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PREPARATION OF SPECIFIC ANTISERA TO ESTRADIOL 17-GLUCURONIDE*

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SUMMARY

The preparation and antigenic properties of three different types of estradiol 17-glucuronide-bovine serum albumin (BSA) conjugates in which the haptens are linked to the carrier protein through the C-2 (or C-4) position have been described. All antibodies raised against these antigens in rabbits possessed high affinity ($K_A = 2.22 \times 10^9$–$2.58 \times 10^9\ M^{-1}$) and excellent specificity to estradiol 17-glucuronide, exhibiting little cross-reactivities for other estrogen glucuronides and sulphates (<1%) and no cross-reactions for free estrogens and other steroids (<0.001%) with an only exception of testosterone 17-glucuronide (0.86–11.5%).

INTRODUCTION

In recent years considerable attention has been focused on the physiological significance of steroid hormone conjugates. The conventional method for determination of conjugated steroids in biological fluids involves prior hydrolysis and separation of the liberated free steroid. Several attempts for radioimmunoassay of the intact steroid glucuronides without hydrolysis have previously been made [1–7]. In these studies the preparation of antisera was carried out with use of antigen in which the steroid conjugate was coupled to a carrier protein through the carboxyl group in the glucuronic acid moiety. However, antisera elicited with these antigens were unsatisfactory in respect of the specificity. For instance, anti-estradiol 17-glucuronide† antisera showed significant cross-reactivities with free estrogens and estriol ring D glucuronides [2]. It is sufficiently substantiated that antibody raised against an immunogen whose hapten is linked to a carrier protein through a preexisting functional group usually lacks the specificity for the group used for coupling and the region of the hapten in its immediate vicinity [3, 8–13]. An urgent need for obtaining specific antisera for use in radioimmunoassay of steroid glucuronides prompted us to develop new antigens. In this paper we report the preparation of anti-estradiol 17-glucuronide antisera with use of three new types of antigens in which the haptens are linked to bovine serum albumin (BSA) through the C-2 (or C-4) position in a steroid molecule.

* Part CXXXII of *Studies on Steroids* by T. Nambara.

† The following trivial names are used in this paper: estrone = 3-hydroxy-1,3,5(10)-estratrien-17-one; estradiol = 1,3,5(10)-estratriene-3,17β-diol; estriol = 1,3,5(10)-estratriene-3,16α,17β-triol; estradiol 17-glucuronide = (3-hydroxy-1,3,5(10)-estratrien-17β-yl-β-D-glucopyranosid)-uronic acid; estradiol 17-glucuronide acetate-methyl ester = methyl (3-hydroxy-1,3,5(10)-estratrien-17β-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate.

MATERIALS AND METHODS

Reagents

[6,7-^{3}H]-Estradiol 17-glucuronide (45.9 Ci/mmol) was supplied by New England Nuclear (Boston, MA). and the radiochemical purity was checked by t.l.c. prior to use. Estradiol 17-glucuronide, its acetate-methyl ester and other conjugated steroids were prepared in these laboratories by known methods. All the free steroids were kindly donated from Teikoku Hormone Mfg. Co., Tokyo, Japan. BSA Fraction V and bovine serum gamma-globulin from Sigma Chemical Co. (St. Louis, MO), Sephadex G-25 from Pharmacia Fine Chemicals (Uppsala, Sweden), Amberlite XAD-2 resin from Rohm and Haas Co. (Philadelphia, PA), complete Freund's adjuvant and other general reagents were purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan), respectively.

