SPECIFICITY OF ANTIBODIES TO OVARIAN HORMONES IN RELATION TO THE SITE OF ATTACHMENT OF THE STEROID HAPTEN TO THE PEPTIDE CARRIER

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ABSTRACT

Estradiol-17β, estriol and progesterone were rendered antigenic by covalent attachment to bovine serum albumin through ring B or C of the steroid molecule. Coupling to lysine residues of the protein was effected via the 6-(O-carboxymethyl)-oximes of the estrogens and via the 6-(carboxymethylene) thioether or the 11α-hemisuccinate of progesterone, by use of the carbodiimide reagent.

Antisera to the estradiol 6-conjugate raised in rabbits or goats distinguished estradiol-17β more efficiently from estrone, estradiol-17α and estriol than did antisera to estradiol-17β-hemisuccinate-BSA; neither serum reacted with non-phenolic steroids or non-steroidal estrogens. Antisera to estriol 6-conjugates showed minimal cross-reaction with estradiol-17β and estrone.

Rabbit antisera against the progesterone 6-conjugate did not react with phenolic or C_{19}-steroids, or with corticosterone, and only feebly with 11-deoxycorticosterone and 20α- or 20β-dihydroprogesterone; significant cross-reaction occurred with 17α-hydroxyprogesterone and 3β,17α-dihydroxypregn-5-en-20-one (3-4%), Δ^5-pregnenolone (8-14%) and with 5α- and 5β-dihydro-progesterone (100%). Sera against the 11-conjugate were better able to recognize changes about the A-ring and failed to cross-react with 5α-dihydro-progesterone (< 3%). Both sera showed greater specificity than reported for antibodies to 20-conjugates of progesterone towards the D-ring and 17-sidechain and were similar in this respect to antibodies elicited by 3-conjugates described in the literature.

It is concluded that the site of attachment of steroid haptens to the peptide carrier importantly affects the specificity of the antibodies produced.

INTRODUCTION

Steroids may be rendered antigenic by attaching them to a macromolecule (1, review). Antibodies to such steroid conjugates have proved valuable in radioimmunoassay procedures (2) and in investigation of hormone action (3). In earlier studies, coupling of the steroid to the peptide carrier was invariably effected through one of the existing functional groups, e.g. in position 3, 17 or 20 of the gonadal hormones. As a result, these groups were masked or modified. It seemed to us that this approach may entail the loss of important antigenic information, since even minor variation in the nature or orientation of substituents in the positions mentioned results in major differences in hormonal specificity and potency. We therefore proposed to couple steroids to peptide carriers through chemical handles (amino or carboxyl groups) attached to the hormone molecule at sites remote from their characteristic functional groups (4).

The 6-position of steroid hormones, being benzylic in the estrogens and allylic in the progestins and androgens, readily lends itself to derivative formation. This feature was exploited by us in the preparation of 6ξ-amino and 6-(O-carboxymethyl)-oximino estrogens (4). Of these, the 6-(O-carboxymethyl)-oximes have the advantage of making the estrogen protrude farther from the carrier molecule, which should favor the production of high titres of antibodies directed against the hapten (1, 4). Furthermore, the oxime derivatives are obtained with greater ease in a state of high purity, and hence were preferred in subsequent immunological studies. This paper describes

in greater detail the preparation and characteristics of estradiol 6-(O-carboxymethyl)-oxime and estriol-6-(O-carboxymethyl)-oxime and compares the specificity of antibodies elicited with an estradiol-6-bovine-serum-albumin (-BSA) complex with that of antibodies to the conventional estradiol-17β-hemisuccinate-BSA conjugate (E_2-17-HS-BSA). In addition, two methods of coupling progesterone to protein, designed to leave the original functional groups of the hormone fully exposed, are described and evaluated. A summary of the results has been presented (5).

MATERIALS AND METHODS

Reagents and apparatus

Steroids used for the preparation of antigens and for the testing of sera were the product of Ikapharm (Ramat Gan, Israel); their purity was confirmed by paper-and gas chromatography. C-carboxymethoxylamine hemihydrochloride was synthesized according to Borek & Clarke (6) and later purchased from Aldrich Chemical Co. Inc. (Milwaukee, Wis.). Crystalline grade bovine serum albumin (BSA) was purchased from Mann Research Laboratories Inc. (New York, N.Y.); thioglycolic acid from British Drug Houses (Poole, U.K.); 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride from Ott Chemical Co. (Muskegon, Mich.); charcoal Norit A from Fisher Scientific Company (Springfield, N.J.) and Dextran T20 from Pharmacia (Uppsala, Sweden); 4-^{14}C-estradiol-17β (8.18 mCi/mmol), 4-^{14}C-estriol (4.5 mCi/mmol), 4-^{14}C-progesterone (58.5 mCi/mmol), 6,7-^{3}H-estradiol-17β (40-50 Ci/mmol), 6,7-^{3}H-estriol (40-50 Ci/mmol) and 1,2-^{3}H-progesterone (40-50 Ci/mmol) from New England Nuclear Corporation (Boston, Mass.). Estradiol-17β-hemisuccinate-BSA (E_2-17-HS) was kindly provided by the Endocrine Evaluation Branch, National Cancer Institute, N.I.H., Bethesda, Md. All solvents used were of reagent grade.

