Estrogen Derivatives for the External Localization of Estrogen-Dependent Malignancy

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Four radioiodinated estrogen derivatives were studied to determine their affinity for the estrogen-binding protein found in the cytosol of rabbit and rat uteri. In vitro determination of the binding properties by competitive-binding experiments and by sucrose-gradient centrifugation indicates that one of the derivatives, iodohexestrol, binds to the cytosol estrogen-binding protein. This in vitro behavior was related to in vivo distribution. Studies in immature female rats showed high uterine uptake of iodohexestrol at 2 hr (1.69% dose/gm). Iodohexestrol also has a high nonspecific binding in both the blood and the uterine cytosol. Thyroxine can diminish the nonspecific binding in vitro; in vivo the prior injection of thyroxine increased the 2-hr uterus-to-blood ratio from 1.9 to 10.4. The in vitro receptor-assay system was helpful in predicting in vivo distribution.

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Tritiated hexestrol and estradiol-17β concentrate in certain experimental and human tumors (*1,2*). The concentration mechanism depends on the presence of specific intracellular estrogen-receptor proteins (*3*). These receptor proteins appear to be the same as those responsible for the concentration of estradiol in natural estrogen-responsive tissues such as the uterus (*4*). Since recent studies have shown a direct positive correlation between the presence of the estrogen receptors in malignancy and the remission of the tumor after endocrine ablation (*5*), the development of an estrogen derivative containing a gamma-emitting nuclide could be useful in detecting metastases and in determining the estrogen dependency of metastatic tumors in sites that are not easily biopsied.

This differentiation of estrogen-dependent tumors from tumors detected by other procedures would provide a noninvasive method to help predict the response to endocrine-ablation therapy. The estrogen derivatives can be evaluated readily with the estrogen-receptor assay. This in vitro system may prove helpful in predicting the in vivo distribution.

MATERIALS AND METHODS

Figure 1 shows the structures of four compounds of potential interest: estradiol-17β (I); hexestrol (II); 1,3,5-estratriene,3-ol,17β-yl N-([1-carbomethoxy-2-(4-hydroxyphenyl)]ethyl)succinamate (III); and 1,3,5-estratriene,3,17β-diol,6-aminoxy acetylamino-3-(4-hydroxyphenyl)propionate (IV). These were either purchased or synthesized by standard techniques (*6,7*).

The compounds were iodinated using equimolar amounts of iodine and chloramine T (*8*). Iodine-125 was used as the radioactive tracer. The location of the iodine is indicated in Fig. 1. In the preparation of compounds IIIa and IVa, the tyrosine methyl ester was iodinated before amide formation. All the compounds were analyzed by thin-layer chromatography, and chemically and radiochemically pure samples were obtained by elution from preparative thin-layer silica-gel plates (*6,7*). Compound Ia was chromatographed in benzene–ethyl acetate (60:40), IIa in methylene chloride, and IIIa and IVa in either benzene–ethyl acetate–acetic acid (60:40:0.5) or chloroform–methanol–water (9:1:0.1).

All chemicals were reagent grade. The ^{3}H-estradiol-17β (54.3 Ci/mmole) and the ^{125}I were ob-

