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Specificity of Antisera Raised against Estradiol 12–Bovine Serum Albumin Conjugates

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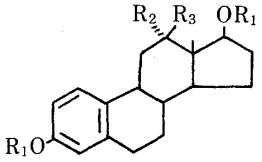
The preparation and antigenic properties of estradiol–bovine serum albumin conjugates in which the haptens are linked to the carrier protein through a glycine amide linkage at the 12α and the 12β positions on the steroid molecule are described. The antigens raised high-titer and reasonably specific antisera in rabbits, exhibiting little or no cross-reactivities with related steroids, with the exceptions of estrone (8.33%, 38.2%) and 16-oxoestradiol (7.90%, 5.65%).

Keywords——estradiol; 12-carboxyesteradiol glycine amide; estradiol [C-12]–bovine serum albumin conjugate; radioimmunoassay; hapten; antisera; cross-reaction

In recent years, the production of highly specific antisera has permitted the assay of estradiol in biological fluids without any chromatographic separation step prior to actual radioimmunoassay (RIA).[1] Such antisera of greater specificity have been obtained by the use of antigens in which the carrier protein was coupled through only C-6,[2] C-7,[3] and C-11[4] in the B or C ring of the estrogen. Conjugation at the C-12 position which leaves the A and D rings available for recognition by antibody, should elicit antisera with enhanced specificity, but so far no report has been published on such antigens, since the synthesis of the haptenic derivatives is a difficult problem. In our previous paper,[5] we reported the efficient synthesis of two new haptens, 12α- and 12β-carboxyestradiol derivatives (**1** and **2**). Immunization with the antigens derived from these haptens might be interesting since hydroxylation at C-12 in the biotransformation of estradiol has not yet been reported[6] and the resulting antibodies should provide information on the effect of epimeric changes on the antigenic properties. The present paper describes the preparation and properties of anti-estradiol antisera elicited by immunizing rabbits with these new hapten–bovine serum albumin (BSA) conjugates (**9** and **10**).

Isomeric 12-methoxycarbonylestradiol 3,17-diacetates (**1** and **2**)[5] were chosen as starting materials for the preparation of the BSA conjugates. Hydrolysis of **1** with potassium hydroxide in methanol and subsequent condensation with glycine ethyl ester in *N*,*N*-dimethylformamide in the presence of diethyl phosphorocyanidate afforded the 12α-glycine amide ester (**4**). Upon exposure to potassium hydroxide in methanol, **4** was transformed into the desired 12α-carboxyestradiol glycine amide (**5**) in good yield. The synthesis of the 12β-isomer was undertaken in a similar fashion. The ester **2** was hydrolyzed and the product was purified as the 3,17-diacetate (**6**). Condensation of **6** with glycine ethyl ester, followed by hydrolysis with potassium in ethanol, yielded the isomeric 12β-glycine amide (**8**). The structures of these haptenic derivatives (**5** and **8**) were confirmed by the proton nuclear magnetic resonance (^{1}H-NMR) spectra in which the characteristics multiplet signal due to the 12α proton in **8** appeared at 3.17 ppm, whereas the 12β proton signal in **5** was shifted downfield to 3.38 ppm.

The steroid haptens (**5** and **8**) were coupled to BSA using the mixed anhydride method of



1: R_1=Ac, R_2=$COOCH_3$, R_3=H
2: R_1=Ac, R_2=H, R_3=$COOCH_3$
3: R_1=R_3=H, R_2=COOH
4: R_1=R_3=H, R_2=$CONHCH_2COOC_2H_5$
5: R_1=R_3=H, R_2=$CONHCH_2COOH$
6: R_1=Ac, R_2=H, R_3=COOH
7: R_1=Ac, R_2=H, R_3=$CONHCH_2COOC_2H_5$
8: R_1=R_2=H, R_3=$CONHCH_2COOH$
9: R_1=R_3=H, R_2=$CONHCH_2CONH$–BSA
10: R_1=R_2=H, R_3=$CONHCH_2CONH$–BSA

