# Characteristics of Anti-testosterone Antisera Produced by Bovine Serum Albumin Conjugates of 15α- and 15β-Carboxymethyltestosterone: Use of [$^{125}$I]Iodinated Tracers

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**Each testosterone-[$^{125}$I]iodinated histamine derivative where [$^{125}$I]iodinated histamines were linked to respective 15α- and 15β-carboxymethyltestosterone (15α- and 15β-CMT), testosterone-3-(*O*-carboxymethyl)oxime (T-3-CMO) and testosterone-17β-hemisuccinate (T-17-HS) were tested for their usefulness as radiotracers in testosterone immunoassay. In the use of anti-15α- and 15β-CMT antisera produced in rabbits against 15α- and 15β-CMT–bovine serum albumin (BSA) conjugates, the antisera with 15α- and 15β-CMT-[$^{125}$I]iodinated tracers showed low sensitivity and somewhat low specificity in comparison with those of the antisera with tritiated testosterone (T-$^{3}$H). On the other hand, the antisera with T-3-CMO-[$^{125}$I]iodinated tracer showed high sensitivity but low specificity for 5α-dihydrotestosterone (5α-DHT) in comparison with T-$^{3}$H. The T-17-HS-[$^{125}$I]iodinated tracer was not bound to the antisera. In the use of anti-15α- and 15β-CMT antisera produced in rabbits by pretreatment with 15α-carboxymethyl-5α-DHT linked to a copolymer of D-glutamic acid and D-lysine followed by immunization with 15α- and 15β-CMT–BSA, the antisera with homologous [$^{125}$I]iodinated tracer showed high sensitivity and specificity.**

**Keywords** anti-testosterone antiserum; 15α-carboxymethyltestosterone–BSA; 15β-carboxymethyltestosterone–BSA; [$^{125}$I]-iodinated tracer; radioimmunoassay

## Introduction

It is well known that the antibody shows an affinity not only for the hapten but for the bridge through which it was attached to the carrier protein. The recognition of the bridge by the antibody has been referred to as bridge-binding.[2-5] In steroid radioimmunoassays, the assay system with radioiodinated tracer, in which a radioiodinated moiety is attached to the steroid through the same bridge that was used to attach the hapten to carrier protein in the immunogen (homologous system) is much less sensitive than the corresponding tritiated system.[2-6] Because of the homology of the bridge, the antibody recognizes both the steroid and the bridge and will bind the tracer with a greater affinity than that of native steroid. Consequently, the amount of native steroid required to displace the radioiodinated tracer is so great that the standard curve is shifted to the right, and a loss sensitivity results.

Two types of heterologous radioligands have been used to circumvent bridge-binding. The radioligands are as follows; 1) heterologous bridge radioligands where the immunogen and the tracer differ in the nature of the bridging groups,[5-7] 2) heterologous site radioligands where the carrier protein and radioiodinated moiety are attached to a different site on the steroid.[4,8] The selection of radioligands is of importance in establishing steroid radioimmunoassay (RIA).

In our previous paper, we produced the antisera (anti-15α- and 15β-CMT antisera) with high specificity for testosterone in rabbits by immunization with the bovine serum albumin (BSA) conjugates of 15α- and 15β-carboxymethyltestosterone (15α- and 15β-CMT), respectively,[9] and also the antisera produced by pretreatment with 15α-carboxymethyl-5α-dihydrotestosterone linked to a copolymer of D-glutamic acid and D-lysine (D-GL) followed by immunization with the corresponding BSA conjugates[10] (pretreatment method). The present paper deals with the sensitivity and specificity of the assay systems formed by combining [$^{125}$I]iodinated tracers and the anti-15α- and 15β-CMT antisera produced by us.[9,10]

## Materials and Methods

**Reagents** Anti-15α- and 15β-CMT antisera produced by us[9,10] were used in this assay. Na[$^{125}$I]I was a gift from Daiichi Radioisotope Laboratories Ltd. (Tokyo, Japan). [1α,2α-$^{3}$H(n)]Testosterone (T-$^{3}$H), 1.8 TBq/mmol and the scintillation solution (Atomlight) were purchased from New England Nuclear and all unlabeled steroids from Steraloids Inc. (Wilton, NH). Other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). Preparative thin layer chromatography (TLC)