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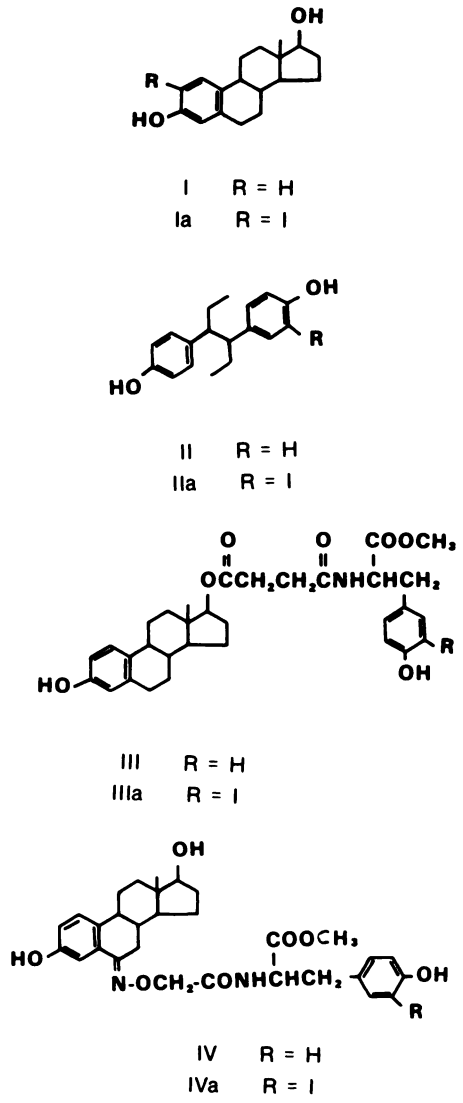

FIG. 1. Structures of estradiol (I); hexestrol (II); 1,3,5-estratriene,3-ol,17β-yl N-([1-carbomethoxy-2-(4-hydroxyphenyl)]ethyl) succinamate (III); and 1,3,5-estratriene,3,17β-diol,6-aminoxy acetylamino-3-(4-hydroxyphenyl)proprionate (IV).

tained commercially and used without further purification. The protein concentration was measured by Lowry's method (*9*).

Frozen uteri from 6-day-pregnant rabbits or from 23–25-day-old immature rats were homogenized in two volumes of TED buffer (0.01 *M* Tris–HCl, pH 7.5, containing 1.5 m*M* EDTA and 0.5 m*M* dithiothreitol) with a Teflon homogenizer. The homogenate was ultracentrifuged at 105,000 g at 4°C for 1 hr.*

Competitive-binding experiment. A volume of 200 μl of rabbit cytosol was incubated with approximately 1 pmole of tritiated estradiol-17β (^{3}H-E$_2$) at 4°C for 1 hr. The incubation was terminated by adding 0.1 ml of a suspension of dextran-coated charcoal (2.5 mg of Norit A and 0.025 mg of dextran in 0.1 ml of Tris–HCl buffer, pH 8.0). The mixture was kept on ice for 20 min, and the charcoal was then spun down at 5,000 rpm for 10 min. A 100-μl aliquot of the supernatant was pipetted into counting vials and the radioactivity was measured in 10 ml of Aquasol.

For competitive-inhibition experiments, various amounts of nonradioactive estradiol-17β or its derivatives were added to the reaction mixture together with ^{3}H-E$_2$ and incubated as described. The incubation mixture containing only ^{3}H-E$_2$ was used as the control, representing 100% binding.

Sucrose-gradient centrifugation and receptor binding. Approximately 1 pmole of ^{3}H-E$_2$ or ^{125}I-estrogen derivative was incubated with 250 μl of uterine cytosol from 6-day-pregnant rabbits (2.1 mg of protein) or from 23–25-day-old rats (2.3 mg of protein) for 1 hr at 4°C, with or without preincubation with 200 pmole of unlabeled estradiol.

Two hundred microliters of the cytosol were layered on 4.6 ml of a linear sucrose gradient (10–30% in TED buffer) and centrifuged at 224,000 g for 18 hr at 4°C. The gradient was fractionated† in 0.1-ml portions and transferred to liquid-scintillation vials.

Heat treatment of uterine cytosol. The uterine cytosol from 6-day-pregnant rabbits was pretreated by heating at 60°C for 5 min before being incubated with ^{3}H-E$_2$ or ^{125}I-labeled compound I. The distribution of radioactivity was then determined using the sucrose-gradient assay.

