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Preparation and Antigenic Properties of 5α-Dihydrotestosterone-15α-Bovine Serum Albumin Conjugate[1]

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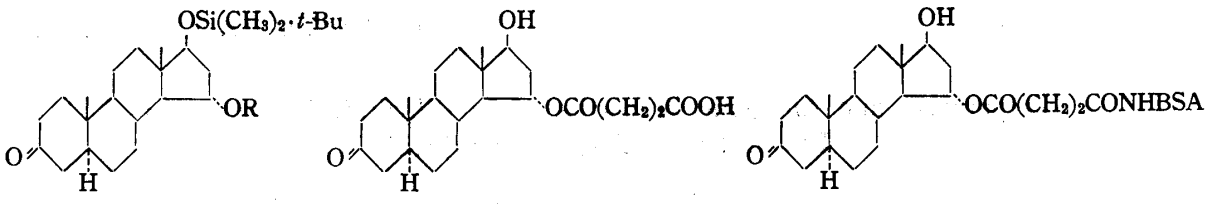
In order to obtain the specific antiserum for the use of radioimmunoassay of 5α-dihydrotestosterone a new hapten-carrier conjugate was prepared from 15α-hydroxy-5α-dihydrotestosterone 15-hemisuccinate by coupling with bovine serum albumin employing the mixed anhydride technique. The specificity of anti-5α-dihydrotestosterone antiserum elicited in the rabbit by immunization with this antigen was tested by cross-reaction studies with the closely related steroids. The results indicated that specific antiserum which is capable of differentiating 5α-dihydrotestosterone from testosterone and 5α-androstane-3β,17β-diol to a certain extent would be produced by antigen whose steroidal moiety is linked to an immunogenic protein through the position remote from the inherent functional groups on the steroid nucleus.

Keywords——radioimmunoassay; 5α-dihydrotestosterone; 15α-hydroxy-5α-dihydrotestosterone 15-hemisuccinate; mixed anhydride method; 5α-dihydrotestosterone-BSA conjugate; anti-5α-dihydrotestosterone antiserum; cross-reactivity

Several attempts have been made on the preparation of anti-5α-dihydrotestosterone antisera for the use of radioimmunoassay by immunization with hapten-carrier protein conjugates. Antisera so far obtained, however, are not yet satisfactory in respect of the specificity. It is sufficiently substantiated that the site through which the steroid molecule is linked to an immunogenic carrier influences the specificity of antibody raised against the hapten-carrier protein conjugate. As a series of our studies on the preparation of more specific antiserum for the use of immunoassay of steroid hormones, we have attempted to couple 5α-dihydrotestosterone hapten to bovine serum albumim (BSA) through the 15α position remote from the principal antigenic determinant in ring A without disturbing the β-side of a steroid molecule.[3] The present paper describes the synthesis of 15α-hydroxy-5α-dihydrotestosterone 15-hemisuccinate as a new hapten, preparation of its BSA conjugate, production of anti-5α-dihydrotestosterone antibody in the rabbit and specificity of this antiserum for 5α-dihydrotestosterone in the radioimmunoassay procedure.

An initial project was directed to the synthesis of 15α-hydroxy-5α-dihydrotestosterone 15-hemisuccinate. For this purpose 17β-*tert*-butyldimethylsilyloxy-15α-hydroxy-5α-androstan-3-one acetate (**1**) whose preparation has been reported in the previous paper,[4] was chosen as a pertinent starting material. Hydrolysis with potassium hydroxide in methanol–tetrahydrofuran under mild conditions provided the 15α-hydroxylic compound (**2**). Being refluxed with succinic anhydride in pyridine, **2** was transformed into the 15-hemisuccinate (**3**). On brief exposure to hydrogen chloride in aqueous acetone elimination of the protecting group at C-17 was readily attained to provide the desired 15α-hydroxy-5α-dihydrotestosterone 15-hemisuccinate (**4**) in a satisfactory yield.

