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Preparation and Antigenic Properties of Testosterone-15α-Protein Conjugate[1]

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In order to obtain the specific antiserum used for radioimmunoassay of testosterone a new hapten-carrier conjugate was prepared from 15α-hydroxytestosterone 15-hemisuccinate by coupling with bovine serum albumin employing the mixed anhydride technique. The specificity of anti-testosterone antiserum elicited in the rabbit by immunization with this antigen was tested by cross-reaction studies with the related steroids. The results indicated that specific antiserum which is capable of differentiating testosterone from 5α-dihydrotestosterone and androstenedione to a certain extent would be produced by antigen whose steroidal moiety is coupled to a protein through the position remote from the inherent functional groups.

Keywords——testosterone radioimmunoassay; hapten; 15α-hydroxytestosterone 15-hemisuccinate; mixed anhydride method; testosterone-15α-BSA conjugate; anti-testosterone antiserum; cross-reactivity

Since the first report by Nugent and his co-workers,[3] a number of attempts have been made on the preparation of anti-testosterone antisera used for radioimmunoassay by coupling the hapten to the carrier protein. The antisera so far obtained, however, are not yet satisfacory in respect of the specificity. It is sufficiently substantiated that the site through which the steroid molecule is conjugated with a carrier protein influences the specificity of the antibody raised against the hapten-protein conjugate. As a series of studies on the preparation of more specific antiserum for radioimmunoassay of steroid hormones,[4] we have attempted to couple the testosterone hapten to bovine serum albumin (BSA) through the 15α position remote from the principal antigenic determinant in ring A without disturbing the β-side of steroid molecule.[5] The present paper deals with the synthesis of 15α-hydroxytestosterone 15-hemisuccinate as a new hapten, the preparation of its BSA conjugate, the production of anti-testosterone antibody in the rabbit, and the specificity of this antiserum for testosterone in the radioimmunoassay procedure.

An initial effort was directed to the synthesis of 15α-hydroxytestosterone 15-hemisuccinate. For this purpose 5-androstene-3β,15α,17β-triol 17-*tert*-butyldimethylsilyl ether (**1**) whose preparation has been reported in a preceding paper,[7] was chosen as a pertinent starting material. Oppenauer oxidation with methyl ethyl ketone and aluminum isopropoxide occurred at C-3 with concomitant migration of the double bond to provide the Δ^4-3-ketone (**2**) in a fairly good yield. Being refluxed with succinic anhydride in pyridine, **2** was trans-

1) Part CXXVI of "Studies on Steroids" by T. Nambara; Part CXXV: T. Nambara, S. Ikegawa, T. Hirayama, and H. Hosoda, *Chem. Pharm. Bull.* (Tokyo), **25**, 3093 (1977).
2) Location: *Aobayama, Sendai.*
3) S. Furuyama, D.M. Mayes, and C.A. Nugent, *Steroids*, **16**, 415 (1970).
4) T. Nambara, M. Takahashi, Y. Tsuchida, and M. Numazawa, *Chem. Pharm. Bull.* (Tokyo), **22**, 2176 (1974); T. Nambara, M. Numazawa, Y. Tsuchida, and T. Tanaka, *Chem. Pharm. Bull.* (Tokyo), **24**, 1510 (1976).
5) After completion of this work the preparation of anti-testosterone antiserum by coupling the testosterone molecule to a protein through the 15β position has been reported.[6]
6) P.N. Rao and P.H. Moore, Jr., *Steroids*, **28**, 101 (1976).
7) H. Hosoda, K. Yamashita, K. Tadano, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **25**, 2650 (1977).

formed 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 yielding the desired 15α-hydroxytestosterone 15-hemisuccinate (**4**).