Effect of L-thyroxine and its derivatives on the binding of iodinated compounds. One picomole of radioiodinated II was added to rabbit uterine cytosol with or without 10^{-4} *M* L-thyroxine or its derivatives. The mixture was incubated at 4°C for 60 min, and dextran-coated charcoal was added to remove unbound iodohexestrol. The remainder of the procedure was carried out as described in the competitive-binding experiment. The experimental procedure was repeated using rat plasma or uterine cytosol to determine whether the binding protein was present in these compartments.

Tissue distribution of radioiodinated compounds I, II, and III. Ten picomoles of each radioiodinated compound, in 0.1 ml of 30% ethanol in saline, was injected into the femoral vein of immature female rats under light ether anesthesia. Two hours after the injection, each rat was killed and 1 ml of blood was immediately collected from the heart. The liver, kidneys, stomach with contents, thyroid, uterus, and ovaries were removed, weighed, and transferred to

counting vials. The radioactivity was expressed as percent dose/gm tissue or percent dose/organ.

In separate experiments with rats, a suspension of L-thyroxine was administered (100 mg/kg) by intraperitoneal injection 1 hr before injection of the radioiodinated compounds. Two hours after the injection, each rat was killed and the tissue samples were analyzed as described.

RESULTS

Tritiated estradiol was chromatographed periodically and shown to be radiochemically pure. All iodinated compounds were purified before use by preparative thin-layer chromatography.

Competitive-binding experiment. The estrogen-receptor binding affinity of the four compounds (Fig. 1) was determined by competitive-binding procedures. Quantities of I, II, III, or IV were added to the receptor-assay system containing ^{3}H-E_2 and rabbit uterine cytosol (Fig. 2). Compound I was most effective in displacing ^{3}H-E_2 (50% inhibition at 2.9 times the amount of ^{3}H-E_2), followed by II (50% inhibition at 6.4×) and III (50% inhibition at 200×). Compound IV could not displace ^{3}H-E_2 from the receptor even at 200-fold excess.

In contrast to the uniodinated molecule, iodinated I could not displace ^{3}H-E_2 from the receptor. Monoiodinated II could displace ^{3}H-E_2, although a 100-fold excess was required to decrease the binding of ^{3}H-E_2 by 50%. Compounds III and IV were not tested in the iodinated form because of the poor inhibitory properties of the uniodinated molecule.

Sucrose-gradient centrifugation and receptor binding. The centrifugation patterns of ^{3}H-E_2 and the radioiodinated derivative of compound II, incubated with rabbit uterine cytosols, are shown in Fig. 3. The distinct peak at fraction 36 in the ^{3}H-E_2 experiment corresponds to the previously reported (*10*) heavy peak (8S) of estrogen-binding protein. This peak disappeared when excess unlabeled estradiol (200×) was added to the cytosol before ^{3}H-E_2 addition. When 0.3 *M* KCl was added to the cytosol, the ^{3}H activity shifted from fraction 36 to fraction 22, which has been identified as a light (4S) estrogen-binding protein (*10*).

As shown in Fig. 3b, no 8S peak was detected for

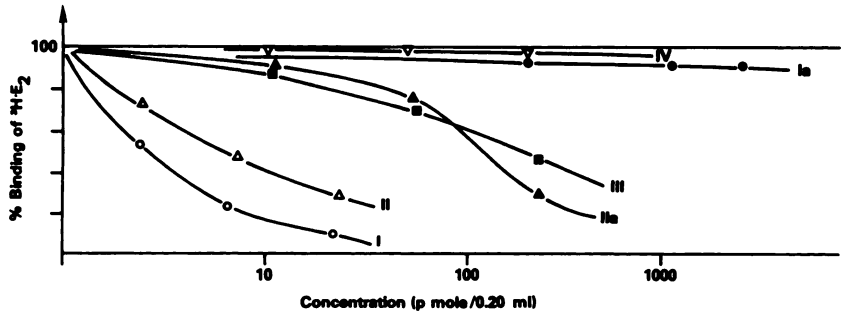

FIG. 2. Competitive-binding experiment using radioreceptor assay with charcoal. Estradiol (I), hexestrol (II), compound III (III), compound IV (IV), iodinated estradiol (Ia), and iodinated hexestrol (IIa). See text for experimental details.