Chart 1

TABLE I. Specificity of Antisera Raised in Rabbits against Estradiol-12–BSA Conjugates

Steroids	% cross-reactivity (50%)	
	12α[a)]	12β[a)]
Estradiol	100	100
17α-Estradiol	2.39	0.150
Estrone	38.2	8.33
2-Methoxyestradiol	0.775	0.500
2-Methoxyestrone	0.217	0.057
Estriol	1.34	0.370
16-Epiestriol	3.02	2.59
17-Epiestriol	0.516	0.088
16,17-Epiestriol	0.318	0.004
16-Oxoestradiol	5.65	7.90
16α-Hydroxyestrone	0.886	1.88
16β-Hydroxyestrone	3.33	1.11
Testosterone	0.002	0.001
5α-Dihydrotestosterone	<0.001	0.001
4-Androstene-3,17-dione	0.001	0.001
Dehydroepiandrosterone	0.001	0.001
Progesterone	<0.001	<0.001
Pregnenolone	<0.001	<0.001
Corticosterone	<0.001	<0.001
Hydrocortisone	<0.001	<0.001
Cholic acid	<0.001	<0.001
Cholesterol	<0.001	<0.001

a) 12α=12α-Carboxyestradiol glycine amide–BSA, 12β=12β-Carboxyestradiol glycine amide–BSA.

Erlanger *et al.*[7] to give the hapten–BSA conjugates (**9** and **10**); satisfactory numbers of haptens were covalently bounded to each BSA. The sera obtained from two rabbits immunized with these antigens for 5 months showed remarkably increased binding affinity for naturally occurring estradiol. Determination of saturation curves for various dilutions of each serum with the highest titer values indicated that the dilutions providing a suitable standard curve in the 0—100 pg range were 1 : 40000 for the 12α and 1 : 70000 for the 12β. Both antisera exhibited high affinity for estradiol with association constants (K_a) of 2.36×10^{10} and $1.90 \times 10^{10}\,M^{-1}$ obtained from conventional Scatchard plots.[8] The dose–response curves obtained by log-logit transformation exhibited linear relationships from 1 pg to 1 ng.

The cross reactions of the antisera with 23 kinds of closely related steroids are listed in Table I. It should be noted that antiserum raised against the estradiol 12β-equatorial–BSA

conjugate is more specific than that elicited with the 12α-axial isomer, and in particular, the former exhibited only 8.3% cross reaction with estrone relative to estradiol, while the latter showed lower specificity (38.2% cross reaction). These findings can be explained by the hypothesis that antigens maintaining the coplanarity of the steroid molecule tend to give specific antibodies.[9] Slight cross reactions of both antisera with compounds having epimeric α-ketol on ring D of the estrogens and their triols was observed, but these antisera well recognized the structure of ring A in the 2-methoxyestrogens. All of the non-estrogens exhibited negligible competition (less than 0.001%).

These results may be ascribable to the structural features at C-17, which is relatively close to the site of conjugation of estradiol with BSA. It is of interest that the cross reactivities of our antisera with related hormones are comparable to those reported in the literature[2-4] in cases where the antisera were elicited by antigens coupled through other positions in the B and C rings. To the best of our knowledge, hydroxylation at C-12 in the biotransformation of estradiol and other aromatic steroids, such as 2-hydroxyestradiol and estriol, has not yet been reported. In these respects, the C-12 position appears to be a suitable site for attachment of a carrier protein in the preparation of the hapten–carrier conjugate. Further studies on the application of these techniques to the production of specific antisera to biologically important catecholestrogens for use in RIA will be reported in the near future.

Experimental

All melting points were taken on a micro hot-stage apparatus and are uncorrected. Ultraviolet (UV) spectra were measured on a Shimadzu model UV-200 spectrophotometer. NMR spectra were recorded using tetramethylsilane as an internal standard on a Hitachi model R-40 spectrometer at 90 MHz. Abbreviations used: s=singlet, d=doublet, dd=doublet of doublets, t=triplet, q=quartet, m=multiplet. For column chromatography, silica gel (70—230 mesh) was used.