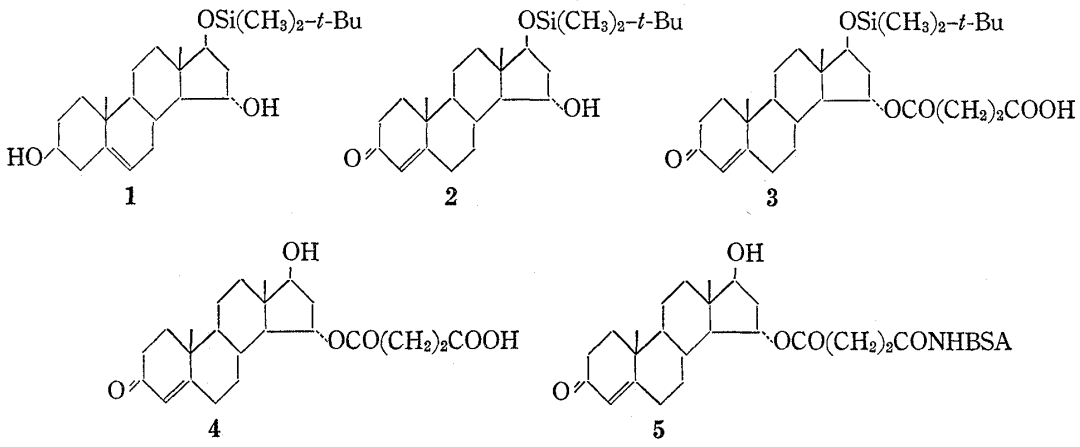

Chart 1

The steroid hemisuccinate was covalently linked to BSA yielding testosterone-15α-BSA conjugate (**5**) by the mixed anhydride method developed by Erlanger, *et al.*[8] As judged from the ultraviolet absorption due to the α,β-unsaturated ketone structure, it proved that satisfactory number of steroid molecules were joined to each BSA molecule in **5**.

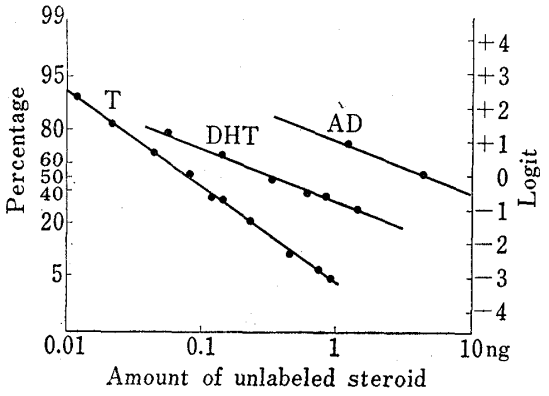

Fig. 1. Dose-Response Curves for Testosterone (T) and Two Cross-Reacting Steroids, 5α-Dihydrotestosterone (DHT) and Androstenedione (AD)

The rabbit was immunized with the testosterone-15α-BSA conjugate emulsified in Freund's complete adjuvant. The titer was determined from the ability of antibody to bind a certain amount of ^{3}H-testosterone. 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 significantly increased activity to testosterone. The dose-response curves were constructed with 1:210000 dilution of the antiserum raised against the testosterone-15α-BSA conjugate. When logit transformation was used to construct the curves, plots of logit per cent bound radioactivity *vs.* logarithm of the amount of unlabeled testosterone and two cross-reacting steroids, 5α-dihydrotestosterone and androstenedione, showed a linear relationship, respectively (Fig. 1).

The specificity of the resulting antiserum was assessed by ascertaining the ability of various related steroids to compete with ^{3}H-testosterone for binding to antibody. The per cent cross-reaction of antibody was determined according to the method of Abraham.[9] The results on cross-reactivities of anti-testosterone antiserum with 32 kinds of related compounds are listed in Table I. 5β-Dihydrotestosterone exhibited only 0.004% of the relative activity of testosterone and the four C-3 and C-5 epimeric androstanediols showed the values less than 0.1%. This antiserum, however, was incapable of discriminating 5α-dihydrotestosterone (22

8) B.F. Erlanger, F. Borek, S.M. Beiser, and S. Liebermann, *J. Biol. Chem.*, **228**, 713 (1957).
9) G.E. Abraham, *J. Clin. Endocrinol.*, **29**, 866 (1969).

%) clearly from the Δ^4-3-ketone. Androstenedione (1.97%) and progesterone (1.9%) showed somewhat high level of cross-reactivities. This result may be ascribable to their structural features at C-17 which are relatively close to the site used for conjugation of the testosterone molecule with BSA. The remarkable cross-reaction with 15α-hydroxytestosterone (22%) seems to be inevitable, since the hapten is coupled to a protein through a derivative at the 15α position. This novel metabolite, however, may not exert any significant influence on the determination of testosterone in the biological material because of its negligible amount.