2-Nitroestradiol 17-glucuronide acetate-methyl ester

To a solution of estradiol 17-glucuronide acetate-methyl ester (400 mg) in acetic acid (10 ml)-chloroform (5 ml) was added conc. HNO_3 ($d = 1.42$) (0.07 ml) dropwise and the mixture was stirred at room temperature for 20 min. The resulting solution was poured onto ice-water, neutralized with $NaHCO_3$ solution and extracted with ethyl acetate. The organic phase was washed with water, dried (Na_2SO_4) and evaporated. An oily residue was purified by column chromatography on silica gel. Elution with benzene–ether (95:5, v/v) and recrystallization of the eluate from methanol gave 2-nitroestradiol 17-glucuronide acetate-methyl ester (159 mg) as pale yellow needles. m.p. 201–204°C. $[\alpha]_D^{20} + 23.6°$ ($c = 0.89$, $CHCl_3$). Anal. Calcd. for $C_{31}H_{39}O_{13}N$: C, 58.76; H, 6.20; N, 2.21. Found: C, 58.84; H, 6.22; N, 2.06. U.V. $\lambda_{max}^{95\%\ EtOH}$ nm: 275 (ε 4600), 342 (ε 3250). I.R. ν_{max}^{KBr} cm^{-1}: 1760, 1740 (C=O), 1525, 1370 (NO_2). NMR ($CDCl_3$)δ: 0.78 (3H, *s*, 18-CH_3), 2.00 (6H, *s*, —$OCOCH_3$), 2.05 (3H, *s*, —$OCOCH_3$), 3.74 (3H, *s*,

—$COOCH_3$), 4.60 (1H, *d*, *J*=7 Hz, pyranose-C_1—H), 4.85–5.35 (4H, *m*, pyranose—CH—OAc), 6.82 (1H, *s*, 4-H), 7.95 (1H, *s*, 1-H).

2-Nitroestradiol 17-glucuronide

To a solution of 2-nitroestradiol 17-glucuronide acetate-methyl ester (150 mg) in methanol (10 ml) was added 1N NaOH solution (1.4 ml) and the mixture was allowed to stand at 4°C for 3 days. The resulting precipitate was collected by filtration and recrystallized from methanol to give Na salt of 2-nitroestradiol 17-glucuronide as red plates. m.p. > 300°C. $[\alpha]_D^{20}$ − 7.9°C (c = 0.81, H_2O). Anal. Calcd. for $C_{24}H_{29}O_{10}NNa_2 \cdot 2H_2O$:C, 50.26; H, 5.80; N, 2.44. Found: C, 50.68; H, 5.22; N, 2.03. U.V. $\lambda_{max}^{H_2O}$ nm: 277 (ε 5000), 346 (ε 3450). I.R. ν_{max}^{KBr} cm^{-1}: 3200–3600 (OH), 1600 (COO^-), 1520 (NO_2). NMR (D_2O) δ: 1.19 (3H, *s*, 18-CH_3), 3.90–4.50 (4H, *m*, pyranose—CH—OH), 4.39 (1H, *d*, *J* = 7 Hz, pyranose—C_1—H), 6.92 (1H, *s*, 4-H), 8.12 (1H, *s*, 1-H).

p-Aminobenzoylated BSA

To a solution of BSA (50 mg) and triethylamine (100 mg) in 50% (v/v) aq. dioxane (20 ml) was added *p*-nitrobenzoyl chloride (40 mg) and the mixture was gently stirred at room temperature for 8 days. The resulting solution was dialyzed against running water at 4°C for 2 days. The precipitate formed was removed by centrifugation at 3000 *g* for 20 min. The supernatant was lyophilized to give a crude *p*-nitrobenzoylated BSA (55 mg). To a solution of this product in 0.4M $NaHCO_3$ (30 ml) was added $Na_2S_2O_4$ (500 mg) and the mixture was gently stirred at 37°C overnight. The resulting solution was dialyzed and then lyophilized to give a fluffy powder (57 mg). Purification by gel filtration on a Sephadex G-25 column (2 × 50 cm) gave *p*-aminobenzoylated BSA (50 mg). Measurement of absorbance at 275 nm with aq. solutions of *p*-aminobenzoylated BSA, *p*-aminobenzoic acid and BSA revealed that 31 moles of *p*-aminobenzoic acid was bound to each mole of modified protein.