Thin layer chromatography (TLC) was carried out on precoated sheets (Eastman 6060 Silica Gel), using the system ethyl acetate / n-hexane / ethanol/ acetic acid (72 : 13.5 : 4.5 : 10). The steroids were stained with iodine vapor. Gas-liquid chromatography was performed with a Packard Model 7821 Gas Chromatograph (7) and radioactivity was measured in a Liquid Scintillation Spectrometer Model 3310 (Packard Instrument Co., Downers Grove, Ill.).

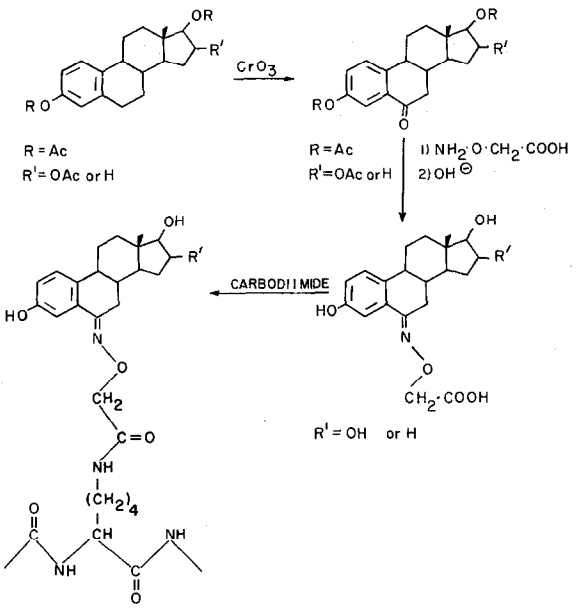

Figure 1. Reaction sequence for coupling estradiol or estriol to protein through carbon-6 of the steroid.

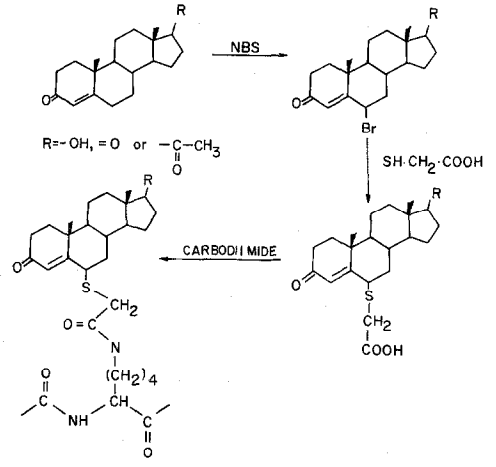

Figure 2. Reaction sequence for coupling 4, 5-unsaturated 3-ketosteroids to protein through carbon-6 of the steroid.

Melting points were determined on a Fisher-Johns apparatus and recorded without correction. Spectra were obtained with a Perkin-Elmer Model 237 Grating Spectrophotometer in the infrared range and Model 402 in the ultraviolet range. NMR spectra were scanned on a Varian A-60 spectrometer using tetramethylsilane as internal standard. Mass spectra were determined with an Atlas CH-4 spectrometer.

Preparation of antigens

General outline. - The 6-(O-carboxymethyl)-oximes of estradiol-17β (E_2-6CMO) and of estriol (E_3-6-CMO) were synthesized as shown schematically in Fig. 1. 6-Bromoprogesterone was converted to progesterone-6-(carboxymethylene)-thioether (P-6-CMTE) as shown schematically in Fig. 2. The steroids used as starting materials for the synthesis of these haptens were weakly labeled by addition of the corresponding ^{14}C-labeled compound (0.2 to 0.5 μCi/g). This facilitated determination of the number of hapten residues eventually bound to each carrier molecule.

6-Oxoestradiol-17β, prepared according to Longwell & Wintersteiner (8), had m.p. 281° [lit. (8) 281-283°]; M^+ at m/e 286; $\lambda_{max}^{ethanol}$ 220, 255 and 327 nm, ε 20 000, 8 800 and 3 000 (lit. 221, 255 and 327 nm, ε 20 200, 8 800 and 3 000); λ_{max}^{KBr} 6.1 μ (C=O). On treatment with ethanedithiol it formed a thioketal with a change in gas-chromatographic retention time (ΔR_{Mr} = 0.42 on SE30) indicative of the presence of a keto group (7). It formed a diacetate with an ultraviolet spectrum ($\lambda_{max}^{ethanol}$ 247 and 298 nm) and m.p. (175-177°) conforming with the literature (8).

