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Preparation and Antigenic Properties of 2-Hydroxyestrone-[C-15]– Bovine Serum Albumin Conjugate¹⁾

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A new hapten-carrier conjugate was prepared from 15 β -(2-carboxyethylthio)-2-hydroxyestrone by coupling to bovine serum albumin employing the mixed anhydride technique. The specificity of anti-2-hydroxyestrone antiserum elicited in rabbits by immunization with this antigen was assessed by cross-reaction studies with related steroids in the radioimmunoassay procedure and the results are discussed from the structural point of view.

Keywords—catechol estrogen; 2-hydroxyestrone; 15 β -(2-carboxyethylthio)-2-hydroxyestrone; hapten-bovine serum albumin conjugate; anti-2-hydroxyestrone antiserum; radioimmunoassay; cross-reactivity

In recent years, considerable attention has been focused on the potential physiological significance of catechol estrogens.^{2–4)} Several attempts have been made to prepare anti-2-hydroxyestrone antisera for use in immunoassays by coupling the hapten to the carrier protein. The antisera so far obtained, however, are still unsatisfactory with respect to the specificity. The urgent need for more specific anti-2-hydroxyestrone antiserum prompted us to develop a new immunogen. This paper describes the preparation of anti-2-hydroxyestrone antiserum with the use of a new type of immunogen where the carrier protein is linked to the steroid hapten through the C-15 position.

An initial project was directed to the synthesis of 15 β -(2-carboxyethylthio)-2-hydroxyestrone (**10**) as a new hapten. First, 2,4,16 α -tribromoestrone⁵⁾ (**1**) readily obtainable from estrone was transformed into the 17,17-ethyleneketal (**2**) by condensation with ethylene glycol in the presence of *p*-toluenesulfonic acid. Subsequent dehydrobromination was readily attained by treatment with potassium *tert*-butoxide in dimethyl sulfoxide, providing the Δ^{15} compound (**3**). Upon exposure to *p*-toluenesulfonic acid in acetone, the protecting group at C-17 was readily removed without disturbing the Δ^{15} -double bond.

The resulting 2,4-dibromo-3-hydroxy-1,3,5(10),15-estratetraen-17-one (**4**) was then treated with sodium nitrite in the presence of acetic acid. A regiospecific reaction occurred at C-2 to afford the 2-nitro compound (**5**) as a sole product.⁶⁾ Subsequent reduction with zinc dust in acetic acid followed by periodate oxidation furnished the 4-bromo-2,3-catechol (**7**) in a reasonable yield. When **7** was treated with 3-mercaptopropionic acid in tetrahydrofuran (THF)-aqueous borax solution, Michael reaction proceeded toward the Δ^{15} -17-ketone, yielding the thiol adduct (**8**). On treatment with diazomethane, **8** was converted to 2-methoxy-15 β -(2-methoxycarbonylethylthio)-estrone 3-methyl ether (**9**). The stereochemistry at C-15 was justified by inspection of the nuclear magnetic resonance (NMR) spectra of the thiol adducts. A C-18 proton signal appeared at lower field as compared with that of the parent compound, due to the 1,3-diaxial interaction.⁷⁾ Finally, reductive elimination of a bromine substituent at C-4 was effected by treatment with triphenyltin hydride to provide the desired