Preparation of antigens

(i) *Glutaraldehyde method.* To a solution of 2-nitroestradiol 17-glucuronide (80 mg) in 0.1N NaOH (5 ml) was added $Na_2S_2O_4$ (240 mg) and the mixture was stirred at room temperature for 1 h. The resulting solution was passed through a column of Amberlite XAD-2 resin. After thorough washing with distilled water the glucuronide was eluted with methanol. The eluate was further purified by column chromatography on Sephadex G-25. The desired fractions were collected and lyophilized to give the crude 2-aminoestradiol 17-glucuronide whose purity was more than 90% as judged from t.l.c. U.V. $\lambda_{max}^{95\%EtOH}$ nm: 285, 280. I.R. ν_{max}^{KBr} cm^{-1}: 3200–3600 (OH, NH_2), 1600 (COO^-). NMR (pyridine-d_5) δ: 1.15 (3H, *s* 18-CH_3), 3.91–4.60 (4H, *m*, pyranose—CH—OH), 4.90 (1H, *d*, *J*=7 Hz, pyranose—C_1—H), 6.92 (1H, *s*, 4-H), 8.02 (1H, *s*, 1-H). The crude product was submitted to further elaboration without purification.

To a solution of the crude 2-aminoestradiol 17-glucuronide (50 mg) and BSA (110 mg) in 0.1M phosphate buffer (pH 6.8) (10 ml) was added 1% (w/v) aq. glutaraldehyde solution (1 ml) dropwise and the reaction mixture was allowed to stand overnight at room temperature [12]. The resulting solution was dialyzed against two changes of 3 liters of distilled water at 4°C overnight. After removal of the precipitate by centrifugation the supernatant was passed through a column of Sephadex G-25 and then lyophilized to give a fluffy powder (130 mg). To a solution of this product in 0.1M borate buffer (pH 8.0) (10 ml) was added $NaBH_4$ (20 mg) and the mixture was allowed to stand at 4°C overnight. The resulting solution was dialyzed and then lyophilized in the manner described above to give a hapten-BSA conjugate (**I**) (120 mg).

(ii) *Mannich reaction method.* To a solution of estradiol 17-glucuronide (100 mg) and BSA (102 mg) in 1M phosphate buffer (pH 10.2) was added 7.5% formalin (0.08 ml) and the mixture was stirred at 37°C for 3 weeks. The resulting solution was dialyzed, purified by gel filtration and then lyophilized in the manner described above to give a hapten-BSA conjugate (**II**) (85 mg).

(iii) *Diazo coupling method.* To a solution of *p*-aminobenzoylated BSA (108 mg) in 10% (v/v) aq. dimethylformamide (20 ml) adjusted to pH 1 with 1N HCl was added 20% (w/v) $NaNO_2$ solution (0.045 ml) under ice-cooling and the mixture was stirred at 0–4°C for 15 min. After addition of sulphamic acid (50 mg) to decompose excess nitrous acid the precipitate was removed by centrifugation at 3000 rev./min for 10 min. The supernatant was added to a solution of estradiol 17-glucuronide (50 mg) in 2M phosphate buffer (pH 10.2) (50 ml) and the mixture was stirred gently at 4°C for 4 h. The resulting solution was dialyzed, purified by gel filtration and then lyophilized in the manner described above to give a hapten-BSA conjugate (**III**) (114 mg).

The number of moles of steroid hapten per mole of estradiol 17-glucuronide-BSA conjugate was determined by the U.V. method using the following references: 2-aminoestradiol for **I**; estradiol 17-glucuronide for **II**; 2- and 4-(*p*-carboxyphenylazo)-estradiol for **III** (Table 1).

Immunization of rabbits

Three male albino rabbits were used for immunization with each of three different types of antigens. The antigen (2 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with complete Freund's adjuvant (0.5 ml). This emulsion was injected into rabbits subcutaneously at the multiple sites over the scapulae and in the thighs. This procedure was repeated at intervals of two weeks for further 2 months and then once a month. The rabbits were bled 10 days after the booster injection. The sera were separated by centrifugation at 3000 rev./min for