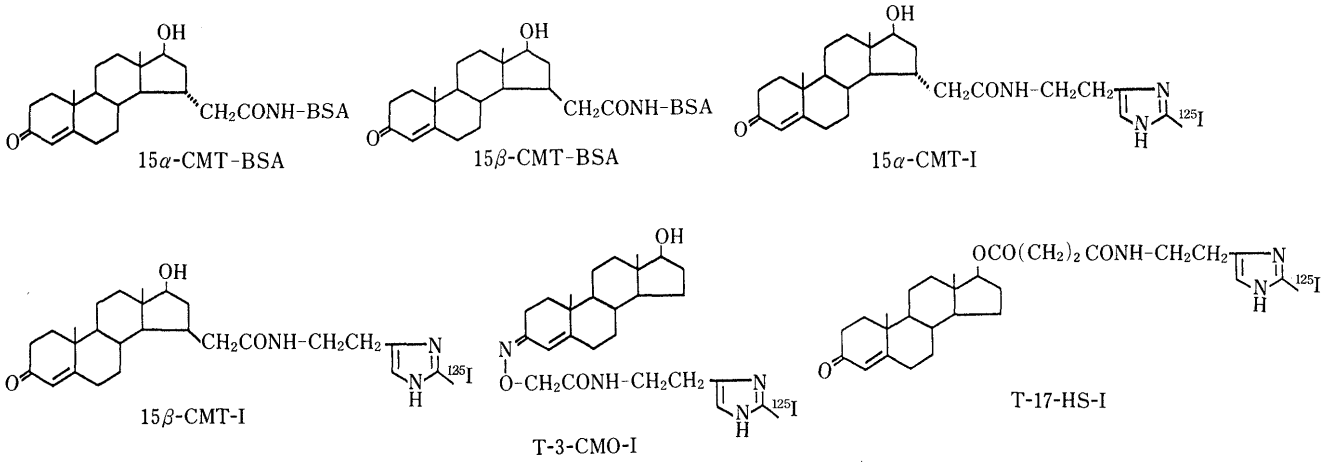


Fig. 1. Structure of 15α- and 15β-CMT–BSA Conjugates and [$^{125}$I]Iodinated Tracers

was carried out on plates prepared with 0.5 mm layer of Silica gel 60 $F_{254}$ (E. Merck).

**Preparation of [$^{125}$I]-Iodinated Tracers** The structure of radioiodinated tracers are shown in Fig. 1.

**Preparation of 15α-[2-([$^{125}$I]Iodoimidazol-4′-yl)ethylcarbamoyl]methyltestosterone (15α-CMT-I)** Tri-*n*-butylamine (10 μl) and isobutyl chloroformate (5 μl) were added to a solution of 15α-CMT (1 mg) in dry tetrahydrofuran (THF) (50 μl) at 0 °C. The mixture was stirred for 15 min and then diluted 50-fold with THF at 0 °C. A solution of [$^{125}$I]-iodohistamine (25 μl) (18.5 MBq) prepared according to the procedure of Tantchou *et al.*[11] was added to the diluted THF solution (50 μl) at 0 °C. To the resulting solution was added 0.2 M NaOH (10 μl). The mixture was stirred for 1 h at 0 °C and acidified with 0.1 M HCl (0.9 ml) and extracted with toluene (1.0 ml). The organic layer was discarded and 0.1 M NaOH (0.9 ml) and sodium metabisulfite (1 ml, 1 mg) was added. The mixture was then extracted with toluene (0.5 ml) by vortex mixing for 2 min. The toluene extract was concentrated *in vacuo* under nitrogen moisture and purified by TLC on a silica gel plate in $CHCl_3$–MeOH (3 : 1). The thin-layer plate was scanned with a radiochromatogram scanner (Aloka TRM-1B) and a major peak of radioactive material (*Rf* 0.45) was revealed. The radioactive material was eluted with EtOH. After removal of silica gel particles by centrifugation, the eluate was stored at −20 °C. The maximal binding of the material (active fraction) to anti-15α-CMT antibody was 99%. The material was considered to be 15α-CMT-I. The radioactivity of 15α-CMT-I obtained was 2.96 MBq using a CRC-5 Radioisotope Calibrator (Capintec, Inc).

**Preparation of 15β-[2-([$^{125}$I]Iodoimidazol-4′-yl)ethylcarbamoyl]methyltestosterone (15β-CMT-I)** [$^{125}$I]Iodinated histamine (18.5 MBq) was linked to 15β-CMT by treatment similar to that described above, to give 15β-CMT-I (2.70 MBq, *Rf* 0.45 in $CHCl_3$–MeOH, 3 : 1). The maximal binding of the radioactive fraction to anti-15β-CMT antibody was 99%.