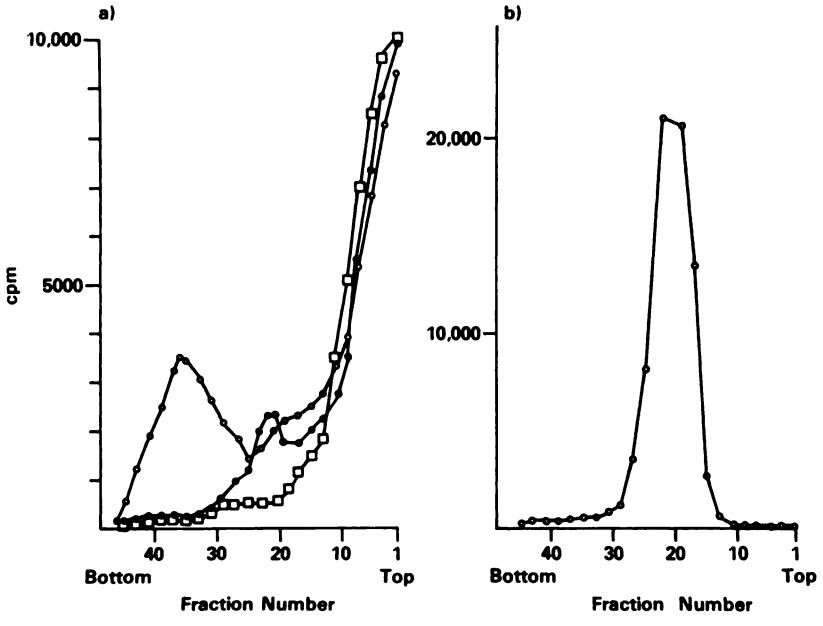

FIG. 3. Sucrose-gradient centrifugation patterns of ^{3}H-estradiol and iodinated hexestrol with rabbit-uterus cytosol. (a) ^{3}H-estradiol alone (○); with 200 pmole of estradiol added (□); and with 0.3 *M* KCl added (●). (b) Iodinated hexestrol. See text for experimental details.

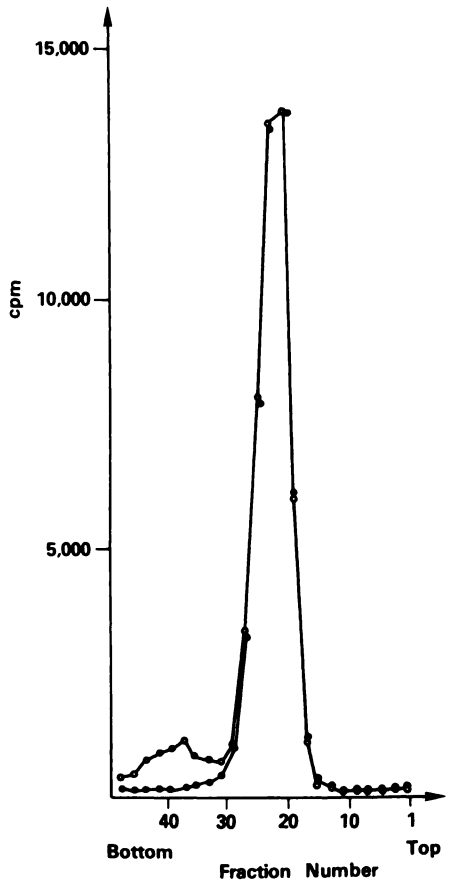

FIG. 4. Binding of iodinated hexestrol to cytosol from immature rat uterus: Sucrose-gradient centrifugation pattern without (○), and with (●) addition of 200 pmole of estradiol. See text for experimental details.

