen and pulverized. The pulverized frozen tissue was lyophilized, and the desiccated powder was stored under vacuum in sealed vials at −80°C. The uterine powders were usually used within 1 month of pulverization. When needed, the powders were suspended in 0.1 M Tris·HCl, pH 7.4/2.5 mM EDTA/5 mM dithiotreithol (TED buffer) at 2.5 mg/ml and homogenized at 0°C in a Polytron homogenizer with two or three pulses of 15 sec each. The homogenate was then centrifuged at 150,000 × g for 60 min and the resulting supernatant (cytosol), containing approximately 1 mg of protein per ml, was used for binding studies on the estrogen receptor.

The binding of ^{3}H-E_2 and ^{125}I-E_2 to the estrogen receptor was determined by adsorbtion of the free steroid with dextran-coated charcoal (10). In brief, varying amounts of the radioactive ligands, with or without 0.7 μM diethylstilbestrol (DES), were added in duplicate to 10 × 75 mm test tubes. After the solvent was removed under a stream of N_2, 250 μl of cytosol was added and the samples were incubated at 0–2°C overnight. The following morning, 250 μl of a 0.5% suspension of dextran-coated charcoal in TED buffer was added to the tubes which then were mixed and centrifuged at 5000 × g for 10 min. The supernatant was assayed to determine bound radioactivity.

Radioimmunoassays. Varying amounts of E_2 dissolved in methanol were added to test tubes and evaporated to dryness.
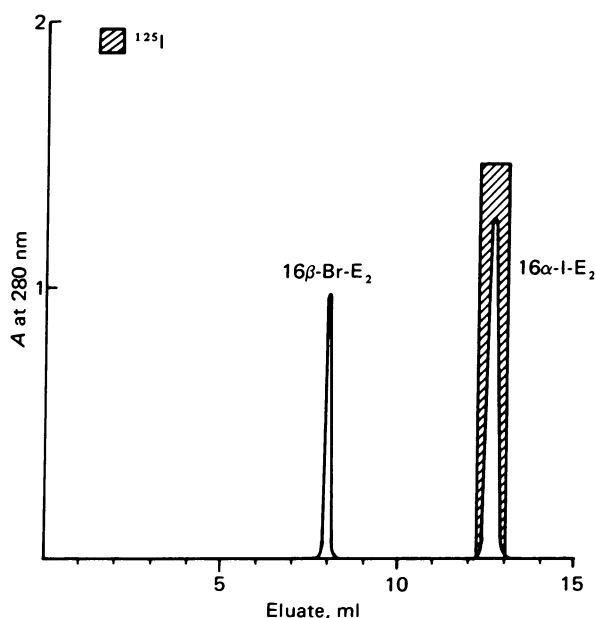

FIG. 1. High-pressure liquid chromatography of the products of the halogen exchange with Na ^{125}I. The reaction mixture resulting from the halogen exchange reaction was subjected to chromatography on silica gel (9) and the ^{125}I-E_2-containing fractions were pooled and chromatographed on a column of Lichrosorb Diol (1 ml/min). In this experiment, authentic 16α-I-E_2 and 16β-Br-E_2 were added before the sample was injected.

The residues were dissolved in 100 μl of antiserum that had been diluted in 0.1 M phosphate, pH 7.0/0.015 M sodium azide/0.15 M NaCl/0.1% gelatin (PBS buffer). After the mixture had incubated at room temperature for 30 min, 100 μl of PBS buffer containing 10,000 cpm of either ^{125}I-E_2 or ^{3}H-E_2 was added to the tubes and the incubation was continued for 1 hr at room temperature and then for 30 min at 4°C. Upon completion of the incubation, 100 μl of 0.5% gelatin in PBS buffer was added, followed by 1 ml of PBS buffer containing 2.5 mg of dextran-coated charcoal. The mixture was agitated in a vortex mixer and, after 10 min at 4°C, it was centrifuged at 5000 × g for 10 min at 4°C. Aliquots (0.8 ml) were assayed for radioactivity.