1) Part CXLI of "Studies on Steroids" by T. Nambara; Part CXL: M. Numazawa, T. Tanaka, and T. Nambara, *Clin. Chim. Acta*, in press.
2) Location: *Aobayama, Sendai, 980, Japan.*
3) T. Nambara, H. Hosoda, K. Tadano, K. Yamashita, and N. Chino, *Chem. Pharm. Bull.* (Tokyo), **25**, 2969 (1977).
4) H. Hosoda, K. Yamashita, K. Tadano, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **25**, 2650 (1977).



1: R=$COCH_3$
2: R=H
3: R=$CO(CH_2)_2COOH$

4 5

Chart 1

The steroid hemisuccinate was covalently coupled to BSA by the mixed anhydride method[5] yielding 5α-dihydrotestosterone-15α-BSA conjugate (**5**). Number of steroid molecules bound to each BSA molecule was determined by the use of [1,4-^{14}C]succinic anhydride for the formation of the 15-hemisuccinate. Counting of radioactivity incorporated into the conjugate revealed that 31 molecules of the steroid hapten were joined to each BSA molecule in **5**.

Rabbtis were immunized with the 5α-dihydrotestosterone-15α-BSA conjugate emulsified with complete Freund's adjuvant. The titer was determined from the ability of antibody to bind a certain amount of ^{3}H-5α-dihydrotestosterone. The antibody was tested after each bleeding at dilutions to determine the 50% binding level. A serum sample obtained from the rabbit immunized with the hapten-carrier conjugate for six months showed remarkably increased activity to 5α-dihydrotestosterone.

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[6] in Fig. 1. The antiserum raised in the rabbit exhibited the high affinity for 5α-dihydrotestosterone with the association constant of $3.05\times10^9\,\text{M}^{-1}$. The dose-response curves were constructed with 1:20000 dilution of anti-5α-dihydrotestosterone antiserum. When logit transformation was used to construct the curves, plots of logit per cent bound radioactivity *vs.* logarithm of the amount of unlabeled 5α-dihydrotestosterone and three cross-reacting steroids, testosterone, 5α-androstane-3β, 17β-diol and epidihydrotestosterone (17α-hydroxy-5α-androstan-3-one) showed a linear relationship, respectively (Fig. 2).

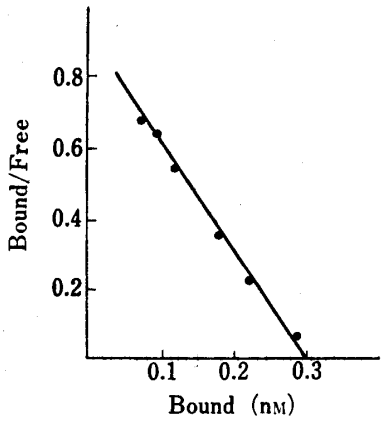


Fig. 1. Scatchard Plot for Anti-5α-dihydrotestosterone Antiserum
$Ka=3.05\times10^9\,\text{M}^{-1}$.

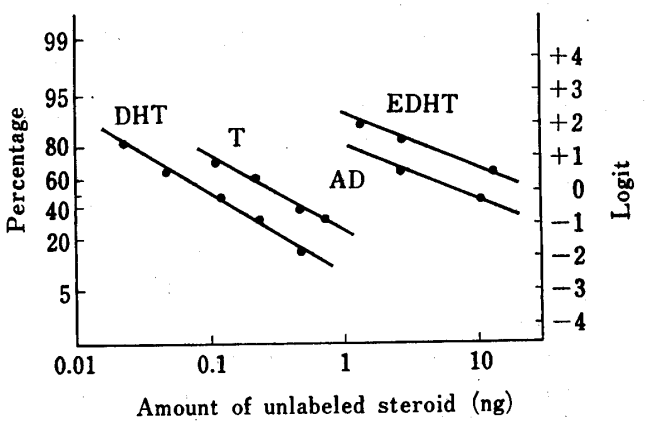


Fig. 2. Dose-Response Curves for 5α-Dihydrotestosterone (DHT) and Three Cross-Reacting Steroids, Testosterone (T), 5α-Androstane-3β,17β-diol (AD) and Epidihydrotestosterone (EDHT)