Chemicals and Reagents——[2,4,6,7-^{3}H] Estradiol (90 Ci/mmol) was supplied by New England Nuclear Corp. (Boston, MA). BSA and complete Freund's adjuvant were purchased from Sigma Chemical Co., (St. Louis MO) and Iatron Laboratories (Tokyo), respectively. The steroid specimens were either synthesized in our laboratory or were obtained commercially. All solvents and chemicals used were of analytical reagent grade.

12α-Carboxyestradiol (3)——A solution of 12α-methoxycarbonylestradiol 3,17-diacetate[5] (**1**, 116 mg) in MeOH–H_2O (1 : 1, 8 ml) was mixed with 2 N KOH–MeOH (8 ml) and the whole was stirred at room temperature for 21 h. After evaporation of the MeOH under reduced pressure, the resulting solution was adjusted to pH 1 with conc. HCl and extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crystalline product (90 mg) was subjected to further elaboration without purification. A portion of the product was recrystallized from MeOH to give **3** as colorless needles. mp 250 °C (dec.). NMR (CD_3OD) δ: 0.97 (3H, s, 18-CH_3), 3.20 (1H, m, 12β-H), 3.96 (1H, t, J=8 Hz, 17α-H), 6.46 (1H, d, J=2 Hz, C_4-H), 6.52 (1H, dd, J=2, 8 Hz, C_2-H), 6.87 (1H, d, J=8 Hz, C_1-H). *Anal.* Calcd for $C_{19}H_{24}O_4$: C, 72.12; H, 7.65. Found: C, 71.85; H, 7.65.

12α-Carboxyestradiol Glycine Amide Ethyl Ester (4)——Glycine ethyl ester (49 mg), triethylamine (79 mg), and diethyl phosphorocyanidate (81 mg) in dimethylformamide (DMF, 3 ml) were added to a solution of **3** (91 mg) in DMF (8 ml) under ice cooling, and the reaction mixture was stirred at 0 °C for 30 min then at room temperature for a further 4 h. The resulting solution was diluted with AcOEt, washed with 5% HCl, 5% $NaHCO_3$, brine, and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the crude product obtained was chromatographed on silica gel (5 g). Elution with hexane–AcOEt (3 : 2, v/v) and recrystallization of the product from AcOEt gave **4** (78 mg) as colorless leaflets. mp 135.5—139.5 °C. NMR ($CDCl_3$) δ: 0.98 (3H, s, 18-CH_3), 1.31 (3H, t, J=6 Hz, –$COOCH_2\underline{CH_3}$), 3.45 (1H, m, 12β-H), 3.87 (3H, m, 17α-H and –$NHC\underline{H_2}COO$–), 4.21 (2H, q, J=6 Hz, –$COO\underline{CH_2}CH_3$), 6.46 (1H, d, J=2 Hz, C_4-H), 6.53 (1H, dd, J=2, 8 Hz, C_2-H), 6.92 (1H, d, J=8 Hz, C_1-H). *Anal.* Calcd for $C_{23}H_{31}NO_5 \cdot H_2O$: C, 65.85; H, 7.93; N, 3.34.Found: C, 65.79; H, 7.90; N, 3.34.