Testosterone-E_2-Binding Globulin (TE$_2$BG). Binding to TE$_2$BG was determined as described (11). Plasma from pregnant women diluted 1:20 was incubated in triplicate with the indicated radioactive steroids for 15 min at 22°C and then for 15 min at 0–2°C. The bound radioactivity was precipitated with 50% (final concentration) ammonium sulfate, and the free radioactivity remaining in solution was assayed. Nonspecific binding was determined for each ligand by conducting parallel incubations with an excess of 5α-dihydrotestosterone.

Mammary Tumors. Estrogen receptor-positive human mammary tumors were kindly provided by J. Wittliff (University of Louisville School of Medicine). They were shipped frozen in dry ice in a pulverized form. The cytosol was prepared and assayed as described for the calf uteri with the exception that 50 mg of tumor tissue was homogenized in 1 ml of TED buffer. The final concentration of cytosolic protein used in the assay was 1.5 mg/ml.

Estrogen Bioassay. Estrogenic potency was assayed (12) in 21-day-old female rats. Groups of six rats received daily subcutaneous injections of various doses of E_2, I-E_2, or sesame oil (control) for 3 days. On the fourth day, the uteri were removed, trimmed, blotted, and weighed.

RESULTS

Binding of ^{3}H-E_2 to the Calf Uterine Receptor. We studied binding of I-E_2 to the calf uterine estrogen receptor for several purposes. By doing experiments in which unlabeled I-E_2 was used to displace a small (subsaturating) constant amount of ^{125}I-E_2 and parallel experiments in which variable amounts of ^{125}I-E_2 were used, we could determine the specific activity of the ^{125}I-E_2 (13). In the same experiment we could ascertain directly the association constant for I-E_2 with the receptor. Using the same uterine preparation, we could also compare these values to those obtained with ^{3}H-E_2. The results illustrated in Figs. 2 and 3 are representative of many such experiments. The specific activity of the ^{125}I-E_2 used in these experiments, determined from its UV absorbtion on high-pressure liquid chromatography was 1250 Ci/mmol, which was in excellent agreement with the value, 1310 Ci/mmol, determined from binding experiments.

Whereas the ratio of the specific activity of the ^{125}I-E_2 to that of the ^{3}H-E_2 in this experiment was about 30, the ratio of binding capacities is close to 45 (122,000 cpm of ^{125}I-E_2 bound compared to 2800 cpm of ^{3}H-E_2) (Fig. 2). This apparent discrepancy is accounted for by the difference in counting efficiencies for the two isotopes. The high counting efficiency of ^{125}I is one of its important advantages. We stress that the specific activity of the ^{125}I-E_2 was arrived at without reference to the ^{3}H-E_2 and that the Scatchard plot shown in Fig. 3 therefore demonstrates that the number of specific binding sites in uterine cytosol for both compounds is precisely the same. It is also apparent that the dissociation constant (which is based on the slope) for both compounds is also the same. Finally, Fig. 2 il-
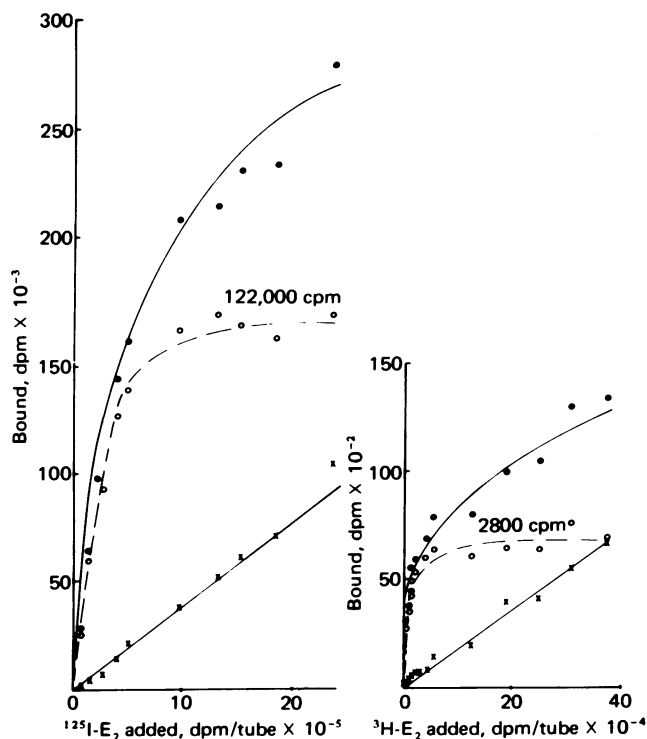
FIG. 2. Saturation analysis of the estrogen receptor from calf uterus. Calf uterine estrogen receptor was prepared and assayed with increasing concentrations of either ^{3}H-E$_2$ (*Right*) or ^{125}I-E$_2$ (*Left*). Nonspecific binding (X) was determined in parallel incubations containing 0.7 μM DES. O, Specific binding; ●, total binding. Although the units on both the abscissa and the ordinate are in dpm, the plateaus of specific binding are shown in cpm. This is done in order to stress the increase in sensitivity and decrease in counting time that can be achieved because of the more efficient detection of ^{125}I compared to ^{3}H.