5) B.F. Erlanger, F. Borek, S.M. Beiser, and S. Liebermann, *J. Biol. Chem.*, **228**, 713 (1957).
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The specificity of antiserum was assessed by testing the ability of selected steroids to compete with ^{3}H-5α-dihydrotestosterone for binding sites on the antibody. The per cent cross-reaction was determined according to the method of Abraham.[7] The results on cross-reactivities of anti-5α-dihydrotestosterone antiserum with 21 kinds of closely related compounds are listed in Table I. The antiserum proved to be sufficiently specific with a single exception of testosterone which showed a 33% cross-reaction. 5β-Dihydrotestosterone exhibited only 0.48% of the activity relative to 5α-dihydrotestosterone. It is of interest that 5α-androstane-3α,17β-diol provided a low value (0.20%) while its C-3 epimer a somewhat higher level of cross-reactivity (1.6%). Other steroids that showed minor cross-reactivity include androstenedione (0.51%), androstanedione (0.98%) and epidihydrotestosterone (0.27%).

Table I. Per Cent Cross-Reaction of Anti-5α-dihydrotestosterone Antiserum with Selected Steroids

Steroid	Cross-reaction (%)	Steroid	Cross-reaction (%)
5α-Dihydrotestosterone	100	5-Androstene-3β,17β-diol	0.054
5β-Dihydrotestosterone	0.48	5α-Androstane-3,17-dione	0.98
17α-Hydroxy-5α-androstan-3-one	0.27	4-Androstene-3,17-dione	0.51
Testosterone	33	5α-Pregnane-3,20-dione	0.16
Epitestosterone	0.069	Progesterone	0.16
Androsterone	0.006	Deoxycorticosterone	0.22
Epiandrosterone	0.13	Cortisol	0.002
Dehydroepiandrosterone	0.009	Estrone	<0.001
5α-Androstane-3β,17β-diol	1.6	Estradiol	<0.001
5α-Androstane-3α,17β-diol	0.20	Estriol	<0.001
5β-Androstane-3α,17β-diol	0.001	Cholesterol	<0.001

Numerous studies have been carried out on the preparation of anti-5α-dihydrotestosterone antisera with conjugates obtained by coupling the steroid to an immunogenic carrier through the C-1,[8–10] C-3,[11–13] C-6,[10,14] C-7,[10,15] C-11,[16] C-15,[17] or C-19[18] position. The antisera raised against the 5α-dihydrotestosterone-15-BSA conjugate are much more specific for 5α-dihydrotestosterone than those elicited with antigens whose haptens are linked to a carrier protein through the positions other than C-15. As for the cross-reactivities of antisera with testosterone Rao *et al.*[17] demonstrated that immunization with the 15β-carboxyethylmercapto-5α-dihydrotestosterone-BSA conjugate in a group of five rabbits produced highly specific antisera exhibiting cross-reactions with testosterone in the range of 7.78% to 33.17%. In addition, Condom *et al.*[19] reported that anti-testosterone antisera differentiating the saturated 3-ketosteroid were elicited in rabbits with the 15α-carboxymethyltestosterone-protein conjugate. The present results together with the previous findings reveal that specific anti-

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9) S. Bauminger, F. Kohen, H.R. Lindner, and A. Weinstein, *Steroids*, **24**, 477 (1974).
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18) J.A. Bermùdez, V. Coronado, A. Mijares, C. Leòn, A. Velàzquez, P. Noble, and J.L. Mateos, *J. Steroid Biochem.*, **6**, 283 (1975).
19) R. Condom and B. Desfosses, *J. Steroid Biochem.*, **8**, 1165 (1977).

serum which is capable of discriminating 5α-dihydrotestosterone from the closely related C_{19} steroids can be produced with the conjugate obtained by coupling the hapten to an immunogenic carrier through the C-15 position. It seems likely that there would be no substantial difference in the cross-reactivity with testosterone between antisera generated with the 15α-BSA and 15β-BSA conjugates. Further studies in progress will provide the more precise knowledge on this problem.