the iodinated form of compound II. Instead, a prominent peak was detected at fraction 22, where the 4S form of the estrogen-binding protein is located. Similar patterns were observed for the iodinated forms of compounds I and III. The distribution of radioiodine, however, was not affected by preincubation with excess estradiol. On the other hand, the 4S binding of ^{3}H-E_2 produced by high salt (KCl) concentration was inhibited totally by the addition of excess estradiol. Therefore, the binding of the iodinated species of compounds I, II, and III does not appear to involve the same protein that binds ^{3}H-E_2 under high-salt conditions. When ^{3}H-E_2 and compound IIa were incubated in the same cytosol preparation, ^{3}H-E_2 was bound to the 8S component, whereas radioiodinated II was bound to the 4S component. This further verified that the estrogen-binding protein was intact but did not bind the iodinated form of compound II.

Despite the inhibitory effect of the iodinated compound II on ^{3}H-E_2 in the radioreceptor assay (Fig. 2), the binding of the radioiodinated form to the 8S component in the sucrose-gradient assay was not evident when uterine cytosol from pregnant rabbits was used. Although this animal model has been used for the study of estradiol-binding protein with ^{3}H-E_2 (*11*), the estradiol produced in pregnancy may be sufficient to prevent low-affinity estrogen derivatives from binding to the receptors. Therefore, the uteri from immature rats were tested as a source of unsaturated estrogen-binding protein.

Figure 4 shows the sucrose-gradient centrifugation

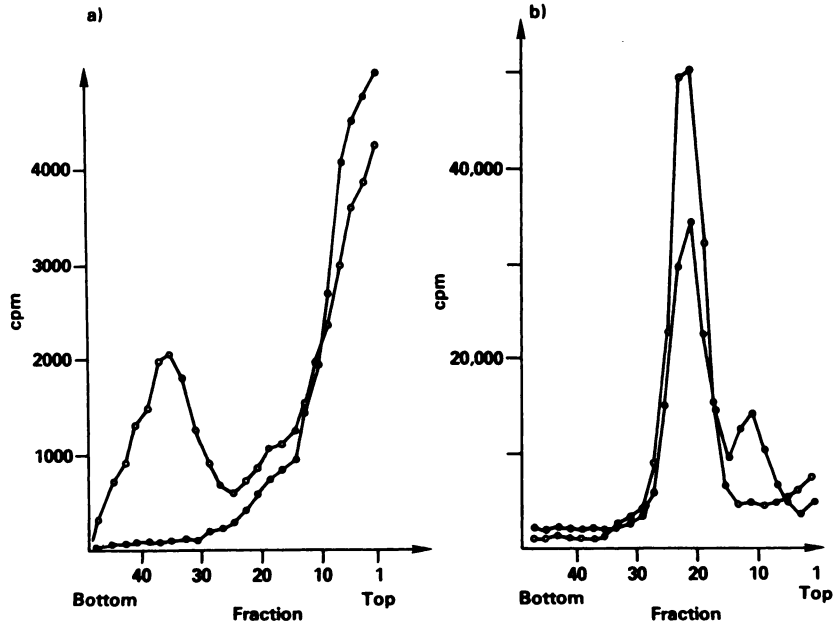

FIG. 5. Effect of previous heating on sucrose-gradient centrifugation pattern of ^{3}H-estradiol (a) and ^{125}I-estradiol (b) with (●) and without (○) heating.

TABLE 1. EFFECT OF L-THYROXINE AND ITS DERIVATIVES ON BINDING OF ^{125}I-HEXESTROL TO UTERINE CYTOSOL AND PLASMA PROTEIN IN THE RAT

Derivatives	Inhibition (%)	
	Uterine cytosol*	Blood plasma†
10^{-4} M L-thyroxine	62.25	38.57
10^{-4} M D-thyroxine	51.17	16.81
10^{-4} M L-diiodotyrosine	34.52	33.48
10^{-4} M-L-monoiodotyrosine	0	0

* Homogenized in 10 volumes of TED buffer.
† Diluted with 20 volumes of TED buffer.

pattern of the radioiodinated form of compound II using immature rat uteri. The radioiodinated compounds I and III showed the same binding patterns as were observed with rabbit uteri (Fig. 3), that is, no binding in the 8S region. However, the radioiodinated compound II showed a small peak in the 8S region where ^{3}H-E_2 binds. This peak diminished to zero in the presence of excess estradiol.