Table I. Per Cent Cross-Reaction of Anti-Testosterone Antiserum with Selected Steroids

Steroid	Cross-reaction (%)	Steroid	Cross-reaction (%)
Testosterone	100	16α-Hydroxydehydroepiandrosterone	0.005
5α-Dihydrotestosterone	22	3β,17β-Dihydroxy-5-androsten-16-one	0.045
Androstenedione	1.97	5-Androstene-3β,15α,17β-triol	0.021
19-Nortestosterone	3.21	15α-Hydroxyandrostenedione	1.1
Epitestosterone	0.55	3α-Hydroxy-5β-androstan-17-one	0.012
Progesterone	1.9	3β-Hydroxy-5α-androstan-17-one	0.038
Dehydroepiandrosterone	0.028	5α-Androstane-3,17-dione	0.53
5-Androstene-3β,17β-diol	0.032	Adrenosterone	0.062
Androsterone	0.015	19-Norandrostenedione	0.14
5β-Dihydrotestosterone	0.004	Estrone	<0.001
5α-Androstane-3β,17β-diol	0.098	Estradiol	<0.001
5β-Androstane-3α,17β-diol	0.004	Estriol	<0.001
5α-Androstane-3α,17β-diol	0.052	Estetrol	<0.001
5β-Androstane-3β,17β-diol	0.031	Cholesterol	<0.001
15α-Hydroxytestosterone	22	Cortisol	0.009
15α-Hydroxydehydroepiandrosterone	0.006	Cortisone	0.009
16β-Hydroxydehydroepiandrosterone	0.004		

A number of investigators have carried out the preparation of specific antiserum by coupling the testosterone molecule to a carrier protein through the position C-3, C-6, C-7, C-11, C-17, or C-19.[10-20] The antiserum raised against the testosterone-15α-BSA conjugate is much more specific for testosterone than those elicited by antigens whose haptens are linked to the carrier protein through other positions than C-15. Rao, *et al.*, however, have recently reported that highly specific anti-testosterone antiserum was generated from both rabbits and sheep immunized with the 15β-carboxyethylmercaptotestosterone-BSA conjugate.[6] It is surprising that this specific antiserum for testosterone exhibited less than 2% cross-reaction with both 5α- and 5β-dihydrotestosterone. Although no plausible explanation is now available, it is of interest that a marked difference in the cross-reactivity with the related steroids, in particular 5α-dihydrotestosterone, was observed between antisera elicited by the 15α-BSA and 15β-BSA conjugates.

10) W.J. Riley, E.R. Smith, D.M. Robertson, and A.E. Kellie, *J. Steroid Biochem.*, **3**, 357 (1972).
11) H.R. Lindner, E. Perel, A. Friedlander, and A. Zeitlin, *Steroids*, **19**, 357 (1972).
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18) J.A. Bermúdez, V. Coronado, A. Mijares, C. León, A. Velázouez, P. Noble, and J.L. Mateos, *J. Steroid Biochem.*, **6**, 283 (1975).
19) C.D. Jones and N.R. Mason, *Steroids*, **25**, 23 (1975).
20) H. Sone, H. Yoshimasu, and M. Kojima, *Yakugaku Zasshi*, **96**, 199 (1976).

Most of radioimmunoassay procedures in current use require preliminary chromatographic separation to achieve its specificity. The availability of more specific antiserum described in this paper may eliminate the step for chromatographic separation, provided the concentration of 5α-dihydrotestosterone in the sample to be assayed is relatively low.

Experimental

Synthesis of Hapten[21]

15α,17β-Dihydroxy-4-androsten-3-one 17-*tert*-Butyldimethylsilyl Ether (2)——A solution of 5-androstene-3β,15α,17β-triol 17-*tert*-butyldimethylsilyl ether (1) (328 mg) and Al (iso-PrO)$_3$ (220 mg) in anhydrous benzene (50 ml) was concentrated to its half volume to remove the moisture. After addition of methyl ethyl ketone (10 ml) the reaction mixture was refluxed for 3 hr and concentrated. To this solution was added methyl ethyl ketone (10 ml) in anhydrous benzene (25 ml), refluxed for 4 hr, and then concentrated. The resulting solution was diluted with ether, washed with 25% Rochelle salt solution and H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product obtained was purified by preparative TLC using benzene–EtOH (13: 1) as developing solvent. Recrystallization of the eluate from aq. acetone gave **2** (200 mg) as colorless needles. mp 169—171.5°. $[\alpha]_D^{18}$ +110.3° (c=0.32). NMR (CCl_4) δ: 0.01 (6H, s, 17-OSi(CH_3)$_2$), 0.70 (3H, s, 18-CH_3), 0.86 (9H, s, 17-OSi-*t*-Bu), 1.18 (3H, s, 19-CH_3), 3.5—4.1 (2H, 15β- and 17α-H), 5.59 (1H, s, 4-H). *Anal.* Calcd. for $C_{25}H_{42}O_3Si$: C, 71.72; H, 10.11. Found: C, 71.64; H, 10.27.