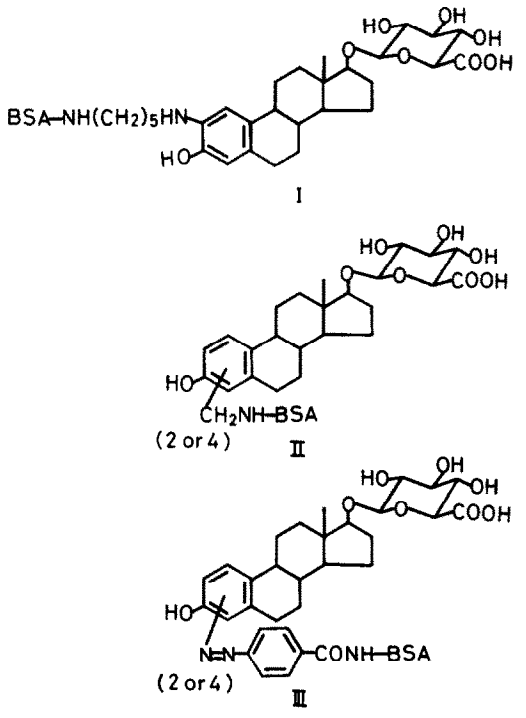

Fig. 1. Structures of three new types of estradiol 17-glucuronide-BSA conjugates used for immunization.

10 min and stored at −20°C. The antisera were thawed and diluted with 0.05M borate buffer (pH 8.0) containing 0.06% BSA and 0.05% bovine serum gamma-globulin.

Assay procedure

A standard curve was constructed in each case by setting up duplicate centrifuge tubes (7 ml) containing 0, 20, 40, 100, 200, 500, and 1000 pg of non-labeled estradiol 17-glucuronide and ^{3}H-labeled estradiol 17-glucuronide (40 pg, 8500 d.p.m.). The diluted antiserum (0.25 ml) was added to all the tubes and incubated at 4°C for 1 h. After addition of a 50% saturated $(NH_4)_2SO_4$ solution (0.25 ml) the resulting suspension was allowed to stand at 4°C for 15 min and then centrifuged at 3000 rev./min for 10 min. A two-tenth ml aliquot of each supernatant was taken into a counting vial, added with 10 ml of Bray's scintillator [15] and counted in a Packard Model 3380 liquid scintillation spectrometer.

Characterization of antisera

Association constants and binding capacity were derived from a Scatchard plot [16] of data by incubating a constant amount of antiserum with increasing amounts of ^{3}H-labeled estradiol 17-glucuronide at 4°C for 1 h. Specificity of antisera was tested by cross-reaction studies with 34 kinds of steroids related to estradiol 17-glucuronide. The relative amounts required to reduce the initial binding of labeled steroid by half, where the mass of non-labeled estradiol 17-glucuronide was arbitrarily chosen as 100%, were calculated by the standard curve.

RESULTS

An initial effort was directed to the preparation of three new types of antigens for production of anti-estradiol 17-glucuronide antisera (Fig. 1). First, 2-aminoestradiol 17-glucuronide, obtained from estradiol 17-glucuronide acetate-methyl ester via 2-nitroestradiol 17-glucuronide in three steps, was coupled to BSA employing glutaraldehyde as a cross-linking agent to yield a hapten-BSA conjugate (**I**). This route was somewhat tedious and therefore coupling of estradiol 17-glucuronide to BSA with the aid of formaldehyde was undertaken. Mannich reaction under basic conditions occurred at the *ortho* positions to the phenolic group in ring A [17] to give a hapten-BSA conjugate (**II**) as the second antigen. The third antigen was prepared by the use of modified BSA. Treatment of BSA with *p*-nitrobenzoyl chloride in the presence of triethylamine, followed by reduction with hydrosulfite provided *p*-aminobenzoylated BSA. Diazo coupling of this modified protein to estradiol 17-glucuronide afforded a hapten-BSA conjugate (**III**) having an azo linkage through the C-2 (or C-4) position. Measurement of the U.V. spectra revealed that satisfactory numbers of steroid molecules were covalently bound to each BSA in these three antigens (Table 1).

Three different types of hapten-BSA conjugates thus prepared were used for immunization of animals. Several months after an initial injection of antigens all the rabbits immunized yielded antibodies exhibiting a remarkably increased binding activity to estradiol 17-glucuronide. Preliminary tests indicated that sera obtained from rabbits immunized with the same antigen did not differ significantly in affinity and specificity. Accordingly, for each group, the serum of the rabbit that had attained the highest titer was selected for detailed characterization. Evaluation of the titer was performed by incubating various dilutions of antiserum with a constant amount of ^{3}H-labeled estradiol 17-glucuronide. The dilution of antiserum which was able to bind 50% of the labeled antigen was defined as a titer.