Estradiol 6-(O-carboxymethyl)-oxime (E_2-6CMO) was prepared by condensation of 6-oxoestradiol-17β with C-carboxymethoxylamine hemihydrochloride according to the general method of Erlanger et al. (9). The product formed was recrystallized from acetone-hexane (yield 65%). It moved as a single component on TLC (R_{st} = 0.82 relative to 6-oxoestradiol) and had the following properties: m.p. 196-200° (decomp.); M^+ at m/e 359; anal. calc. for $C_{20}H_{25}O_5N$: C, 66.83: H, 7.01; found: C, 66.33; H, 7.30; $\lambda_{max}^{ethanol}$ 265 and 313 nm (ε 7 540 and 2 330); λ_{max}^{KBr} 5.81 μ (COOH) and 5.99 μ (oxime); NMR δ [$(CD_3)_2SO$] 0.75 (18-CH_3), 3.56 (17α-H) and 4.66 (=N-O-CH_3). [The difference between the chemical shift of the 17α-hydrogen in E_2-6CMO (3.56 δ) and in estradiol-17β (4.57 δ) is probably explained by an intermolecular interaction between the 17β-hydroxyl group and the carboxyl group in E_2-6CMO, since a similar change in the chemical shift (to 3.50 δ) was observed when estradiol was scanned in the presence of trifluoroacetic acid]. E_2-6CMO formed a diacetate (pyridine and acetic anhydride) with m.p. 86-87°; M^+ at m/e 443; NMR δ [$(CD_3)_2SO$] 0.75 (18-CH_3), 2.0 (C_{17}-OAc), 2.3 (C_3-OAc), 4.68 (3 protons: 17α-H and =N-OCH_2).

6-Oxoestriol was prepared from the triacetate of estriol in the same way as 6-oxoestradiol. The product, M^+ at m/e 302, λ_{max}^{KBr} 5.95, had an ultraviolet spectrum ($\lambda_{max}^{ethanol}$ 257 and 325 nm) and m.p. (240°) in agreement with the literature (10).

Estriol 6-(O-carboxymethyl)oxime (E_3-6CMO) was prepared from 6-oxoestriol as described for E_2-6CMO and recrystallized from acetone-hexane in 69% yield: m.p. 220-222° (decomp.); M^+ at m/e 375; anal. calc. for $C_{20}H_{25}O_6N$: C, 63.98; H, 6.71; found: C, 63.80; H, 6.88; $\lambda_{max}^{ethanol}$ 264 and 316 nm (ϵ 7525 and 2475); λ_{max}^{KBr} 5.85 μ (COOH) and 6.05 (oxime); NMR δ [$(CD_3)_2SO$] 0.75 (18-CH_3), 3.93 (17α-H?) and 4.70 (3 protons: 16β-H? and =N-OCH_2). [The 16β and 17α hydrogens of estriol appeared at 4.60 and 4.73 δ (unassigned). The change in the chemical shift of one of these methine protons to 3.93 δ in E_3-6CMO is analogous to the phenomenon observed in E_2-6CMO (see above), and likewise is mimicked by adding trifluoracetic acid to a solution of estriol in $(CD_3)_2SO$]. E_3-6CMO formed a triacetate with m.p. 98-99°; M^+ at m/e 501; NMR δ [$(CD_3)_2SO$] 0.75 (18-CH_3) 2.0 and 2.08 (C17-OAc and C16-OAc, unassigned), 2.25 (C3-OAc), 4.67 (-OCH_2) and 4.94 (2 protons: C16β-H and C17α-H, unassigned).

6-Bromoprogesterone was prepared from progesterone (11) by treatment with N-bromosuccinimide (NBS). The product was recrystallized from acetone-hexane (M^+ at m/e 393) and appeared homogeneous when run on TLC (R_f = 0.81). Its m.p. (145-146° decomp.) and ultraviolet spectrum ($\lambda_{max}^{ethanol}$ 248 nm) agreed with the literature (11).

Progesterone 6-(carboxymethylene) thioether (P-6-CMTE). A mixture of 6-bromoprogesterone (0.6 g) and thioglycolic acid (0.2 g) was refluxed for 2 h in a solution of KOH (0.25 g) in methanol (25 ml). The reaction mixture was diluted with water (25 ml), acidified with 10% HCl to pH 1 and extracted with ether. The ether phase was extracted repeatedly with 0.1N $NaHCO_3$. Acidification of the alkaline extract with 10% HCl yielded a precipitate that was collected by filtration, thoroughly washed with water, dried and recrystallized from ethyl acetate-petroleum ether, yielding 0.27 g (42%) of white crystals with m.p. 148-150°, M^+ at m/e 404. Anal. calc. for $C_{23}H_{32}O_4S$: C, 68.29; H, 7.97; found: C, 67.97; H, 7.92. The product moved as a single component on TLC (R_{st} 0.87 relative to 6-bromoprogesterone) and showed $\lambda_{max}^{ethanol}$ 245 nm (ϵ 10500); λ_{max}^{KBr} 5.83 (COOH), 5.91 (C=O), 6.03 (-C=C-CO); NMR δ ($CDCl_3$) 0.66 (18-CH_3), 1.22 (19-CH_3), 2.12 (21-CH_3), 3.17-3.82 (C6-H). The proton at position 4 did not appear in the vinylic region of the NMR spectrum. We tend to attribute this to an intramolecular interaction with the substituent at C-6. A rearrangement of the substituent from C-6 to C-4 seems unlikely in view of the appearance of the broad band between 3.17-3.82 δ which can be assigned to a methine proton at C-6.