12α-Carboxyestradiol Glycine Amide (5)——A solution of **4** (37 mg) in EtOH (4.5 ml) was treated with 2.5 N methanolic KOH (0.5 ml), and the reaction mixture was stirred at room temperature for 30 min. After evaporation of the solvent, the residue was adjusted to pH 3 with 1 N HCl and extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crystalline product was recrystallized from acetone–MeOH to give **5** (32 mg) as colorless needles. mp 264.5—272 °C (dec.). NMR ($CDCl_3$–CD_3OD=4 : 1, v/v) δ: 0.97 (3H, s, 18-CH_3), 3.38 (1H, m, 12β-H), 3.93 (2H, d, J=13 Hz, –$NHC\underline{H_2}COOH$), 3.89 (1H, m, 17α-H), 6.43 (1H, d, J=2 Hz, C_4-H), 6.49 (1H, dd, J=2, 8 Hz, C_2-H), 6.89 (1H, d, J=8 Hz, C_1-H). *Anal.* Calcd for $C_{23}H_{31}NO_5 \cdot H_2O$: C,

65.85; H, 7.93; N, 3.34. Found: C, 65.84; H, 8.00; N, 3.60.

12β-Carboxyestradiol 3,17-Diacetate (6)——12β-Methoxycarbonylestradiol 3,17-diacetate[5] (**2**, 500 mg) was hydrolyzed with 2 N methanolic KOH (20 ml) by the same method as described for **3**. After usual work-up, the crude product was acetylated with Ac_2O (2 ml) and pyridine (4 ml) in the usual manner. The crystalline product was recrystallized from AcOEt to give **6** (325 mg) as colorless needles. mp 277—278 °C (dec.). NMR ($CDCl_3$) δ: 0.93 (3H, s, 18-CH_3), 2.00 (3H, s, 17-$OCOCH_3$), 2.26 (3H, s, 3-$OCOCH_3$), 4.93 (1H, t, J=7 Hz, 17α-H), 6.74 (1H, d, J=2 Hz, C_4-H), 6.80 (1H, dd, J=2, 8 Hz, C_2-H), 7.20 (1H, d, J=8 Hz, C_1-H). *Anal.* Calcd for $C_{23}H_{28}O_6$: C, 69.98; H, 7.05. Found: C, 69.03; H, 7.15.

12β-Carboxyestradiol 3,17-Diacetate Glycine Amide Ethyl Ester (7)——**6** (300 mg) was treated with glycine ethyl ester (116 mg), diethyl phosphorocyanidate (136 mg), and triethylamine (165 mg) in DMF (15 ml) by the same method as described for **4**. The crude product was recrystallized from AcOEt to give **7** (280 mg) as colorless leaflets. mp 177.5—179 °C. NMR ($CDCl_3$) δ: 0.99 (3H, s, 18-CH_3), 1.30 (3H, t, J=6 Hz, $-COOCH_2\underline{CH_3}$), 1.96 (3H, s, 17-$OCOCH_3$), 2.26 (3H, s, 3-$OCOCH_3$), 3.71, 4.16 (2H, dd, J=5, 18 Hz, $-NH\underline{CH_2}COO-$), 4.20 (2H, q, J=6 Hz, $-COO\underline{CH_2}CH_3$), 4.90 (1H, m, 17α-H), 6.12 (1H, t, J=5 Hz, –NH–), 6.73 (1H, d, J=2 Hz, C_4-H), 6.87 (1H, dd, J=2, 8 Hz, C_2-H), 7.20 (1H, d, J=8 Hz, C_1-H). *Anal.* Calcd for $C_{27}H_{35}NO_7$: C, 66.78; H, 7.27; N, 2.88. Found: C, 66.70; H, 7.22; N, 3.03.

12β-Carboxyestradiol Glycine Amide (8)——Potassium was added to a solution of **7** (82 mg) in anhydrous EtOH (10 ml) under a stream of Ar gas and the reaction mixture was stirred at room temperature for 19.5 h. After neutralization of the mixture with 1 N HCl, the EtOH was evaporated off under reduced pressure. The resulting solution was diluted with H_2O and extracted with AcOEt. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated. The crystalline product was recrystallized from aqueous EtOH to give **8** (26 mg) as colorless granules. mp 261.5—275 °C (dec.). NMR ($CDCl_3$–DMSO-d_6=1:1, v/v) δ: 0.78 (3H, s, 18-CH_3), 3.17 (1H, m, 12α-H), 3.76, 4.10 (2H, each d, J=18 Hz, $-NH\underline{CH_2}COO-$), 4.02 (1H, t, J=8 Hz, 17α-H), 6.45 (1H, d, J=2 Hz, C_4-H), 6.56 (1H, dd, J=2, 8 Hz, C_2-H), 7.12 (1H, d, J=8 Hz, C_1-H). *Anal.* Calcd for $C_{21}H_{27}NO_5$: C, 67.54; H, 7.29; N, 3.75. Found: C, 67.27; H, 7.51; N, 3.62.