The binding affinity was determined by incubating a constant amount of antiserum with increasing amounts of the labeled antigen. The binding of labeled antigen by homologous antiserum was inhibited when the corresponding cold antigen was added

Table 1. Moles of steroid per mole of estradiol 17-glucuronide-BSA conjugate as determined by U.V. method

Preparation of hapten-BSA conjugate	Moles of steroid
Glutaraldehyde method	15
Mannich reaction method	20
Diazo coupling method	16

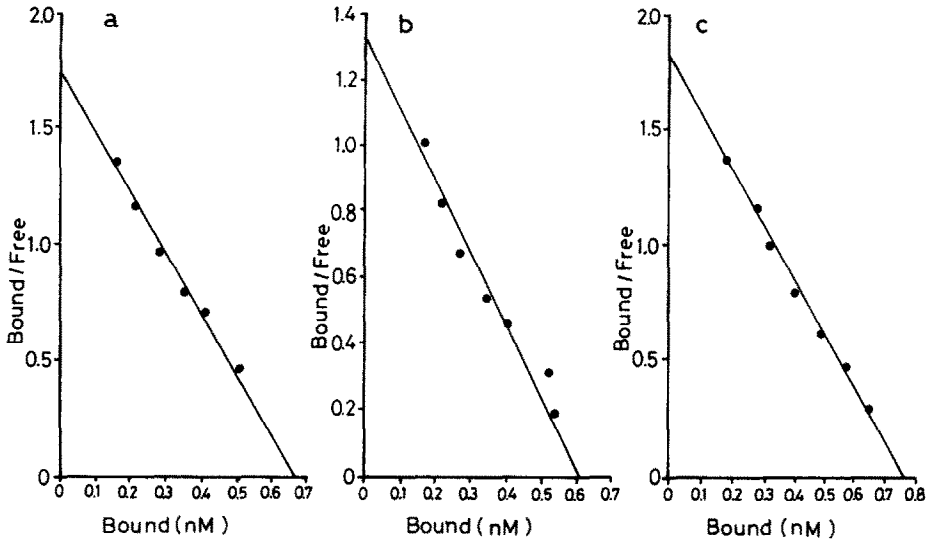

Fig. 2. Scatchard plots for antisera raised against three new types of estradiol 17-glucuronide-BSA conjugates prepared by the different methods. (a) glutaraldehyde method; (b) Mannich reaction method; (c) diazo coupling method.

to the system. The relationship between the concentration of antigen and the ratio of the bound to free (B/F) observed with each antiserum is illustrated as a Scatchard plot in Fig. 2. The titer and binding affinity of antibody which was produced with each of the three immunogens are listed in Table 2. The specificity of antiserum was assessed by testing the ability of the closely related compounds to compete for binding sites on the antibody. The per cent cross-reactions determined by the method of Abraham[18] with each antiserum are collected in Table 3.

DISCUSSION

In recent years several investigators have attempted to develop a direct radioimmunoassay of steroid glucuronides in human plasma and urine without hydrolysis. Contrary to the expectations, however, almost all antisera so far obtained are not always satisfactory in respect of the specificity. For instance, antisera raised against the estrogen 3-glucuronide-BSA conjugates showed cross-reactions with the corresponding aglycones by 33–100% [2]. Also antisera elicited with the estriol 16-glucuronide-BSA conjugate exhibited cross-reactivities with estrone and estriol by 46% and 53%, respectively [3]. On the other hand Soares *et al.*[5] reported the linking of carboxyphenylazoestriol 16-glucuronide to BSA through the C-2 (or C-4) position and the use of a resulting conjugate for immunization. The antiserum raised against this hapten-BSA conjugates showed the relatively low cross-reactivity with free estrogens (<2.2%). As for the structure of this antigen both carboxyl groups in the benzoyl and glucuronyl residues would possibly be occupied for condensation with BSA. The insufficient specificity appears to be ascribable in part to the linkage of antigen where the hapten was coupled to a carrier protein through carboxylic acid in the glucuronyl moiety.