11α-Hydroxyprogesterone hemisuccinate (P-11-HS) was initially prepared using succinic anhydride in pyridine according to the general method of Buzby et al. (12), m.p. 150-153°, $\lambda_{max}^{0.1N\ NaOH}$ 243 nm (ϵ 15200). Later this compound was generously made available by Dr. A. Schuurs of N.V. Organon, Oss, Holland.

Coupling of haptens to BSA. (i) Estrogens. - A mixture of E_2-6CMO (100 mg) or E_3-6CMO (100 mg) in dioxane (10 ml), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (100 mg) in water (5 ml) and crystalline BSA (200 mg) in 0.05N phosphate buffer, pH 7.8 (10 ml) was stirred for 20 h at room temperature and then dialyzed against water for 48 h. The retentate was centrifuged (12 000 x g, 15 min) and the supernatant was lyophilized, yielding 150 mg of fluffy material. Examination by TLC did not reveal any free hapten in this material. The amount of hapten in each preparation was calculated from the absorbancy in 0.1N NaOH at 340 nm and also from the amount of radioactivity due to ^{14}C per unit weight. The two estimates were in good agreement. E_2-6CMO-BSA was found to contain 10.2% E_2-6CMO, indicating that on the average 20 residues of hapten were attached to each BSA molecule. The corresponding molar ratio for E_3-6CMO-BSA was only 8 : 1.

(ii) Progesterone-6-CMTE-BSA. - P-6-CMTE (100 mg) in dimethylformamide (9 ml) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (100 mg) in water (10 ml) were stirred for 20 min before adding a solution of BSA (200 mg) in water (10 ml). The mixture was stirred for 3 days at 4°C (a precipitate formed). The material was then dialyzed for 1 day against a 0.05M solution of $NaHCO_3$ (pH 8), during which time the precipitate redissolved, and 4 days against water. The retentate was lyophilized and examination by TLC indicated it was free of unbound steroid. The product contained, on the average, 30 residues of P-6-CMTE per molecule of BSA.

(iii) Progesterone-11-HS-BSA was prepared by condensing P-11-HS with BSA as described for (ii), except that BSA was added to the reaction mixture in 0.05M phosphate buffer, pH 7.8. The absorbance of the product at 243 nm in 0.1N NaOH indicated that it contained, on the average, 28 residues of P-11-HS per carrier molecule.

Immunological procedures

Immunization. - Antigen (steroid-BSA conjugate, 5 to 15 mg per animal) was dissolved in 2 ml saline and emulsified with an equal volume of complete Freund's adjuvant (CFA). This emulsion was injected once into multiple intradermal and subcutaneous sites along both sides of the back of 4-month-old male rabbits. The rabbits were bled weekly from the marginal ear vein, starting two weeks after the injection. Goats (mature females, intact or ovariectomized) received 4 subcutaneous injections of 3 mg antigen emulsified in CFA at weekly intervals, followed by booster injections at 6 to 7 week intervals. Blood samples were drawn from the jugular vein 5 weeks after the first injection and two weeks after each booster injection. Undiluted sera were stored at 4°C for up to 9 months.

Radioimmunoassay. - Sera were diluted with 0.05M Tris-HCl buffer (pH 8.0) containing 0.1M NaCl and 0.1% NaN_3 to the extent required, so that 40-45% of a fixed amount of the homologous tritiated steroid (12 - 18 pg) was bound to antibody, as indicated by a preliminary titration. To 0.4 ml lots of the diluted serum placed in 10 x 75 mm disposable test tubes, varying

amounts (0.5×10^{-11} to 10^{-8} g) of unlabeled hapten or of heterologous steroids were added in 0.1ml of the same buffer (10 µg/ml ethanolic solutions of the cold steroids were diluted with buffer to the required concentration). This mixture was incubated for 30 min at 0°C before adding a fixed amount (12 - 18 pg) of the homologous tritiated steroid in 0.1 ml Tris buffer, and then kept for another 3 h at 0°C. (This "pre-emptive" method of adding the cold steroid or unknown sample to the antiserum before the labeled steroid in our hands slightly enhances the sensitivity of the assay, compared to the "equilibrium" technique of adding the two steroid species simultaneously). The remaining free steroid was then removed by adding 0.1 ml of a suspension of dextran-coated charcoal in Tris buffer (0.5% w/v Norit A activated charcoal and 0.05% w/v Dextran T20), stirring for 10 min at 0°C and centrifugation at 2 200 x g for 20 min at 4°C. A portion (0.5 ml) of the supernatant was withdrawn into a counting vial containing Insta-Gel (Packard Instrument Co.) for determination of the bound radioactive steroid by liquid scintillation counting.