Heat treatment of uterine cytosol. Since the estrogen-binding protein is very unstable under heat treatment (*12*), the stability of the 4S binding component was examined. As shown in Fig. 5, the binding affinity of the 8S estrogen-binding protein for ^{3}H-E_2 was the binding of the ^{125}I-labeled compound I with the light component was retained, although the binding capacity had decreased and a new binding component had appeared.

Effect of L-thyroxine and its derivatives on the binding of iodinated compounds. The observation that the three compounds Ia, IIa, and IIIa have an iodophenol structure in common suggests that the 4S component could be a thyroxine-binding protein; this is not the 4S component that binds ^{3}H-E_2 under high-salt conditions. To strengthen the belief that the 4S component might be a thyroxine-binding protein, the binding of iodinated II was tested with rat plasma. One picomole of iodinated II bound completely with a plasma component, as well as with uterine cytosols from rabbits or immature rats. The binding of iodinated II with plasma or uterine cytosols was markedly inhibited by L-thyroxine and moderately by D-thyroxine or L-diiodotyrosine (Table 1). L-Monoiodotyrosine had no effect on the binding of iodinated II. Since the inhibiting effect of these thyroxine analogs on iodinated II was the same as their effect on the binding of labeled thyroxine to thyroxine-binding proteins (*13*), the iodinated forms of I, II, and III most likely bind to thyroxine-binding proteins.

Tissue distribution of radioiodinated compounds I, II, and III. The radioiodinated forms of compounds, I, II, and III were then evaluated as diagnostic radiopharmaceuticals by comparing their distributions in immature female rats (Table 2). The uterus-to-blood ratio was used as an index of their value as a diagnostic agent. In agreement with the in vitro experiments, the radioiodinated form of compound II showed a higher uterine concentration (1.69% dose/gm) than the radioiodinated forms of either compound I (0.29% dose/gm) or compound III (0.17% dose/gm). However, the uterus-to-blood ratio for compound II, although the highest of the three compounds, was still less than 2.

As determined in the in vitro studies, binding to a thyroxine-binding protein constituted a major part of the interaction. Thus, just as the addition of thyroxine to the in vitro systems decreased the binding to the thyroxine-binding protein, so did the intraperitoneal injection of thyroxine in the animal-distribution studies. As shown in Table 3, the injection of thyroxine before the injection of the radioiodinated compound II produced an increase in the uterine concentration and a decrease in that of the blood. The uterus-to-blood ratio was 10.4 at 2 hr after injection. At 6 hr the uterus-to-blood ratio of the radioiodinated form of compound II had dropped to 2.7 (Table 2), but prior injection with thyroxine increased this ratio to 5.5. For the radioiodinated form of compound I, by contrast, the effect of thyroxine on the uterus-to-blood ratio was minimal. A similar competition study, performed in vitro, also reports inhibition of binding by thyroxine (*14*).

DISCUSSION

Iodinated estrogens have been suggested as tracers of estradiol. Monoiodinated estradiol was the first compound prepared, but it did not achieve a high uterus-to-blood ratio (*15*), suggesting a lack of ability to concentrate in estrogen-sensitive tissues. The in vitro receptor-assay studies reported here suggest that iodination in the ortho position of the A ring interferes with the binding to the estrogen-binding protein.

Compounds IIIa and IVa have been used successfully as radioligands in radioimmunoassays for estradiol, but these were not tested as radioligands for radioreceptor studies.