Preparation of Antigens——A solution of hapten (**5** or **8**, 28 mg) in dry dioxane was treated with tri-*n*-butylamine (0.02 ml) and isobutyl chlorocarbonate (0.01 ml) at 11 °C and the whole was stirred for 30 min. Then BSA (90 mg) in H_2O (2.2 ml)–dioxane (1.4 ml)–1 N NaOH (0.08 ml) was added under ice cooling and the mixture was stirred for 3 h. The resulting solution was dialyzed against cold running water overnight and the turbid protein solution was brought to pH 4.5 with 0.1 N HCl. After being allowed to stand at 4 °C overnight, the suspension was centrifuged at 3000 rpm for 20 min. The precipitate was dissolved in 5% $NaHCO_3$ and dialyzed in the manner described above. Lyophilization of the solution afforded the estradiol-12–BSA conjugate (**9** or **10**, approx. 95 mg) as a fluffy powder.

Determination of the Molar Ratio of Hapten to BSA in the Conjugates——Spectrometric analysis was carried out by comparing the absorbance at 279 nm of the conjugates with those of BSA and haptens as a control in 0.1 N NaOH solution and by using the followed constants: molecular weight of BSA, 67000; ε values for **5** and **8**, 3860 and 2832, respectively. The numbers of steroid molecules linked to a BSA molecule were determined to be 14 and 20, respectively.

Immunization of Rabbits——Four domestic albino rabbits were used for immunization. The antigen (2 mg) was dissolved in sterile isotonic saline (1 ml) and emulsified with complete Freund's adjuvant (1 ml). The emulsion was incorporated into rabbits subcutaneously at multiple sites over the scapulae. This procedure was repeated at intervals of one week for a month and then once a month. Two rabbits were used for each conjugate. The rabbits were bled one week after a booster injection. The sera were separated by centrifugation at 3000 rpm for 15 min, and stored at −20 °C. The antisera were thawed and diluted with 0.01 M phosphate buffer (pH 7.4) containing 0.9% NaCl, 0.1% gelatin, and 0.06% NaN_3.

Measurement of Radioactivity——The samples were counted on a Delta 300 liquid scintillation spectrometer (model 6890) employing EX-H as a scintillator.

Assay Procedure——A standard curve was constructed by setting up duplicate centrifuge tubes (7 ml) containing 0, 5, 10, 25, 50, 100 and 250 pg of non-labeled estradiol in buffer (0.1 ml) and ^{3}H-labeled estradiol (14000 dpm in 0.1 ml). The diluted antiserum (0.1 ml) was added and the mixture was incubated at 4 °C overnight. After addition of dextran (0.06% (w/v))–charcoal (0.1% (w/v), 0.5 ml), the resulting solution was vortex-mixed and allowed to stand at 0 °C for 15 min, then centrifuged at 4 °C, 3000 rpm for 15 min. The supernatant was transferred by decantation into a vial containing a scintillation cocktail (10 ml) and the radioactivity was counted.

Cross-Reaction Study——The specificity of antisera raised against the estradiol–BSA conjugates was tested by studies of the reactivities with 23 kinds of purified steroids related to estradiol (Table I). The relative amounts required to reduce the initial binding of [^{3}H]estradiol by half, where the mass of unlabeled estradiol was arbitrarily set at 100%, were calculated from the standard curves.

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