**Preparation of Testosterone 3-{*O*-[2-([$^{125}$I]Iodoimidazol-4′-yl)ethylcarbamoyl]}oxime (T-3-CMO-I)** [$^{125}$I]Iodinated histamine (18.5 MBq). was linked to testosterone 3-(*O*-carboxymethyl)oxime (T-3-CMO) by treatment similar to that as described above to give T-3-CMO-I (1.81 MBq, *Rf* 0.38 in $CHCl_3$–MeOH, 3 : 1).

**Preparation of 3-Oxo-4-androsten-17β-yl *N*-2-([$^{125}$I]Iodoimidazol-4′-yl)ethyl Succinamate (T-17-HS-I)** [$^{125}$I]Iodinated histamine (18.5 MBq) was linked to testosterone-17β-hemisuccinate (T-17-HS) by treatment similar to that as described above to give 15β-CMT-I (2.29 MBq, *Rf* 0.42 in $CHCl_3$–MeOH, 4 : 1).

**Immunization Procedures and Collection of Antisera** Four groups of female New Zealand white rabbits, with 3 animals per group, received primary immunization subcutaneously at multiple site along the back with 1 mg of respective 15α-CMT–BSA-conjugate (#13, 14 and 15 in group 1 and #22, 23 and 24 in group 3) and 15β-CMT–BSA conjugate (#16, 17 and 18 in group 2 and #25, 26 and 27 in group 4) emulsified with complete Freund's adjuvant (CFA). The former groups and the latter groups were boosted with 0.5 mg of 15α- and 15β-CMT–BSA emulsified with CFA every 4 weeks, respectively. Group 2 and 4 were pretreated with a single intraperitoneal injection of 5 mg of 15α-carboxymethyl-5α-dihydrotestosterone–D-GL conjugate in saline 3 d before the primary immunization. Blood was collected 3 months after the primary immunization and centrifuged at 2500 rpm for 10 min. The serum was diluted to bind 50% of the radiotracer. Titres were shown as the reciprocal of dilution of the serum dilution that bound corresponding homologous [$^{125}$I]iodinated tracer (*ca.* 40000 dpm) or T-$^3$H (*ca.* 20000 dpm). Titres are shown in Table I.

**Assay Procedure** The assay systems were composed as follows: Homologous system; combinations of 15α- and 15β-CMT-I tracers and anti-15α- and 15β-CMT antisera, respectively. Heterologous bridge system; combinations of 15α- and 15β-CMT-I tracers and anti-15β- and 15α-CMT antisera, respectively. Heterologous site system; combinations of T-3-CMO-I tracer and the antisera, and of T-17-HS-I and the antisera.

All dilutions of the standard labeled tracer and antiserum were prepared with 0.1 M borate buffer (pH 8.0) containing 0.1% gelatin, 0.9% NaCl and 0.01% $NaN_3$. Standard testosterone (0.2 ml, 1 pg to 10 ng/tube) or cross reactant steroids (0.2 ml, 1 pg to 1 μg/tube) dissolved in assay buffer were added to duplicate tubes. [$^{125}$I]Iodinated tracers (0.1 ml) (*ca.* 40000 dpm: 4.8, 4.4, 4.7 and 3.7 pg/tube for 15α-CMT-I, 15β-CMT-I, T-3-CMO-I and T-17-HS-I, respectively) or T-$^3$H (0.1 ml) (*ca.* 20000 dpm, 45 pg/tube) and antiserum diluted to bind 50% of the radiotracer (0.1 ml) were added to the assay tubes. The mixture was incubated for 2 h at room temperature. After addition of dextran-coated charcoal containing gelatin (0.5 ml) (dextran 0.05%, charcoal 0.5%, gelatin 0.1%) (0.5 ml), the suspension was vortex-mixed, allowed to stand at 4 °C for 10 min and then centrifuged for 30 min at 2500 rpm. The radioactivity of the supernatant solutions (0.5 ml) containing tritiated or [$^{125}$I]iodinated tracer were measured with an Aloka ES-700 liquid scintillation spectrometer and a Shimadzu RAW-300 auto-well scintillation spectrometer, respectively.