RESULTS

Antisera to estrogenic conjugates

Radioimmunoassay calibration curves. - Sera of rabbits immunized with E_2-6-CMO-BSA, diluted 1 : 8 000 to 1 : 12 000, bound 40 - 45% of added tritiated estradiol-17β (18 pg), under the assay conditions described. Addition of 5 to 10 pg of pure estradiol-17β (E_2) produced significant inhibition of the binding of the labeled steroid; the degree of inhibition increased with the amount of cold steroid added over the range of 10 to 100-200 pg (Fig. 3). The potential sensitivity and working range of the assay using antisera to E_2-6-CMO-BSA is thus comparable to that obtainable with antisera to E_2-17-HS-BSA (2). The actual sensitivity and precision of the assay when applied to biological material is still under investigation.

Specificity in relation to site of conjugation. - Antisera raised in goats with E_2 coupled to BSA through carbon-17 (E_2-17-HS-BSA; ref. 12) showed equal affinity for E_2 and estrone (E_1), and showed 10% cross-reaction

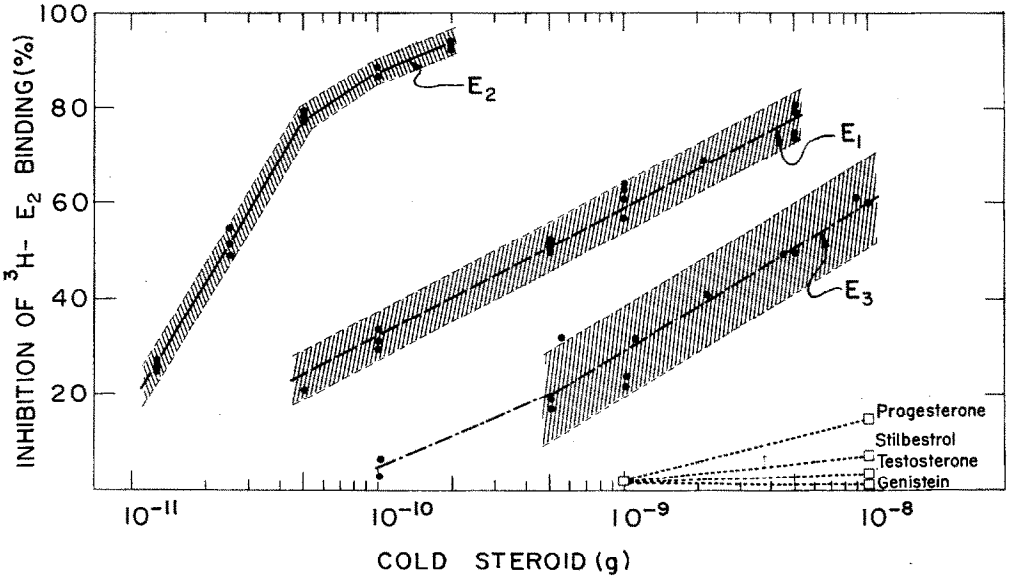

Figure 3. Effect of various steroids and non-steroidal estrogens on the binding of tritiated estradiol (12 pg) by rabbit anti-E_2-6-CMO-BSA (1 : 9 000 dilution). Shaded areas represent 95% fiducial limits of the regression lines. E_1, estrone; E_2, estradiol-17β; E_3, estriol.