It is evident from the data that no substantial difference in the affinity and specificity was observed among antisera elicited by immunization with the three types of antigens. The antisera raised against these antigens exhibited the high affinity for estradiol 17-glucuronide with the association constants in the range of $2.22 \times 10^9\ M^{-1}$ to $2.58 \times 10^9\ M^{-1}$. As can be seen from the results on the per cent cross-reaction all antisera were highly specific to estradiol 17-glucuronide. There was no significant cross-reaction with estrogen ring D and A glucuronides (<1%) and no

Table 2. Antibody characterization*

Preparation of hapten-BSA conjugate	Titer	Production time	Binding affinity (K_A)
Glutaraldehyde method	1:25,000	6 months	$2.58 \times 10^9\ M^{-1}$
Mannich reaction method	1:150,000	5 months	$2.39 \times 10^9\ M^{-1}$
Diazo coupling method	1:2,000	6 months	$2.22 \times 10^9\ M^{-1}$

* Antiserum of the rabbit that had attained the highest titer of each group (three rabbits) was selected for detailed characterization.

Table 3. Per cent cross-reactions of anti-estradiol 17-glucuronide antisera with selected steroids

Steroid	Antisera*		
	A	B	C
Estradiol 17-glucuronide	100	100	100
Testosterone 17-glucuronide	2.94	11.5	0.86
Estrone 3-glucuronide	0.10	0.24	0.06
Estradiol 3-glucuronide	0.10	0.19	0.04
16-Epiestriol 3-glucuronide	0.19	0.57	0.02
Estriol 3-glucuronide	<0.001	<0.001	<0.001
Estriol 16-glucuronide	<0.001	<0.001	<0.001
Estriol 17-glucuronide	0.06	0.38	<0.001
2-Hydroxyestradiol 2-glucuronide	<0.001	<0.001	<0.001
Androsterone 3-glucuronide	<0.001	<0.001	<0.001
Pregnanediol 3-glucuronide	<0.001	<0.001	<0.001
Estradiol 17-sulphate	<0.001	<0.001	<0.001
Estriol 3-sulphate	<0.001	<0.001	<0.001
2-Hydroxyestradiol 3-sulphate	<0.001	<0.001	<0.001
Dehydroepiandrosterone 3-sulphate	<0.001	<0.001	<0.001
2-Hydroxyestrone glutathione 1-thioether	<0.001	<0.001	<0.001
2-Hydroxyestrone glutathione 4-thioether	<0.001	<0.001	<0.001
Estrone	<0.001	<0.001	<0.001
Estradiol	<0.001	<0.001	<0.001
Estriol	<0.001	<0.001	<0.001
Estetrol	<0.001	<0.001	<0.001
16-Epiestriol	<0.001	<0.001	<0.001
17-Epiestriol	<0.001	<0.001	<0.001
16,17-Epiestriol	<0.001	<0.001	<0.001
2-Hydroxyestrone	<0.001	<0.001	<0.001
2-Hydroxyestradiol	<0.001	<0.001	<0.001
2-Methoxyestrone	<0.001	<0.001	<0.001
16-Ketoestradiol	<0.001	<0.001	<0.001
16α-Hydroxyestrone	<0.001	<0.001	<0.001
Testosterone	<0.001	<0.001	<0.001
Progesterone	<0.001	<0.001	<0.001
Pregnenolone	<0.001	<0.001	<0.001
Cortisol	<0.001	<0.001	<0.001
Cortisone	<0.001	<0.001	<0.001
Cholesterol	<0.001	<0.001	<0.001

* Preparation of hapten-BSA conjugates used for immunization: (A) glutaraldehyde method; (B) Mannich reaction method; (C) diazo coupling method.

cross-reactivity with free estrogens, their sulphates and other steroids (<0.001%). Of the selected steroids an exception was testosterone 17-glucuronide with a cross-reaction of 0.86–11.5%. This may reflect the close similarity in the ring D structure of testosterone 17-glucuronide to estradiol 17-glucuronide.

It is to be noted that antibody elicited with antigen in which the steroid hapten is linked to the carrier protein through the C-2 (or C-4) position remote from the glucuronyl moiety in ring D, is capable of discriminating estradiol 17-glucuronide from the closely related free and conjugated estrogens. The design of hapten-carrier conjugate in similar fashion may be applicable to the preparation of specific antisera to other steroid conjugates.

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