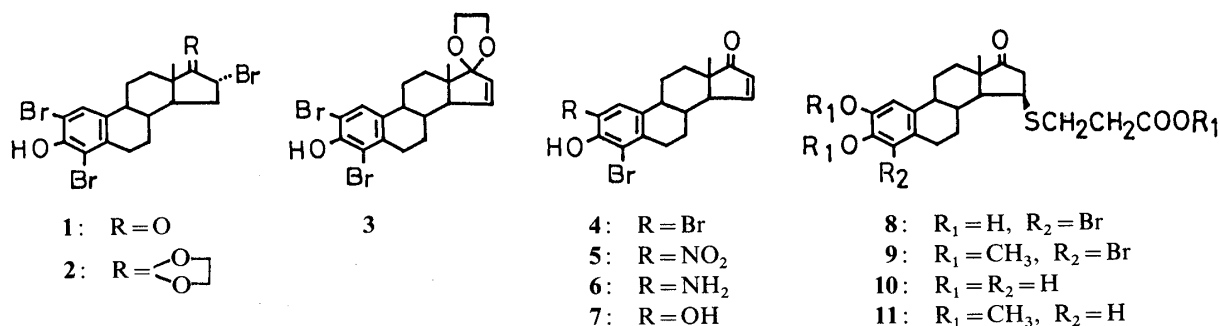


Chart 1

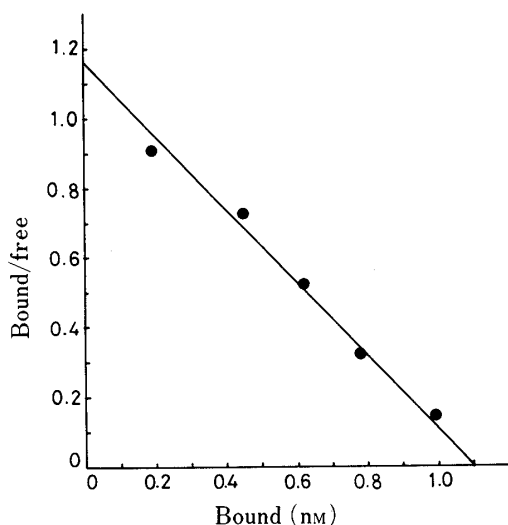


Fig. 1. Scatchard Plots for Antiserum Raised against 2-Hydroxyestrone-[C-15]-BSA Conjugate in the Rabbit

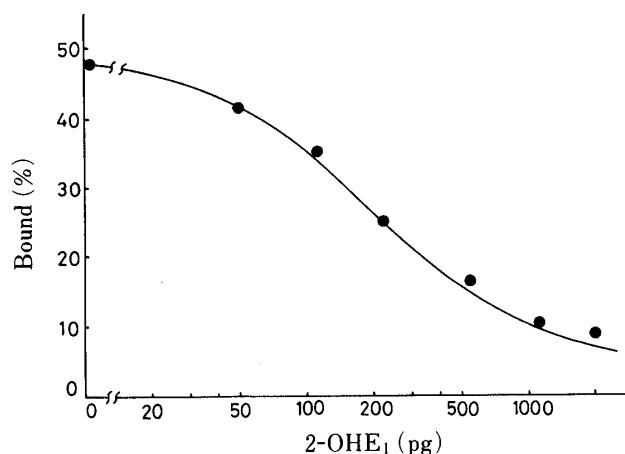


Fig. 2. Dose-Response Curve for Radioimmunoassay of 2-Hydroxyestrone Using Anti-2-hydroxyestrone Antiserum (1:3000 Dilution)

15 β -(2-carboxyethylthio)-2-hydroxyestrone (**10**). Usual methylation of **10** with diazomethane provided the 2,3-dimethyl ether-methyl ester (**11**).

The hapten was covalently linked to bovine serum albumin (BSA), yielding the 2-hydroxyestrone-[C-15]-BSA conjugate by the mixed anhydride method developed by Erlanger *et al.*⁸⁾ In radioimmunoassay, [6,7-³H]2-hydroxyestrone was used as a labeled antigen, and the separation of bound and free fractions was carried out by a dextran-coated charcoal method. The dilution of antiserum which was able to bind 50% of the labeled antigen was defined as a titer. The sera obtained from two rabbits immunized with the antigen for six months showed significantly increased binding activity to 2-hydroxyestrone. A preliminary test indicated that there was no substantial difference in the affinity of specificity between antisera elicited in the two rabbits. The relationship between the concentration of bound antigen and the ratio of the bound to free (B/F) observed with antiserum is illustrated as a Scatchard plot in Fig. 1. The antiserum exhibited the high affinity for 2-hydroxyestrone with the association constant of $1.05 \times 10^9 \text{ M}^{-1}$. The dose-response curve was constructed by incubating 50–1000 pg of unlabeled 2-hydroxyestrone and a constant amount of the labeled antigen with the 1:3000 diluted antiserum. A typical standard curve is shown in Fig. 2.

The specificity of antisera was assessed by ascertaining the ability of various related steroids to compete with [³H]2-hydroxyestrone for binding to antibody. The per cent cross-reaction of antisera was determined according to the method of Abraham.⁹⁾ The results on

TABLE I. Per Cent Cross-Reactions of Anti-2-hydroxyestrone Antiserum with Selected Steroids