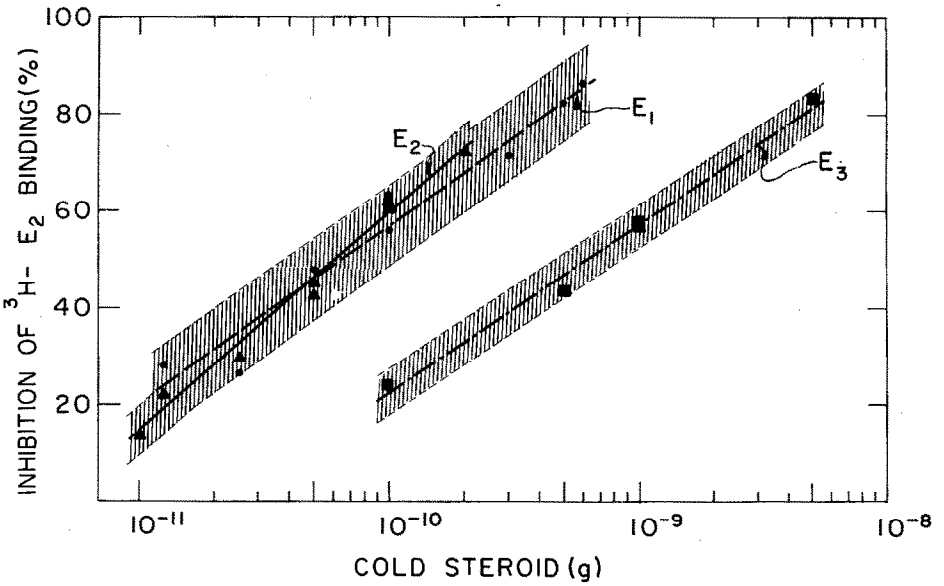

Figure 4. Effect of unlabeled estradiol (▲), estrone (●) and estriol (■) on the binding of tritiated estradiol (12 pg) by goat anti-E_2-17-HS-BSA serum (1 : 18 000 dilution). Shaded areas represent 95% fiducial limits of the regression lines. Abbreviations, as in Fig. 3.

TABLE 1

Comparison of specificity of antisera raised with different estrogen conjugates

COMPOUND	CROSS-REACTION (%)*		
	ANTI E_2-6-CMO-BSA		ANTI E_2-17-HS-BSA
	(Rabbit)	(Goat)	(Goat)
Estradiol-17 β	100	100	100
Estrone	4.6	8.0	100
Estradiol-17 α	1		60 (ref. 2)
Estriol	0.4	< 0.3	10
Stilbestrol, Genistein	< 0.2		< 0.2
Testosterone, Progesterone	< 0.2		⩽ 0.2

*Defined (2) as $\frac{100\ X}{Y}$, where X is the mass of unlabeled estradiol and Y the mass of the heterologous compound required to produce 50% inhibition of the binding of tritiated estradiol by antibody. For abbreviation of antigen formulae see Methods section.

with estriol (E_3). Representative inhibition curves are shown in Fig. 4. Marked cross-reaction (60%) of such sera with estradiol-17α (E_2-17α) has also been reported (2). By contrast, antisera to E_2-6-CMO, whether produced in goats or in rabbits, clearly distinguished E_2 from E_1 and E_2-17α, and showed much less cross-reaction (0.4%) with E_3 (Fig. 3 & Table 1). Conversely, sera to E_3-6-CMO showed minimal cross-reaction with E_2 or E_1 (< 3%). Antisera to E_2-6-CMO-BSA and to E_2-17-HS-BSA both failed to cross-react significantly with the non-phenolic steroids testosterone and progesterone, and with the non-steroidal estrogens diethylstilbestrol and genistein (5, 7, 4'-trihydroxyisoflavone, a phyto-estrogen).

The degree of cross-reaction has been expressed numerically in Table 1 on the basis of the mass of each steroid required to produce 50% inhibition of the binding of the labeled hapten as proposed by Thorneycroft et al. (2). These values must be regarded as approximations only, since the inhibition curves of heterologous steroids were not always parallel to that of E_2. Furthermore, the extent of cross-reaction with heterologous estrogens could be reduced somewhat by equilibrating the antiserum with the steroid at room temperature or at 37°C, instead of 0°C as in the routine procedure.

Antisera to progesterone

Radioimmunoassay calibration curves. - Rabbit antisera to P-6-CMTE-BSA used at 1 : 9 000 dilution gave useful radioimmunoassay calibration curves with pure progesterone over the range 10 - 200 pg (Fig. 5); an approximately linear relationship obtained between the log