lustrates the fact that the level of nonspecific binding not only is acceptably low for ^{125}I-E$_2$ but also is less than that of the ^{3}H-labeled hormone.

Specificity of the Estrogen Receptor. In order to be sure that both ^{125}I-E$_2$ and ^{3}H-E$_2$ bound with similar specificities, experiments were undertaken in which a series of compounds were allowed to compete with the two differently labeled estrogens for sites on the estrogen receptor. The results of this experiment are shown in Table 1. Only compounds with estrogenic activity were active in displacing E$_2$ labeled with either isotope; none of the C$_{19}$ or C$_{21}$ steroids competed. Because of the difference in concentration between ^{125}I-E$_2$ and ^{3}H-E$_2$ and the consequent difference in initial binding (40 and 11%, respectively), precise quantitative comparison between the two compounds is not possible; it is apparent, however, that, regardless of which radioactive ligand was used, E$_2$ and I-E$_2$ were about equipotent in competing for the estrogen receptor. This is consonant with, and confirms, the independently arrived at data which show (see Fig. 3) that the binding constant for the two isotopically labeled estrogens are identical.

Radioimmunoassay. Both ^{3}H-E$_2$ (42 Ci/mmol) and ^{125}I-E$_2$ (1300 Ci/mmol) were incubated with various dilutions of antisera produced against bovine serum albumin complexes with E$_2$ 17-hemisuccinate, E$_2$-6-carboxymethyloxime, and E$_2$ 3-carboxymethyl ether; displacement of the bound tracer by picogram amounts of E$_2$ was measured. Whereas both the ^{3}H-E$_2$ and ^{125}I-E$_2$ bound to all three antisera, the importance of the great difference in the specific activities became apparent at very high dilutions of the antibodies. These antisera were capable of binding sizeable amounts of the iodinated steroid at
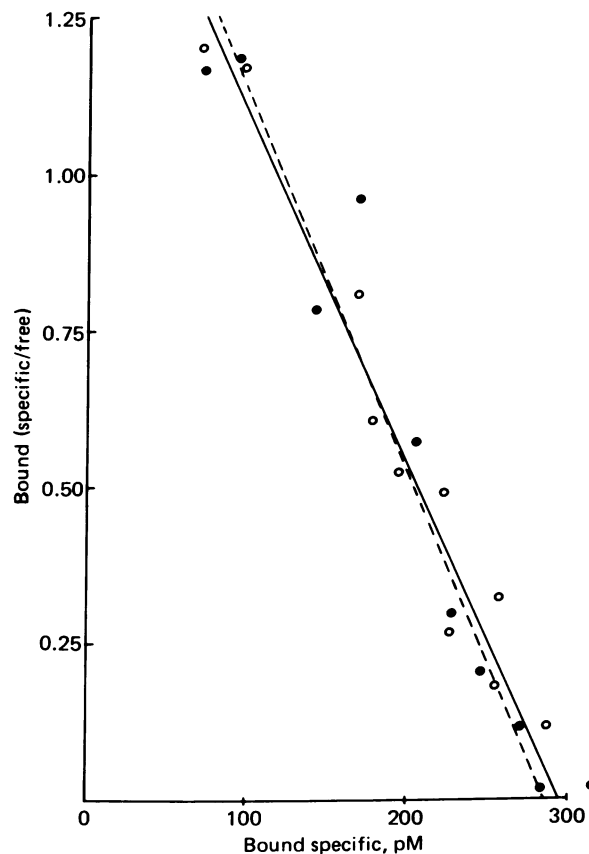