17β-*tert*-Butyldimethylsilyloxy-15α-hydroxy-4-androsten-3-one Hemisuccinate (3)——To a solution of **2** (135 mg) in pyridine (9 ml) was added succinic anhydride (380 mg), refluxed for 21 hr, and then concentrated. The resulting solution was diluted with ether and the insoluble material was removed by filtration. The filtrate was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. Purification of the residue by preparative TLC using $CHCl_3$–MeOH (8: 1) as developing solvent gave **3** (142 mg) as colorless oil. NMR ($CDCl_3$) δ: 0 (6H, s, 17-OSi(CH_3)$_2$), 0.78 (3H, s, 18-CH_3), 0.86 (9H, s, 17-OSi-*t*-Bu), 1.18 (3H, s, 19-CH_3), 2.59 (4H, s, -$COCH_2CH_2CO$-), 3.74 (1H, t, J=8 Hz, 17α-H), 4.6—5.2 (1H, m, 15β-H), 5.72 (1H, s, 4-H).

15α,17β-Dihydroxy-4-androsten-3-one 15-Hemisuccinate (4)——To a solution of **3** (140 mg) in acetone (4.5 ml) was added 5 N HCl (1.3 ml) and allowed to stand at room temperature for 1 hr. The resulting solution was diluted with AcOEt, washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. Recrystallization of the residue from MeOH–AcOEt gave **4** (66 mg) as colorless leaflets. mp 197—198°. $[\alpha]_D^{17}$ +80.0° (c=0.10). NMR ($CDCl_3$–CD_3OD (3: 1)) δ: 0.84 (3H, s, 18-CH_3), 1.22 (3H, s, 19-CH_3), 2.56 (4H, s, -$COCH_2$-CH_2CO-), 3.79 (1H, t, J=8 Hz, 17α-H), 4.6—5.2 (1H, m, 15β-H), 5.72 (1H, s, 4-H). *Anal.* Calcd. for $C_{23}H_{32}O_6$: C, 68.29; H, 7.97. Found: C, 68.12; H, 8.01.

Conjugation of 4 with BSA——To a solution of **4** (30 mg) in dry dioxane (0.7 ml) were added (n-C_4H_9)$_3$N (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 afforded testosterone-15α-BSA conjugate (**5**) (90 mg) as fluffy powder. Number of steroid molecules linked to a BSA molecule was elucidated to be 24 by measuring the ultraviolet absorption spectra of testosterone, BSA, and testosterone-15α-BSA conjugate.

Animals——Domestic strain female albino rabbits weighing 2.5—3.0 kg were used.

Materials——[1,2,6,7-^{3}H]-Testosterone (86.4 Ci/mmol) was supplied from the Radiochemical Centre (Amersham). BSA and Freund's complete adjuvant were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Iatron Laboratories (Tokyo), respectively. Epitestosterone and adrenosterone were kindly donated from Teikoku Hormone Mfg. Co. (Tokyo). Other 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 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——The antigen (**5**) (1 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with Freund's complete adjuvant (0.5 ml). This emulsion was injected into a rabbit subcutaneously at multiple sites along the back. This procedure was repeated once a week for 3 weeks and then once every fortnight. Blood was collected 6 months after the initial injection from the rabbit and centrifuged

21) 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. For preparative TLC silica gel H (E. Merck AG, Darmstadt) was used as an adsorbent.

at 3000 rpm for 10 min. The antiserum thus prepared was stored at 4° and used in the assay at an initial dilution of 1 : 30000.

Assay Procedures——All dilutions of standards, tracer, and antiserum were performed in 0.01 M phosphate buffer (pH 7.4) containing gelatin (0.1%), NaCl (0.9%), and NaN_3 (0.01%). To a series of standard solutions (0, 12, 23, 45, 86, 123, 157, 235, 470, 705, and 940 pg of testosterone) in buffer (0.1 ml) were added ^{3}H-testosterone (*ca.* 10000 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% w/v)-charcoal (1% w/v) 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 scintillation solution (10 ml) and submitted to counting of radioactivity.

Cross-Reaction Study——The specificity of antiserum raised against the testosterone-BSA conjugate was tested by cross-reaction studies with 32 kinds of purified steroids related to testosterone (Table I). The relative amounts required to reduce the initial binding of ^{3}H-testosterone by half, where the mass of unlabeled testosterone was arbitrarily chosen as 100%, were calculated by the standard curves.

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