Katzenellenbogen et al. proposed the use of a series of iodinated hexestrols to trace subcellular amounts of estradiol and to label estrogen-binding proteins (*16*). Using the sucrose-gradient method and electrophoresis, they showed that the iodohexestrol derivative binds to a thyroxine-binding protein as well as to the estrogen-binding protein. Their in

TABLE 2. DISTRIBUTION OF ^{125}I-LABELED DERIVATIVES IN IMMATURE RATS AT 2 HR (% dose/gm ± 1 s.d.)

Organ	Compound Ia (n = 5)	IIa (n = 5)	IIIa (n = 5)	IIa* (n = 3)
Liver	0.91 ± 0.18	1.45 ± 0.12	2.09 ± 0.36	0.72 ± 0.26
Kidney	0.47 ± 0.08	0.55 ± 0.02	0.81 ± 0.09	0.28 ± 0.09
Uterus	0.29 ± 0.03	1.69 ± 0.22	0.17 ± 0.04	0.77 ± 0.10
Blood	0.32 ± 0.00	0.91 ± 0.12	0.63 ± 0.02	0.30 ± 0.04
Uterus-to-blood ratio	0.92	1.87	0.27	2.67
Injected dose (ng)	5.5	3.0	6.0	3.0
Injected dose (pmole)	13.8	7.57	8.87	7.57

* At 6 hr.

vivo study did not include blood levels and therefore does not indicate whether or not the iodinated hexestrol would be a useful imaging agent.

Although many diagnostic radiopharmaceuticals provide high rates of detection of abnormalities, they are generally not specific. In an attempt to develop specific diagnostic agents for the detection of hormone-regulated tumors, four iodinated derivatives of estradiol and hexestrol were prepared to determine their affinity for the estrogen-binding protein present in estrogen-response tissue. For such derivatives to be useful, they should possess certain properties required of site-directed agents (*17*):

1. Exclusive and complete transport to the diseased tissue or target organ without degradation or metabolism of the derivative prior to contact.
2. A high affinity for the binding protein similar to that of the natural compound.
3. Absence of nonspecific binding of the derivative to normal tissue or protein.

Compounds Ia, IIIa, and IVa did not fulfill Criterion 2 in that they could not effectively inhibit the binding of ^{3}H-E_2 as shown by the competitive-binding and the sucrose-gradient assay experiments. To some extent, compound IIa could inhibit the binding of ^{3}H-E_2, but a good target-to-nontarget ratio (i.e., uterus-to-blood) could not be obtained initially because the iodinated derivative was bound to thyroxine-binding proteins. Prior injection with thyroxine did produce satisfactory uterus-to-blood ratios, suggesting that the iodinated hexestrol was bound to a protein that binds thyroxine. The in vitro studies of estrogen-receptor binding gave a good indication of the in vivo distribution to be expected. Further efforts must be made to design derivatives that retain their affinity for estradiol-binding protein but do not contain the o-iodophenol moiety that is bound to proteins in a nonspecific manner.

TABLE 3. EFFECT OF THYROXINE ON DISTRIBUTION OF ^{125}I-LABELED DERIVATIVES IN IMMATURE RATS AT 2 HR (% dose/gm ± 1 s.d.)

Organ	Compound Ia (n = 4)	IIa (n = 5)	IIa* (n = 4)
Liver	0.50 ± 0.03	1.16 ± 0.21	0.62 ± 0.06
Kidney	0.26 ± 0.00	0.39 ± 0.06	0.21 ± 0.01
Uterus	0.23 ± 0.03	2.57 ± 0.42	0.95 ± 0.04
Blood	0.21 ± 0.00	0.25 ± 0.02	0.17 ± 0.00
Uterus-to-blood ratio	1.12	10.4	5.48
Injected dose (ng)	5.5	3.0	3.0
Injected dose (pmole)	13.8	7.57	7.57

* At 6 hr.

ACKNOWLEDGMENTS

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FOOTNOTES

* Beckman L3-50 ultracentrifuge with a Spinco 40 or a SW 50.1 rotor.

† ISCO density-gradient fractionator Model 640.

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