FIG. 3. Scatchard analysis of the estrogen receptor from calf uterus. The experiment with ^{3}H-E$_2$ (●) used the same data as the one in Fig. 2. The ^{125}I-E$_2$ experiment (O) was done with a constant amount (61,000 dpm, 83 pM) of the isotopically labeled steroid and increasing concentrations of unlabeled I-E$_2$. The same result was obtained with increasing concentrations of ^{125}I-E$_2$. For ^{3}H-E$_2$: K_a = 6.4 × 10^9 M^{-1}; binding capacity = 401 fmol/mg of protein. For I-E$_2$: K_a = 7.1 × 10^9 M^{-1}; binding capacity = 411 fmol/mg of protein.

dilutions that bound barely measurable quantities of ^{3}H-E$_2$ (Fig. 4). The data in Fig. 4 also indicate that very sensitive radioimmunoassays could be developed with all three antisera. In fact, with anti-E$_2$-6-carboxymethyloxime–albumin, almost 50% of the tracer was displaced from the antibody with only 1 pg of E$_2$.

Binding to TE$_2$BG. There is a naturally occurring protein in human plasma that binds estrogens specifically and with high affinity (14) and that can interfere with assays for receptors in human tissues, specifically with the assay for estrogen receptors in breast cancers. We therefore examined the binding of ^{125}I-E$_2$ to TE$_2$BG along with two of the steroids, [^{3}H]dihydrotestosterone and ^{3}H-E$_2$, both of which are known to bind to TE$_2$BG. Because it was already known (14) that estriol does not bind to TE$_2$BG, it did not seem likely that ^{125}I-E$_2$ would bind. This is confirmed by the data in Table 2.

Binding to Human Mammary Tumors. A pool of two different estrogen receptor-positive mammary tumors was studied. Each was assayed at a protein concentration of 1.5 mg/ml with eight different concentrations of ^{3}H-E$_2$ and ^{125}I-E$_2$. Using ^{125}I-E$_2$, we found the receptor concentration to be 24 fmol/mg of protein for the first pool (no. 124) and 72 fmol/mg for the second (no. 126). With ^{3}H-E$_2$, the respective concentrations were 36 and 64 fmol/mg of protein. The observed differences are probably insignificant for both clinical and scientific purposes.

Bioassay for Estrogenic Activity. Fig. 5 presents simulta-

Table 1. Competition for the estrogen receptor

| | $B/B_0 \times 10^2$ | | | |
| | $^{125}I\text{-}E_2$ | | $^{3}H\text{-}E_2$ | |
Compound	0.5 nM	5 nM	0.5 nM	5.0 nM
I-E_2	35	2	69	12
E_2	34	1	66	30
Estrone	67	17	99	50
Estriol	70	20	92	47
DES	32	0	60	0
Testosterone	93	93	97	95
5α-Dihydrotestosterone	100	96	99	95
Dehydroisoandrosterone	104	107	98	108
Progesterone	98	102	97	102
17α-Hydroxyprogesterone	106	106	93	91
Corticosterone	100	93	103	97
Cortisol	94	103	105	100
Dexamethasone	100	98	100	100

Each compound, at the two indicated concentrations, was allowed to compete with both labeled steroids for sites in calf uterine cytosol. Incubations were overnight at 4°C, and bound and free steroids were separated with dextran-coated charcoal. The concentrations were: $^{3}H\text{-}E_2$, 1.6 × 10^5 dpm/ml (0.74 nM); $^{125}I\text{-}E_2$, 6.0 × 10^5 dpm/ml (0.22 nM). B_0, dpm specifically bound when only the radioactive compound was present; B, dpm bound in the presence of the indicated concentration of competitor. % B_0 = 40% for $^{125}I\text{-}E_2$ and 11% for $^{3}H\text{-}E_2$. Nonspecific binding was determined by parallel incubations in the presence of 70 nM DES (see Fig. 2).