Experimental

Synthesis of Hapten[20]

17β-*tert*-Butyldimethylsilyloxy-15α-hydroxy-5α-androstan-3-one (2)——To a solution of 17β-*tert*-butyldimethylsilyloxy-15α-hydroxy-5α-androstan-3-one acetate (**1**)[4] (340 mg) in MeOH (4 ml)–tetrahydrofuran (2 ml) was added 30% KOH (1 ml) and stirred at room temperature for 4 hr. After usual work-up the crude product was recrystallized from MeOH to give **2** (295 mg) as colorless needles. mp 144—146°. $[\alpha]_D^{15}$ +56.3° (c=0.08). NMR (CCl_4) δ: 0.02 (6H, s, 17-OSi$(CH_3)_2$), 0.70 (3H, s, 18-CH_3), 0.87 (9H, s, 17-OSi-*t*-Bu), 1.01 (3H, s, 19-CH_3), 3.6—4.1 (2H, 15β- and 17α-H). *Anal.* Calcd. for $C_{25}H_{44}O_3Si$: C, 71.38; H, 10.54. Found: C, 71.28; H, 10.65.

17β-*tert*-Butyldimethylsilyloxy-15α-hydroxy-5α-androstan-3-one Hemisuccinate (3)——To a solution of **2** (34 mg) in pyridine (2 ml) was added succinic anhydride (70 mg) and refluxed for 28 hr. After removal of pyridine by evaporation under reduced pressure the residue obtained was diluted with ether and filtered. The filtrate was washed with NaCl solution and water, dried over anhydrous Na_2SO_4 and evaporated. The residue was purified by preparative TLC using $CHCl_3$–MeOH (10: 1) as developing solvent to give **3** (35 mg) as colorless oil. NMR ($CDCl_3$) δ: 0.01 (6H, s, 17-OSi$(CH_3)_2$), 0.74 (3H, s, 18-CH_3), 0.86 (9H, s, 17-OSi-*t*-Bu), 0.99 (3H, s, 19-CH_3), 2.57 (4H, s, $-COCH_2CH_2CO-$), 3.72 (1H, t, J=8 Hz, 17α-H), 4.88 (1H, m, 15β-H). The crude product was submitted to further elaboration without purification.

15α,17β-Dihydroxy-5α-androstan-3-one 15-Hemisuccinate (4)——To a solution of **3** (35 mg) in acetone (2 ml) was added 5 N HCl (0.3 ml) and allowed to stand at room temperature for 2 hr. The resulting solution was diluted with H_2O (1 ml) and acetone was evaporated under a stream of N_2 gas. After addition of MeOH (5 ml) the solution was extracted with AcOEt. The organic layer was washed with NaCl solution and H_2O, dried over anhydrous Na_2SO_4 and evaporated. The residue obtained was recrystallized from AcOEt to give **4** (30 mg) as colorless needles. mp 177.5—179°. $[\alpha]_D^{15}$ +61.3° (c=0.08). NMR ($CDCl_3$) δ: 0.76 (3H, s, 18-CH_3), 1.01 (3H, s, 19-CH_3), 2.52 (4H, s, $-COCH_2CH_2CO-$), 3.74 (1H, t, J=8 Hz, 17α-H), 4.85 (1H, m, 15β-H). *Anal.* Calcd. for $C_{23}H_{34}O_6$: C, 67.95; H, 8.43. Found: C, 68.10; H, 8.64.