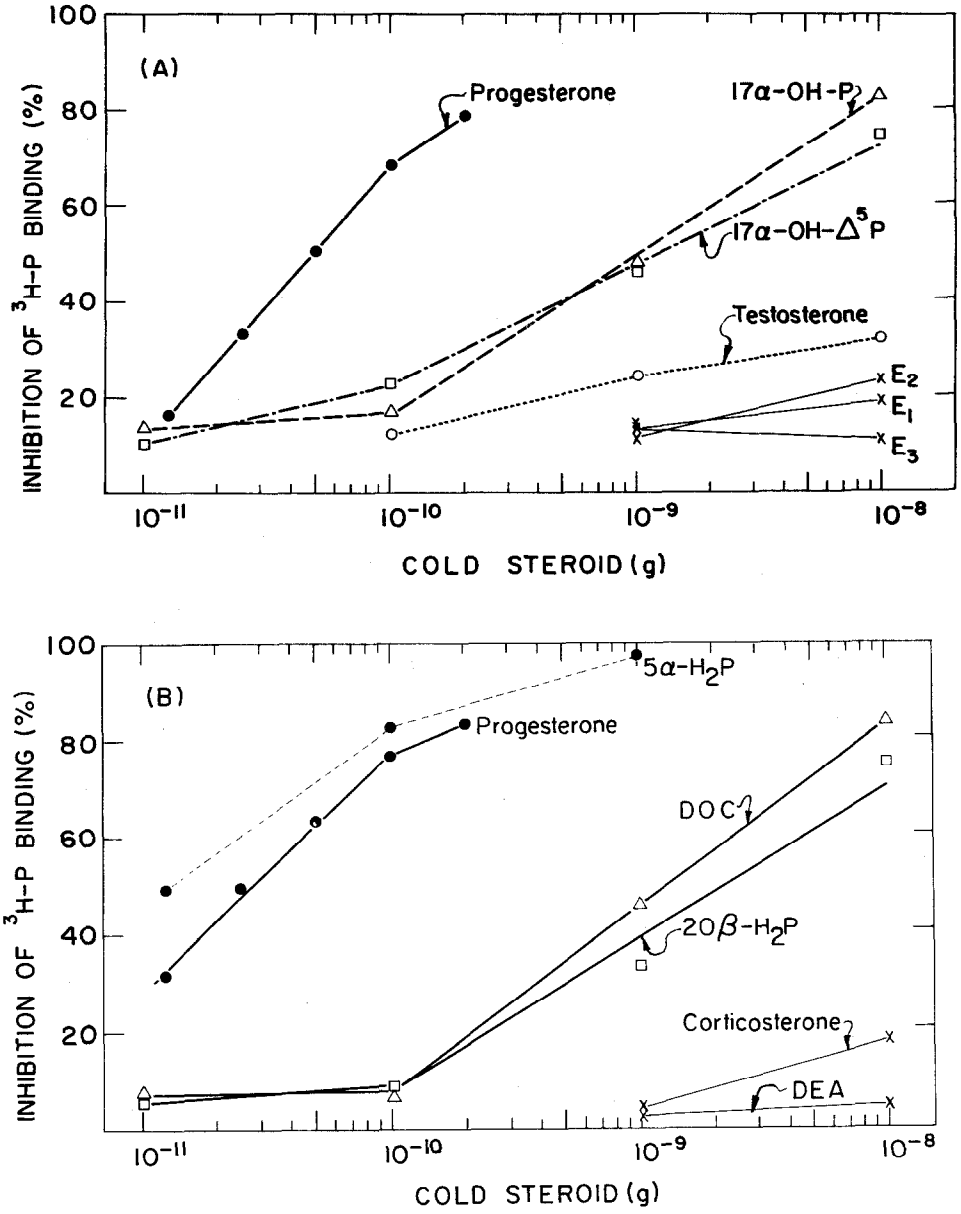

Figure 5. Effect of various steroids on the binding of tritiated progesterone (12 pg) by rabbit anti-P-6-CMTE-BSA serum. A, 1 : 7500 dilution B, 1 : 9000 dilution. Abbreviations: ^{3}H-P = tritiated progesterone; 17α-OH-P = 17α-hydroxy-pregn-4-ene-3, 20-dione; 17α-OH-Δ^{5}-P = 17α-hydroxy-pregn-5-ene-3, 20-dione; 5αH_2P = 5α-pregnane-3, 20-dione; DOC = 21-hydroxypregn-4-ene-3, 20-dione; 20β-H_2P = 20β-hydroxy-pregn-4-ene-3-one; DEA = 3β-hydroxy-androst-5-ene-17-one.

TABLE 2

Comparison of specificity of antisera raised in rabbits with different progesterone conjugates

COMPOUND	CROSS-REACTION (%)*	
	ANTI P-6-CMTE-BSA	ANTI P-11-HS-BSA
Progesterone	100	100
5α-Pregnane-3, 20-dione	≥ 100	3
5β-Pregnane-3, 20-dione	≤ 100	
3β-Hydroxypregn-5-en-20-one	8-14	< 0.3
17α-Hydroxypregn-4-ene-3, 20-dione	4	2
17α-Hydroxypregn-5-en-20-one	3	
20α-Hydroxypregn-4-en-3-one	1-2	8
20β-Hydroxypregn-4-en-3-one	1-2	
11-Deoxycorticosterone	1-2	8.5
11α-Hydroxypregn-4-ene-3, 20-dione	0.4	4.1
Testosterone	< 0.3	
Corticosterone Dehydroepiandrosterone	< 0.3	0.4
Estradiol-17β Estrone Estriol	< 0.3	< 0.3

* See definition under Table 1. For abbreviation of antigen formulae see Methods section.

of the amount of cold progesterone added and percentage inhibition of ^{3}H-progesterone binding by antibody over the range 1 - 100 pg. Antisera to P-11-HS-BSA (1 : 20 000) yielded similar standard curves.

Specificity in relation to site of conjugation. - The extent of cross-reaction with other steroids of the antiserum produced by immunization with P-6-CMTE-BSA is shown in Fig. 5. The specificity of this antiserum to progesterone is compared in Table 2 with that of antisera produced by immunization with P-11-HS-BSA.