Steroids	Cross-reactivity (%)
2-Hydroxyestrone	100
2-Hydroxyestradiol	6.4
2-Hydroxyestriol	0.21
2-Methoxyestrone	<0.01
2-Hydroxyestrone 3-methyl ether	0.03
4-Hydroxyestrone	0.46
4-Hydroxyestrone 3-methyl ether	<0.01
4-Methoxyestrone	<0.01
Estrone	0.31
Estradiol	<0.01
Estriol	<0.01
Estrone methyl ether	<0.01
Estradiol 3-methyl ether	<0.01
Estriol 3-methyl ether	<0.01
2-Hydroxyestrone 2-glucuronide	<0.01
2-Hydroxyestradiol 2-glucuronide	<0.01
2-Hydroxyestradiol 17-glucuronide	<0.01
2-Hydroxyestriol 2-glucuronide	<0.01
4-Hydroxyestrone 3-glucuronide	<0.01
4-Hydroxyestrone 4-glucuronide	<0.01
4-Hydroxyestradiol 4-glucuronide	<0.01
2-Hydroxyestradiol 3-sulfate	<0.01

cross-reactions of anti-2-hydroxyestrone antiserum with 22 kinds of related steroids are listed in Table I. The antiserum proved to be sufficiently specific with the single exception of 2-hydroxyestradiol, which showed a 6.4% cross-reaction. Other related unconjugated and conjugated C₁₈ steroids exhibited less than 0.5% cross-reaction.

Several studies have been carried out on the preparation of anti-2-hydroxyestrone antisera with conjugates obtained by coupling the steroid to an immunogenic carrier through the C-6⁽¹⁰⁾ and C-17⁽¹¹⁾ positions. The antiserum raised against the 2-hydroxyestrone-[C-15]-BSA conjugate is much more specific for 2-hydroxyestrone than those elicited with antigens whose haptens are linked to a carrier protein through positions other than C-15.

It has previously been reported that immunization with testosterone- and 5 α -dihydrotestosterone-[C-15]-BSA conjugates in rabbits produced highly specific antisera differentiating the structural characteristics of ring A.^{7,12-14)} The present result together with the previous findings lends support to the consideration that for the production of specific antiserum the steroid hapten should be coupled to a carrier protein at a site remote from the inherent functional groups, which are left available as antigenic determinants.

It is hoped that the availability of antiserum specific to 2-hydroxyestrone will serve to clarify the metabolic disposition and physiological significance of catechol estrogens in living animals.

Experimental

Synthesis of Hapten—Melting points were measured on a Yanagimoto melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 automatic polarimeter. Proton nuclear magnetic resonance (¹H-NMR) spectra were recorded on a JEOL FX-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard. Abbreviations used are s=singlet, q=quartet and m=multiplet. Mass spectra (MS) were obtained on Hitachi M-52 and JEOL JMS-OISG-2 spectrometers equipped with a JAM-2000 computer. For column

chromatography and preparative thin-layer chromatography (TLC), Silica gel 60 and Silica gel HF₂₅₄ (E. Merck AG, Darmstadt) were used, respectively.

2,4,16 α -Tribromo-17,17-ethylenedioxy-1,3,5(10)-estratrien-3-ol (2)—Ethylene glycol (70 ml) and *p*-toluenesulfonic acid (1.5 g) were added to a solution of 2,4,16 α -tribromoestrone (1)⁵ (8 g) in toluene (300 ml), and the whole was distilled azeotropically over a period of 48 h. The solution was diluted with AcOEt, washed with a saturated NaHCO₃ solution and water, dried over anhydrous Na₂SO₄ and evaporated down. Recrystallization of the crude product from acetone–MeOH gave **2** (6.3 g) as colorless plates. mp 197–200 °C. [α]_D²⁵ + 59.3° (*c* = 0.17, CHCl₃). Anal. Calcd for C₂₀H₂₃Br₃O₃: C, 43.59; H, 4.21; Br, 43.49. Found: C, 43.23; H, 4.21; Br, 43.41. NMR (CDCl₃) δ : 0.91 (3H, s, 18-CH₃), 3.92–4.34 (4H, m, 17-OCH₂CH₂O–), 5.82 (1H, s, 3-OH), 7.36 (1H, s, 1-H). MS *m/z*: 553, 551, 549, 547 [M]⁺.