**Cross-Reaction** The cross-reactivity of antiserum was determined using the method of Abraham.[12]

TABLE I. Titres of Anti-15α- and 15β-CMT Antisera

| Antiserum | Group | Treatment | Rabbit No. | Titre: Homologous 15α- and 15β-CMT-I | Titre: T-$^3$H |
|---|---|---|---|---|---|
| Anti-15α-CMT antiserum | 1 | Saline | #13 | 1: 98000 | 1: 9800 |
| | | | #14 | 1:120000 | 1:12000 |
| | | | #15 | 1:220000 | 1:22000 |
| | 2 | 5α-DHT–15-CM–D-GL | #16 | 1: 76000 | 1: 6400 |
| | | | #17 | 1: 45000 | 1: 4100 |
| | | | #18 | 1: 72000 | 1: 7200 |
| Anti-15β-CMT antiserum | 3 | Saline | #22 | 1:210000 | 1:21000 |
| | | | #23 | 1:140000 | 1:14000 |
| | | | #24 | 1:100000 | 1:10000 |
| | 4 | 5α-DHT–15-CM–D-GL | #25 | 1: 48000 | 1: 3700 |
| | | | #26 | 1: 77000 | 1: 6400 |
| | | | #27 | 1: 70000 | 1: 6400 |

Titres are expressed as the reciprocal of dilution of antisera bound 50% of added homologous [$^{125}$I]iodinated tracer (*ca.* 40000 dpm) or [$^3$H]testosterone (*ca.* 20000 dpm). Abbreviations used are as follows: 15α- and 15β-CMT for 15α- and 15β-carboxymethyltestosterone, respectively, 15α- and 15β-CMT-I for 15α- and 15β-[2-([$^{125}$I]iodoimidazol-4′-yl)ethylcarbonyl]methyltestosterone, respectively, and T-$^3$H for tritiated testosterone.

## Results and Discussion

**Sensitivity and Specificity of Anti-15α- and 15β-CMT Antisera** The amount of unlabeled steroid needed to displace 50% of the bound tracer (50% point)[3,5,6] has been used in order to compare the sensitivities of homologous and heterologous bridge systems in steroid RIAs, because it is very stable and provides an accurate comparison of curve location. On the other hand, total binding[6] which is expressed in the percentage of the amount of radiolabeled tracer bound to antibody in the absence of unlabeled steroid was also used for this purpose, because it reflects affinity of antibody for radiotracer in the same amount of the antibody. We also employed 50% point and total binding for comparison in the assay sensitivities of homol-

TABLE II. The 50% Point of Anti-15α- and 15β-CMT Antisera Produced without Pretreatment

| Antiserum | Rabbit No. | 50% point (pg/ml): 15α-CMT-I | 15β-CMT-I | T-3-CMO-I | T-$^3$H |
|---|---|---|---|---|---|
| Anti-15α-CMT antiserum | #13 | 875 | 893 | 64 | 453 |
| | #14 | 867 | 811 | 57 | 484 |
| | #15 | 889 | 835 | 50 | 487 |
| Mean | | 877 | 846 | 57 | 474 |
| Anti-15β-CMT antiserum | #22 | 856 | 911 | 86 | 466 |
| | #23 | 837 | 903 | 77 | 471 |
| | #24 | 889 | 859 | 53 | 487 |
| Mean | | 860 | 891 | 72 | 459 |

The 50% point is expressed as the amount of unlabeled steroid needed to displace 50% of the bound tracer.

TABLE III. Total Binding of Anti-15α- and 15β-CMT Antisera Produced without Pretreatment

| Antiserum | Rabbit No. | Total binding (%) | | | |
|---|---|---|---|---|---|
| | | 15α-CMT-I | 15β-CMT-I | T-3-CMO-I | T-$^3$H |
| Anti-15α-CMT | #13 | 48.2 | 50.6 | 35.0 | 50.2 |
| antiserum | #14 | 49.1 | 50.3 | 32.4 | 50.1 |
| | #15 | 49.1 | 51.1 | 32.8 | 49.5 |
| Mean | | 48.3 | 50.7 | 33.4 | 49.7 |
| Anti-15β-CMT | #22 | 48.6 | 49.0 | 36.7 | 50.3 |
| antiserum | #23 | 48.3 | 50.1 | 34.0 | 49.8 |
| | #24 | 48.0 | 47.9 | 33.1 | 49.8 |
| Mean | | 48.3 | 49.0 | 34.6 | 50.0 |

Abbreviations for compounds are the same as in Table I.

ogous and heterologous systems. Data of 50% point and total binding are shown in Tables II and III, respectively.