In the similar fashion the ^{14}C-labeled hapten (**4**) was prepared from **2** by the use of [1,4-^{14}C]succinic anhydride.

Conjugation of Hapten with BSA——To a solution of the ^{14}C-labeled hapten (1000 dpm/mg) (30 mg) in dry dioxane (0.7 ml) were added tributylamine (0.02 ml) and isobutyl chlorocarbonate (0.01 ml) at 11° and stirred for 30 min. To this solution was added BSA (90 mg) in H_2O (2.2 ml)–dioxane (1.4 ml) containing 1 N NaOH (0.08 ml) under ice-cooling and stirred for 3 hr. The resulting solution was dialyzed against cold running water overnight and the turbid protein solution was brought to pH 4.5 with 1 N HCl. After being allowed to stand at 4° overnight the suspension was centrifuged at 3000 rpm for 20 min. The precipitate was dissolved in 5% $NaHCO_3$ and dialyzed in the manner as described above. Lyophilization of the solution gave 5α-dihydrotestosterone-15α-BSA conjugate (**5**) (160 dpm/mg) (95 mg) as fluffy powder.

Materials——[1,2-^3H]5α-Dihydrotestosterone (40 Ci/mmol) and [1,4-^{14}C]succinic anhydride (9.32 mCi/mmol) were supplied by New England Nuclear Co. (Boston, Ma.) and used without purification. BSA (crystallized) and complete Freund's adjuvant were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Iatron Laboratories (Tokyo), respectively. Unlabeled steroids were prepared in these laboratories. All solvents and chemicals used were of analytical grade.

Measurement of Radioactivity——The samples were counted on a Packard Tri-Carb Model 3380 liquid scintillation spectrometer employing the modified Bray's scintillant, composed of 2,5-diphenyloxazole (4 g), 1,4-bis[2-(5-phenyloxazolyl)]benzene (200 mg), naphthalene (60 g) and sufficient dioxane to make the total volume 1 l.

Immunization of Rabbits——Two domestic strain female albino rabbits weighing 2.5—3.0 kg were used for immunization. The antigen (1 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with

20) All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were determined in $CHCl_3$ solution. Nuclear magnetic resonance (NMR) spectra were run on a JEOL Model PS-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard. Abbreviation used s=singlet, t=triplet and m=multiplet. For preparative thin-layer chromatography (TLC) silica gel H (E. Merck AG, Darmstadt) was used as an adsorbent.

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 1 month and then once a month. The rabbits were bled 3 weeks after the booster injection. The sera were separated by centrifugation at 3000 rpm for 10 min and stored at 4°. Antisera were used in the assay at a final dilution of 1: 20000.

Assay Procedure——All dilutions of the standard, tracer and antiserum were performed in 0.01 M phosphate buffer (pH 7.4) containing 0.1% gelatin, 0.9% NaCl and 0.01% NaN_3. To a series of standard solution (0, 11, 23, 45, 113, 227, 454 and 907 pg of 5α-dihydrotestosterone) in buffer (0.1 ml) were added ^{3}H-5α-dihydrotestosterone (*ca.* 8000 dpm, 0.5 ml) and diluted antiserum (0.1 ml) and incubated at 4° overnight. To the incubation mixture was added a dextran (0.06%)–charcoal (1%) suspension (0.5 ml), vortexed, allowed to stand at 0° for 10 min and then centrifuged at 4°, 2000 rpm for 10 min. The supernatant was transferred by decantation into a vial containing the scintillation solution (10 ml) and submitted to counting of radioactivity.

Cross-reaction Study——The specificity of antiserum raised against 5α-dihydrotestosterone-15α-BSA conjugate was tested by cross-reaction studies with 21 kinds of steroids related to 5α-dihydrotestosterone. The relative amounts required to reduce the initial binding of ^{3}H-5α-dihydrotestosterone by half where the mass of unlabled 5α-dihydrotestosterone was arbitrarily chosen as 100%, were calculated by the standard curves.

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