neously determined dose–response curves of the estrogenic activity of E_2 and I-E_2. It is apparent that I-E_2 is estrogenic *in vivo*, albeit not as potent as E_2 itself. This difference in potency, despite association constants for the receptor that are equal for the two compounds, should in no way be considered an important surprise. It can be explained either by differences in the peripheral metabolism of the two compounds or by differences in events that occur after binding to the cytosolic receptor has taken place. At this time we believe the former explanation to be correct because the two estrogens are equipotent *in vitro* in inducing growth of an estrogen-dependent human breast tumor cell line (MCF-7) (unpublished data).
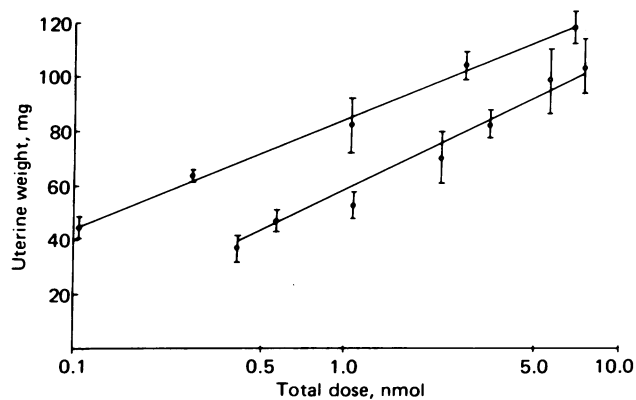
FIG. 5. Uterotropic potency of E_2 (●) and I-E_2 (○). Each point is the mean ± SD of data from six animals.

DISCUSSION

The original report of the synthesis of $^{125}I\text{-}E_2$ described preparations whose specific activities varied from 42 to 150 Ci/mmol. Our current preparations vary from 1000 to 2000 Ci/mmol (the theoretical maximum is 2200 Ci/mmol). This improvement is related mainly to the meticulous purification of the precursor compounds, 16β-Br-E_2, used in the halogen exchange reaction. Specifically, we found that minute amounts of 16α-Br-E_2 contaminated the 16β-Br-E_2 substrate used in the iodination. Because 16α-Br-E_2 binds strongly to the receptors and also cochromatographs with 16α-I-E_2, its presence caused an artifactual decline in the measured specific activity of the $^{125}I\text{-}E_2$.