The 50% points (877 and 891 pg/ml) of anti-15α and 15β-CMT antisera produced without pretreatment using homologous 15α- and 15β-CMT-I were higher than those (474 and 459 pg/ml) of the antisera with T-$^3$H, respectively. On the other hand, the 50% point (846 pg/ml) of the anti-15α-CMT antiserum using heterologous 15β-CMT-I was similar to that (877 pg/ml) of the antiserum using homologous 15α-CMT-I. The total binding (50.7%) of the anti-15α-CMT antiserum using heterologous 15β-CMT-I was similar to that (48.8%) of the antiserum using homologous 15α-CMT-I. These trends were also observed when the anti 15β-CMT antiserum was used (50% point of 860 pg/ml *vs.* 891 pg/ml and total binding of 48.3% *vs.* 49.0%). The anti-15α- and 15β-CMT antibodies weakly recognize the 15-carboxymethyl bridge of the tracers. When the assay sensitivity of a heterologous site system was compared with that of a tritiated system, remarkable difference developed. The 50% points (57 and 72 pg/ml) of the anti-15α- and 15β-CMT antisera using T-3-CMO-I were much lower than those (474 and 459 pg/ml) of the antisera using T-$^3$H, respectively. However, the antisera were not bound to T-17-HS-I. On the other hand, the total bindings (33.4 and 34.6%) of the anti-15α- and 15β-CMT antisera using T-3-CMO-I were lower than those (49.7 and 50.0%) of the antisera using T-$^3$H, respectively.

Accordingly, each of the 15α- and 15β-CMT antisera in the heterologous system using T-3-CMO-I showed high sensitivity. This phenomenon suggests that the antisera will have low affinities to T-3-CMO-I in comparison with those to T-$^3$H.

The specificity of antisera in various systems was evaluated by ascertaining the ability of various related steroids to compete with radioiodinated or tritiated tracer in binding to antibody. The cross-reactivity of antisera were determined using the method of Abraham[12] and the data are shown in Tables IV and V.

The anti-15α-CMT antiserum (#14) using homologous 15α-CMT-I showed 9.78% cross-reaction with 5α-dihydrotestosterone (5α-DHT) but low cross-reactivity for progesterone (1.32%), deoxycorticosterone (0.86%), corticosterone (0.03%) and 4-androstene-3,17-dione (1.01%). The anti-15α-CMT antiserum in a heterologous bridge system, using 15β-CMT-I showed 7.09% cross-reaction with 5α-DHT, but low cross-reactivity for progesterone (0.97%),

TABLE IV. Percentage Cross-Reactivity of Anti-15α-CMT Antisera Produced without Pretreatment

| Steroid | 15α-CMT-I | 15β-CMT-I | T-3-CMO-I | T-$^3$H |
|---|---|---|---|---|
| Testosterone | 100.00 | 100.00 | 100.00 | 100.00 |
| 5α-Dihydrotestosterone | 9.78 | 7.09 | 35.13 | 3.98 |
| 5β-Dihydrotestosterone | 0.46 | 0.45 | 2.92 | 0.23 |
| 4-Androstene-3β,17β-diol | <0.01 | <0.01 | 6.38 | <0.01 |
| 5α-Androstane-3β,17β-diol | <0.01 | <0.01 | 4.10 | <0.01 |
| 4-Androstene-3,17-dione | 1.01 | 1.15 | 2.22 | 0.64 |
| 11β-Hydroxytestosterone | <0.01 | <0.01 | 0.08 | <0.01 |
| Progesterone | 1.32 | 0.97 | 5.81 | 0.40 |
| Deoxycorticosterone | 0.86 | 0.74 | 7.35 | 0.43 |
| Cortisol | <0.01 | <0.01 | 0.04 | <0.01 |
| Corticosterone | 0.03 | 0.03 | 2.07 | 0.02 |
| Estrone | <0.01 | <0.01 | <0.01 | <0.01 |
| Estradiol | <0.01 | <0.01 | <0.01 | <0.01 |

Abbreviations for compounds are the same as in Table I.