2,4-Dibromo-17,17-ethylenedioxy-1,3,5(10),15-estratetraen-3-ol (3)—Potassium *tert*-butoxide (5 g) was added to a solution of **2** (4.5 g) in anhydrous dimethyl sulfoxide (45 ml), and the whole was stirred at 60 °C for 20 h. The mixture was poured into NaCl saturated ice-water and neutralized with 1 N HCl. The precipitate was collected by filtration and recrystallized from acetone–MeOH to give **3** (3.1 g) as colorless needles. mp 192–195 °C (dec.). [α]_D²⁵ + 21.0° (*c* = 0.17, CHCl₃). Anal. Calcd for C₂₀H₂₂Br₂O₃: C, 51.08; H, 4.72; Br, 33.99. Found: C, 50.66; H, 4.73; Br, 33.81. NMR (CDCl₃) δ : 0.94 (3H, s, 18-CH₃), 3.97 (4H, m, 17-OCH₂CH₂O–), 5.75 (1H, q, *J* = 6, 2 Hz, 15-H), 5.82 (1H, s, 3-OH), 6.22 (1H, q, *J* = 6, 1 Hz, 16-H), 7.37 (1H, s, 1-H). MS *m/z*: 472, 470, 468 [M]⁺.

2,4-Dibromo-3-hydroxy-1,3,5(10),15-estratetraen-17-one (4)—*p*-Toluenesulfonic acid (400 mg) in water (20 ml) was added to a solution of **3** (3 g) in acetone (150 ml), and the whole was stirred at 4 °C for 12 h. The resulting solution was extracted with AcOEt, and the organic layer was washed with 1% NaHCO₃ and water, dried over anhydrous Na₂SO₄ and evaporated down. Recrystallization of the crude product from acetone gave **4** (2.5 g) as colorless plates. mp 250–254 °C (dec.). [α]_D²⁵ + 6.7° (*c* = 0.18, CHCl₃). Anal. Calcd for C₁₈H₁₈Br₂O₂: C, 50.73; H, 4.26; Br, 37.50. Found: C, 50.76; H, 4.14; Br, 37.66. NMR (CDCl₃) δ : 1.09 (3H, s, 18-CH₃), 5.87 (1H, s, 3-OH), 6.09 (1H, q, *J* = 2.5, 6 Hz, 15-H), 7.36 (1H, s, 1-H), 7.60 (1H, q, *J* = 1.5, 6 Hz, 16-H). MS *m/z*: 428, 426, 424 [M]⁺.

4-Bromo-3-hydroxy-2-nitro-1,3,5(10),15-estratetraen-17-one (5)—Aqueous NaNO₂ solution (800 mg in 20 ml) was added to a solution of **4** (1.6 g) in AcOH (200 ml), and the whole was stirred at room temperature for 30 min. The reaction mixture was poured into ice-water and the precipitate was extracted with AcOEt. The organic layer was dried over anhydrous Na₂SO₄ and evaporated down. The residue obtained was purified by column chromatography on silica gel. Recrystallization of the product from ether–hexane gave **5** as pale yellow needles (1.34 g). mp 206–208 °C (dec.). [α]_D²⁵ + 5.3° (*c* = 0.18, CHCl₃). Anal. Calcd for C₁₈H₁₈BrNO₄: C, 55.12; H, 4.62; N, 3.57; Br, 20.37. Found: C, 55.00; H, 4.40; N, 3.35; Br, 20.71. NMR (CDCl₃) δ : 1.10 (3H, s, 18-CH₃), 6.08 (1H, q, *J* = 2.5, 6 Hz, 15-H), 7.57 (1H, q, *J* = 1.5, 6 Hz, 16-H), 7.98 (1H, s, 1-H). MS *m/z*: 393, 391 [M]⁺.

4-Bromo-2,3-dihydroxy-1,3,5(10),15-estratetraen-17-one (7)—Zinc dust (10 g) was added to a solution of **5** (1.0 g) in AcOH (150 ml), and the whole was stirred at room temperature for 30 min. After removal of the precipitate by filtration, the filtrate containing the resulting 2-amino derivative (**6**) was added within 3 min to a vigorously stirred solution of sodium metaperiodate (10 g) in 0.1 N HCl (500 ml) at room temperature. The mixture was stirred for 5 min and extracted with CHCl₃, and the organic layer was washed with water. After addition of AcOH (5 ml) and aqueous KI solution (3 g in 50 ml), the whole was vigorously stirred for 10 min. After reduction of I₂ with 5% sodium hydrosulfite (300 ml), the organic layer was dried over anhydrous Na₂SO₄ and evaporated down. The residue obtained was purified by column chromatography on silica gel to give **7** (295 mg) as a colorless amorphous substance. [α]_D²⁵ + 14.5° (*c* = 0.14, MeOH). NMR (CDCl₃) δ : 1.09 (3H, s, 18-CH₃), 6.07 (1H, q, *J* = 3, 6 Hz, 15-H), 6.87 (1H, s, 1-H), 7.60 (1H, q, *J* = 1.5, 6 Hz, 16-H). MS *m/z*: 364, 362 [M]⁺. High MS *m/z*: 362.0473 [M]⁺ (Calcd for C₁₈H₁₉BrO₃, 362.0518). The product was used in the subsequent step without further purification.