It is apparent that the position in the steroid nucleus to which the iodine atom is attached is critical in the synthesis of a radioiodinated ligand that can bind to the estrogen receptor. What may not be as clear is that the mode of synthesis is equally important. Although many reagents and synthetic routes are commonly used for the preparation of organic iodides, most of the reagents are not available labeled with radioiodine and most synthetic routes are not suitable for such radioactive com-
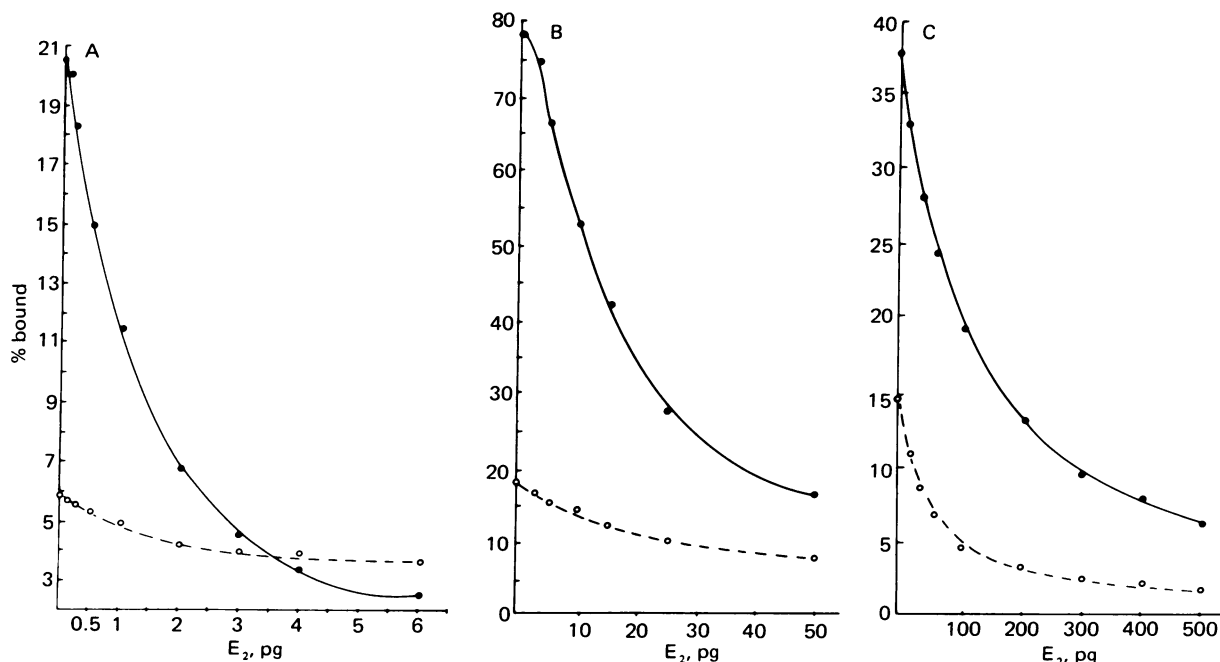
FIG. 4. Standard curves with antibodies against three different E_2 conjugates. (*A*) Against E_2-6-carboxymethyloxime–bovine serum albumin. (*B*) Against E_2-17β-hemisuccinate–bovine serum albumin. (*C*) Against E_2-3-carboxymethylether–bovine serum albumin. ●, $^{125}I\text{-}E_2$, 10^3 cpm (4.8 fmol); ○, $^{3}H\text{-}E_2$, 10^3 cpm (215 fmol).

Table 2.   Binding of dihydrotestosterone (DHT), E_2, and ^{125}I-E_2 to TE_2BG

| Ligand | | | Free, cpm | | Specific binding, cpm |
Compound	Specific activity, Ci/mmol	cpm added	Tracer	Tracer + DHT	
^3H-DHT	55	66,700	5,300	56,100	50,800
^3H-E_2	45	53,500	6,800	48,000	41,200
^{125}I-E_2	105	52,400	38,000	37,100	−900

Diluted pregnancy plasma served as the source of TE_2BG. TE_2BG-Bound steroid was precipitated with ammonium sulfate and the free steroid in the supernatant was assayed for radioactivity. Specifically bound steroid was calculated as the difference between "cpm added" and "tracer + DHT."

pounds. The short half-lives of the various useful isotopes of iodine, as well as the many difficulties in dealing with radioisotopes, preclude long, laborious syntheses. Thus, although usable nonradioactive iodinated compounds can be made (8) by various methods, the technique of synthesis of the same substances with radioiodine may not be transferrable. For the [^{125}I]iodoestrogens, and for other analogous organic compounds (of which hormonal steroids represent only a small group), the halogen exchange procedure is vital.

We have described in some detail the interaction of ^{125}I-E_2 with a number of proteins. It binds specifically and with high affinity to estrogen receptors from calf and rat (9) uterus, rat hypothalamus and pituitary (E. Peck, personal communication), and human breast carcinoma and to antiestrogen antibodies; it does not bind to TE_2BG from human plasma.

The exceedingly high specific activities attainable with ^{125}I-E_2 relative to ^3H-E_2, together with the fact that the binding constants for the estrogen receptor of the two radioactive ligands are generally indistinguishable, makes the iodinated estrogen an extremely attractive tool for experiments on this receptor. Furthermore, the same exchange procedure can be used to synthesize 16α-I-E_2 labeled with ^{123}I and ^{131}I. Estrogens labeled with these isotopes have a great potential in the diagnosis and treatment of estrogen-dependent cancers.

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