TABLE V. Percentage Cross-Reactivity of Anti-15α-CMT Antisera Produced without Pretreatment

| Steroid | 15α-CMT-I | 15β-CMT-I | T-3-CMO-I | T-$^3$H |
|---|---|---|---|---|
| Testosterone | 100.00 | 100.00 | 100.00 | 100.00 |
| 5α-Dihydrotestosterone | 10.04 | 14.88 | 44.37 | 3.05 |
| 5β-Dihydrotestosterone | 0.63 | 0.58 | 1.87 | 0.46 |
| 4-Androstene-3β,17β-diol | <0.01 | <0.01 | 5.46 | <0.01 |
| 5α-Androstane-3β,17β-diol | <0.01 | <0.01 | 5.10 | <0.01 |
| 4-Androstene-3,17-dione | 1.21 | 1.30 | 3.94 | 0.68 |
| 11β-Hydroxytestosterone | <0.01 | <0.01 | 0.15 | <0.01 |
| Progesterone | 1.54 | 1.83 | 4.21 | 0.45 |
| Deoxycorticosterone | 1.10 | 0.92 | 9.48 | 0.20 |
| Cortisol | <0.01 | <0.01 | 0.13 | <0.01 |
| Corticosterone | 0.03 | 0.03 | 3.77 | 0.01 |
| Estrone | <0.01 | <0.01 | <0.01 | <0.01 |
| Estradiol | <0.01 | <0.01 | <0.01 | <0.01 |

Abbreviations for compounds are the same as in Table I.

deoxycorticosterone (0.74%), corticosterone (0.03%) and 4-androstene-3β,17β-dione (1.15%). The 15α-CMT antiserum of a heterologous site system, using T-3-CMO-I, showed 35.13% cross-reaction with 5α-DHT and a somewhat high cross-reactivity for progesterone (5.81%), deoxycorticosterone (7.35%), 4-androstene-3β,17β-diol (6.38%), 5α-androstane-3β,17β-diol (4.10%) and 4-androstene-3,17-dione (2.22%). On the other hand, the anti-15β-CMT antiserum (#24) using homologous 15β-CMT-I showed 14.88% cross-reaction with 5α-DHT but, low cross-reactivity for progesterone (1.83%), deoxycorticosterone (0.92%), corticosterone (0.03%) and 4-androstene-3,17-dione (1.30%). The anti-15β-CMT antiserum in a heterologous bridge system using 15α-CMT-I, showed 10.04% cross-reaction with 5α-DHT but, low cross-reactivity for progesterone (1.54%), deoxycorticosterone (1.10%) corticosterone (0.03%) and 4-androstene-3,17-dione (1.21%). The anti-15β-CMT antiserum in a heterologous site system using T-3-CMO-I, showed 44.37% cross-reaction with 5α-DHT and also a somewhat higher cross-reactivity for progesterone (4.21%), deoxycorticosterone (9.48%), 4-androstene-3β,17β-diol (5.46%), 5α-androstane-3β,17β-diol (5.10%) and 4-androstene-3,17-dione (3.94%).

Respective anti-15α- and 15β-CMT antisera using the corresponding homologous [$^{125}$I]iodinated tracer showed higher cross-reactivity for 5α-DHT and similar cross-

Table VI. The 50% Point of Anti-15α- and 15β-CMT Antisera Produced with Pretreatment

| Antiserum | Rabbit No. | 50% point (pg/ml) | | |
|---|---|---|---|---|
| | | 15α-CMT-I | 15β-CMT-I | T-$^3$H |
| Anti-15α-CMT | #16 | 125 | | 443 |
| antiserum | #17 | 133 | | 470 |
| | #18 | 133 | | 462 |
| Mean | | 130 | | 458 |
| Anti-15β-CMT | #25 | | 125 | 463 |
| antiserum | #26 | | 140 | 476 |
| | #27 | | 148 | 462 |
| Mean | | | 138 | 467 |

Abbreviations for compounds are the same as in Table I.

Table VII. Percentage Cross-Reactivity of Anti-15α- and 15β-CMT Antisera Produced by the Pretreatment Method

| Steroid | Anti-15α-CMT antiserum | Anti-15β-CMT antiserum |
|---|---|---|
| Testosterone | 100.00 | 100.00 |
| 5α-Dihydrotestosterone | 1.43 | 1.34 |
| 5β-Dihydrotestosterone | 0.29 | 0.25 |
| 4-Androstene-3β,17β-diol | <0.01 | <0.01 |
| 5α-Androstane-3β,17β-diol | <0.01 | <0.01 |
| 4-Androstene-3,17-dione | 0.78 | 0.60 |
| 11β-Hydroxytestosterone | <0.01 | <0.01 |
| Progesterone | 0.55 | 0.39 |
| Deoxycorticosterone | 0.39 | 0.26 |
| Cortisol | <0.01 | <0.01 |
| Corticosterone | 0.02 | 0.02 |
| Estrone | <0.01 | <0.01 |
| Estradiol | <0.01 | <0.01 |