4-Bromo-15 β -(2-carboxyethylthio)-2,3-dihydroxy-1,3,5(10)-estratrien-17-one (8)—3-Mercaptopropionic acid (0.6 ml) was added to a solution of **7** (192 mg) in THF (16 ml)–aqueous borax solution¹⁵ (200 mg in 10 ml) (pH 8.0, adjusted with 1 N HCl), and the whole was stirred at room temperature for 60 h under an N₂ gas atmosphere. The resulting solution was acidified with 1 N HCl, concentrated *in vacuo* and extracted with AcOEt. The organic layer was dried over anhydrous Na₂SO₄ and evaporated down. The residue obtained was purified by column chromatography on silica gel. Recrystallization of the eluate from AcOEt gave **8** (161 mg) as colorless needles. mp 193–196 °C (dec.). [α]_D²⁵ + 71.3° (*c* = 0.17, MeOH). Anal. Calcd for C₂₁H₂₅BrO₅S·1/2H₂O: C, 52.72; H, 5.47; Br, 16.70. Found: C, 53.04; H, 5.19; Br, 17.35. NMR (CDCl₃–CD₃OD (5:1)) δ : 1.12 (3H, s, 18-CH₃), 2.75 (4H, m, 15-S(CH₂)₂COOH), 6.80 (1H, s, 1-H).

4-Bromo-2,3-dimethoxy-15 β -(2-methoxycarbonylthio)-1,3,5(10)-estratrien-17-one (9)—Treatment of **8** (10 mg) with CH₂N₂ in ether–MeOH was carried out at room temperature for 3 h. Usual work-up followed by preparative TLC gave **9** (6 mg) as a colorless amorphous substance. NMR (CDCl₃) δ : 1.11 (3H, s, 18-CH₃), 2.80 (4H, m, 15-S(CH₂)₂COOCH₃), 3.71 (3H, 15-S(CH₂)₂COOCH₃), 3.81 (3H, s, 2- or 3-OCH₃), 3.84 (3H, s, 3- or 2-OCH₃), 6.81 (1H, s, 1-H). MS *m/z*: 512, 510 [M]⁺. High MS *m/z*: 510.1081 (Calcd for C₂₄H₃₁BrO₅S, 510.1076).

15 β -(2-Carboxyethylthio)-2,3-dihydroxy-1,3,5(10)-estratrien-17-one (10)—Triphenyltin hydride (300 mg) and α,α' -azobisisobutyronitrile (10 mg) was added to a solution of **8** (141 mg) in toluene–THF (10:1, 20 ml), and the whole was stirred at 90 °C for 2.5 h under an N₂ gas atmosphere. The resulting solution was diluted with AcOEt and

extracted with 2.5% NaHCO₃. The aqueous layer was acidified with 2N HCl and extracted with AcOEt. The organic layer was dried over anhydrous Na₂SO₄ and evaporated down. Recrystallization of the crude product from ether gave **10** (86 mg) as colorless plates. mp 156–160 °C. $[\alpha]_D^{25} + 61.2^\circ$ ($c=0.25$, MeOH). *Anal.* Calcd for C₂₁H₂₆O₅S·3/2H₂O: C, 60.41; H, 7.00. Found: C, 60.51; H, 7.01. NMR (CDCl₃–CD₃OD (5:1)) δ : 1.13 (3H, s, 18-CH₃), 2.76 (4H, m, 15-S(CH₂)₂COOH), 6.56 (1H, s, 4-H), 6.74 (1H, s, 1-H).

2,3-Dimethoxy-15 β -(2-methoxycarbonylethylthio)-1,3,5(10)-estratrien-17-one (11)—Compound **11** (5 mg) was obtained in the same manner as described for **9**. NMR (CDCl₃) δ : 1.15 (3H, s, 18-CH₃), 2.85 (4H, m, 15-S(CH₂)₂COOCH₃), 3.62 (3H, s, 15-S(CH₂)₂COOCH₃), 3.86 (6H, s, 2- and 3-OCH₃), 6.61 (1H, s, 4-H), 6.81 (1H, s, 1-H). MS m/z : 432 [M]⁺. High MS m/z : 432.1946 (Calcd for C₂₄H₃₂O₅S, 432.1970).