DISCUSSION

This paper describes the preparation of antigenic conjugates of a number of ovarian hormones in which the steroid is attached to the carrier protein through its carbon number 6 or 11, and the properties of antibodies elicited by such conjugates. This mode of conjugation was intended to preserve the substituents of rings A and D, including the 17-sidechain if present, as part of the antigenic determinant.

In the case of the estrogens, it seems clear that 6-conjugated antigens indeed give rise to antibodies that are better able to recognize changes in the chemistry of the D-ring than are antibodies raised with the conventional estradiol-17β -hemisuccinate complex. Estrone was readily distinguished from estradiol-17β, and cross-reaction with estradiol-17α and estriol was much reduced (from 60 to 1% for $E_2 17^{\alpha}$ and from 10 to 0.4% for E_3). Furthermore, the anti-estriol-6-CMO serum reacted only feebly with estrone and estradiol-17β, in agreement with a recent report by Kuss & Goebel (13). These authors could distinguish estriol not only from

estrone and estradiol, but also from its 16- and 17-epimers, by use of an antiserum of this type. Specificity for the phenolic A-ring was retained in the sera to 6-conjugates.

The anti-estradiol sera failed to cross-react with non-steroidal estrogens, such as stilbestrol (cf. 14) and the isoflavone genistein. Conversely, antibodies to genistein do not cross-react with steroidal estrogens (15), though both classes of estrogen compete for binding sites on the receptor protein of uterine cytosol (16).

The new method described for coupling progesterone to protein through carbon-6 is likely to be applicable to other 4,5-unsaturated 3-ketosteroids, and its use in the development of an immunoassay of testosterone is currently being explored. When applied to known amounts of pure progesterone, the radioimmunoassay, using either the 6- or the 11-conjugate of progesterone, was more sensitive by an order of magnitude than the competitive protein binding assay using transcortin. However, radioimmunoassay procedures for progesterone of equal sensitivity have recently been proposed by other authors (17, 18) and need be considered.

Compared to antisera to progesterone coupled to BSA through carbon-20 (data from ref. 19), the antiserum to P-6-CMTE-BSA is markedly superior in specificity with respect to changes about the D-ring and the 17-sidechain: it exhibits less cross-reaction towards testosterone (0.3% vs. 95.2%), deoxycorticosterone, 20β-hydroxypregn-4-en-3-one (1-2% vs. 96.5%) and 17α-hydroxyprogesterone (4% vs. 98.1%); but it is somewhat less specific than antibodies to the 20-conjugate towards changes

about the A-ring, as indicated by its cross-reaction with 3β-hydroxypregn-5-en-20-one (8% vs. 1.1%) and its strong cross-reaction with 5α and 5β-pregnane-3, 20-dione (about 100%).

Sera produced with conjugates of progesterone linked to BSA through carbon-3 likewise show marked cross-reaction with 5α-pregnane-3, 20-dione (51%, ref. 18) and 3β-hydroxypregn-5-en-20-one (7.3%, ref. 18; 13%, ref. 19), and are rather similar to our anti-P-6-CMTE-BSA sera in their ability to recognize changes about the 17- sidechain.

Another convenient way of coupling progesterone to protein while leaving the A- and D- rings fully exposed is via carbon-11. This was achieved in the course of a systematic investigation, motivated by arguments similar to our own, by Niswender & Midgley (17), who used the chloro-carbonate derivative (Schotten-Baumann reaction), and by Lindner et al. (ref. 4 and present results) via the 11α-hemisuccinate. The principal feature of the antibodies elicited by this type of antigen, as compared with those produced with conjugates through carbon-6 (Table 2) or carbon-3 (18, 19), is an improved recognition of changes about the A-ring, indicated by the greatly reduced cross-reaction with 5α-pregnane-3, 20-dione and with 3β-hydroxypregn-5-en-20-one. At the same time, specificity towards the D-ring and 17-sidechain appears superior to that of sera raised with the 20-conjugate (19).

The relative merit in a radioimmunoassay procedure of the four types of antisera to progesterone now available will also depend on the relative concentration of potentially cross-reacting steroids in the biological material

to be examined, and the relative ease with which these can be eliminated during the initial extraction or purification steps. For instance, it has been suggested that 5α- and 5β- pregnanediones and 3β-hydroxypregn-5-en-20-one are absent or have very low concentrations in the peripheral plasma of normal men and women (18).

The fact that antisera to the 11-conjugates of progesterone cross-react to some extent with 11α-hydroxyprogesterone (cf. 19) demonstrates that the functional group used for coupling the hapten to the carrier is not entirely deprived of its function as antigenic determinant. Nevertheless, the generalization appears to hold that functional groups remote from the coupling site are more potent determinants.

The results presented confirm our view (4, 5, 15) that the site of attachment of steroids to the peptide carrier importantly affects the specificity of the antibodies produced. Coupling through carbon-6 or carbon-11 made it possible to obtain antibodies that discriminate more efficiently between closely related gonadal steroids of differing hormonal potency than do those produced by conventional antigens (2, 9).

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