Abbreviations for compounds are the same as in Table I.

reactivity for C-21 steroids in comparison with the antiserum using T-$^3$H. The cross-reactivity for DHT and C-21 steroids of the anti-15α- and 15β-CMT antisera, using heterologous [$^{125}$I]iodinated tracers, was similar to that of the antisera using the corresponding homologous [$^{125}$I]-iodinated tracers. On the other hand, the cross-reactivity of the anti-15α- and 15β-CMT antisera for 5α-DHT using the heterologous T-3-CMO-I, showed much higher (35.13 and 44.37%) than those (3.98 and 3.05%) of the antisera using T-$^3$H, respectively.

**Sensitivity and Specificity of Anti-15α- and 15β-CMT Antisera Produced with Pretreatment** The anti-15α- and 15β-CMT antisera produced with pretreatment showed 130 and 138 pg/ml for 50% point using 15α- and 15β-CMT-I, respectively (Table VI). On the other hand, the anti-15α- and 15β-CMT antisera showed 458 and 467 pg/ml for 50% point using T-$^3$H, respectively (Table V). The 50% point (130 pg/ml) of the anti-15α-CMT antiserum using 15α-CMT-I was lower than that (458 pg/ml) of the antiserum using T-$^3$H. A similar trend was also observed when the anti-15β-CMT antiserum was used with 15β-CMT-I (50% point: 138 pg/ml *vs.* 467 pg/ml). As a result, each of the anti-15α- and 15β-CMT antiserum produced with pretreatment using the corresponding homologous [$^{125}$I]iodinated tracers, showed high sensitivity in comparison with the corresponding antiserum using T-$^3$H.

The anti-15α-CMT antiserum (#18) using 15α-CMT-I showed 1.43% cross-reaction with 5α-DHT and also low cross-reactivity for progesterone (0.55%), deoxycorticosterone (0.39%), corticosterone (0.02%) and 4-androstene-3,17-dione (0.78%) (Table VII). The anti-15β-CMT antiserum (#27) using 15β-CMT-I showed 1.34% cross-reaction with 5α-DHT and also low cross-reactivity for progesterone (0.39%), deoxycorticosterone (0.26%), corticosterone (0.02%) and 4-androstene-3,17-dione (0.60%) (Table VII).

Accordingly, anti-15α- and 15β-CMT antisera prepared with pretreatment using the corresponding homologous [$^{125}$I]iodinated tracers showed the highest specificity for 5α-DHT and C-21 steroids in the assay system using [$^{125}$I]-iodinated tracer.

The pretreatment method is based on the clonal selection theory of antibody formation. The mechanism underlying induction of B lymphocyte tolerance by a hapten conjugated to D-GL was characterized by Katz *et al.*[13] and Hamaoka *et al.*[14] When a hapten D-GL conjugate was administered to experimental animals, the conjugate specifically bound to surface immunoglobulin receptors on B lymphocytes, and rendered those cells irreversibly tolerant. Accordingly, high sensitivity and specificity of anti-15α- and 15β-CMT antisera are presumed as followed: When rabbits were pretreated with 5α-DHT–15-CM–D-GL, both reactive clones for 5α-DHT and 15-carboxymethyl bridge (15-CM) were inactivated and anti-15α- and 15β-CMT antisera produced by immunization in the rabbits with 15α- and 15β-CMT did not recognize the 5α-DHT and 15-CM. On the other hand, an advantage of [$^{125}$I]iodinated tracer is the higher dilution of the antibodies in comparison with that of the antibodies using tritiated tracer. The dilutions of anti-15α- and 15β-CMT antisera using homologous 15α- and 15-CMT-I was at least 10-fold greater than that of the antisera using T-$^3$H.

In these results, the anti-15α- and 15β-CMT antisera produced with pretreatment using the corresponding homologous [$^{125}$I]iodinated tracers showed high sensitivity in comparison with the corresponding antisera using T-$^3$H, and the highest specificity for 5α-DHT and C-21 steroids in an assay system using [$^{125}$I]iodinated tracers.

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