Materials—[6,7-³H]Estrone (60 Ci/mmol) was supplied by New England Nuclear (Boston, MA), and its radiochemical purity was checked by TLC prior to use. Mushroom tyrosinase and BSA (fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO), Sephadex LH-20 from Pharmacia Fine Chemicals (Uppsala, Sweden), and complete Freund's adjuvant from Difco Lab. (Detroit, MICH). Conjugated steroids were prepared in these laboratories by the known methods. All free steroids were kindly donated by Teikoku Hormone Mfg. Co. (Tokyo, Japan) and other general reagents were from Nakarai Chemicals (Kyoto, Japan).

Preparation of Antigen—Tri-*n*-butylamine (40 μ l) and isobutyl chlorocarbonate (20 μ l) were added to a solution of 15 β -(2-carboxyethylthio)-2-hydroxyestrone (**10**) (30 mg) in dry dimethylformamide (DMF) (1.5 ml) under ice-cooling, and the whole was stirred for 30 min. The resulting mixture was added to a solution of BSA (80 mg) in water (3 ml)–DMF (3 ml) containing 0.05N NaOH (0.3 ml) under ice-cooling. Stirring was continued for 4 h while maintaining a pH of ca. 7, then the resulting solution was dialyzed for 24 h at 4 °C against a cold running 0.001% ascorbic acid solution (6 l), and the turbid protein solution was brought to pH 4.0 with 0.1N HCl. After stirring at 4 °C overnight, the precipitate was collected by centrifugation at 1400 $\times g$ for 10 min, then dissolved in 0.01% ascorbic acid (5 ml)–1% NaHCO₃ (5 ml) (pH 8.0), and dialyzed in the same manner as described above. Lyophilization of the solution afforded the hapten–BSA conjugate as a fluffy powder (60 mg). The molar steroid–protein ratio of the conjugate as determined spectrophotometrically was 36.*

Immunization of Rabbits—Two male albino rabbits were used for immunization with antigen. The antigen (1 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with complete Freund's adjuvant (0.5 ml). The emulsion was injected into rabbits subcutaneously at multiple sites over the scapulae and in the thighs. This procedure was repeated at intervals of 2 weeks for 2 months and then once a month. The rabbits were bled 10 d after the booster injection. The sera were separated by centrifugation at 1900 $\times g$ and stored at –20 °C.

Preparation of Labeled Antigen—[6,7-³H]2-Hydroxyestrone was prepared enzymatically from [6,7-³H]estrone (250 μ Ci) with mushroom tyrosinase employing the method described in the previous paper¹⁰⁾ and purified by Sephadex LH-20 column chromatography using benzene–MeOH (9:1) saturated with ascorbic acid. The eluate was further purified by TLC using cyclohexane–ethyl acetate–methanol–acetic acid (65:32:2:1) as a development solvent and by Sephadex LH-20 column chromatography using the previous solvent. The resulting labeled compound was stored in MeOH containing 2% ascorbic acid and 1% AcOH at –20 °C.

Radioimmunoassay Procedure—All dilutions of the standard, tracer and antiserum were performed in 0.05M phosphate buffer (pH 7.4) containing 0.5% BSA and 0.1% ascorbic acid. A standard curve was constructed by setting up duplicate centrifuge tubes (7 ml) containing 0, 55, 110, 220, 550, 1100 and 2200 pg of non-labeled 2-hydroxyestrone (0.1 ml) and ³H-labeled 2-hydroxyestrone (6000 cpm) (0.1 ml). The diluted antiserum (0.1 ml) was added and the mixture was incubated at 4 °C for 12 h. After incubation with dextran-coated charcoal (0.5% Norit A and 0.1% dextran T-70 in assay buffer) (0.2 ml) for 5 min at 0 °C, the bound and free steroids were separated by centrifugation at 1900 $\times g$. A 0.25 ml aliquot of each supernatant was taken into a counting vial, and tT 21 scintillation cocktail¹⁶⁾ (4 ml) was added. The radioactivity was counted in a Beckman LS 7000 liquid scintillation spectrometer.

Cross-Reaction Study—The specificity of antiserum raised against the 2-hydroxyestrone–BSA conjugate was assessed by a cross-reaction study with 22 kinds of selected steroids (Table I). The relative amounts required to reduce the initial binding of ³H-labeled steroid by half, where the mass of non-labeled 2-hydroxyestrone was arbitrarily set at 100%, were calculated from the standard curve.⁹⁾

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