ANTIGENIC COMPLEXES OF STEROID HORMONES FORMED BY COUPLING TO PROTEIN THROUGH POSITION 7 : PREPARATION FROM Δ^4-3-OXOSTEROIDS AND CHARACTERIZATION OF ANTIBODIES TO TESTOSTERONE AND ANDROSTENEDIONE

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ABSTRACT

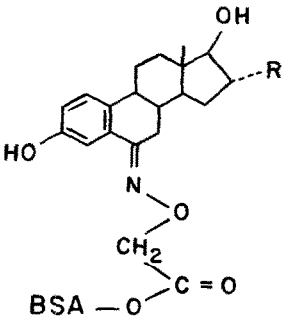
A general method for rendering Δ^4-3-oxosteroids antigenic by coupling to a macromolecule through position 7 is described. It involves nucleophilic attack on the 6,7-dehydroderivatives of the steroids by ambidentate reagents to form 7α-thioether alkanoic acids. These were covalently attached to bovine serum albumin (BSA) by use of the carbodiimide reagent. Addition products with mercapoacetic acid and β-mercaptopropionic acid, and their BSA-conjugates, were thus obtained from testosterone, androst-4-ene-3,17-dione, progesterone and 17α-hydroxyprogesterone through the respective 4,6-dienes.

Immunization of rabbits with testosterone-7α-carboxymethyl-thioether-BSA and the homologous testosterone-7α-carboxyethyl-thioether-BSA gave rise to antisera of high affinity for testosterone (Ka=9.4×10^9 l/mol) that showed little cross-reaction with androstenedione (<1%) and with a variety of 17-oxo-androstane compounds ($\leq 0.5\%$). Conversely, immunization with androstenedione-7α-carboxyethyl-thioether-BSA yielded an antiserum with high affinity for androstenedione (Ka=1.04×10^{10} l/mol) but minimal cross-reaction with testosterone ($< 0.5\%$) and 17β-hydroxy-androstane compounds ($\leq 1\%$). The reaction of anti-testosterone and anti-androstenedione sera with their homologous haptens was not significantly inhibited by the closely related steroids 17α-epitestosterone and dehydroepiandrosterone, or by 11-deoxycorticosterone, progesterone, 17α-hydroxyprogesterone, estrone and estradiol-17β. However, anti-testosterone sera cross-reacted with 5α-dihydrotestosterone (40-50%) and to a lesser extent with 5β-dihydrotestosterone (5%). Analogously, the anti-androstenedione sera cross-reacted with 5α-dihydroandrostenedione (71%) and to a minor extent with 5β-dihydroandrostenedione (8%).

A radioimmunoassay procedure for the determination of testosterone in plasma is described, which makes use of the new anti-testosterone serum. Preliminary results suggest that it can be applied to ether extracts from human sera without chromatographic purification.

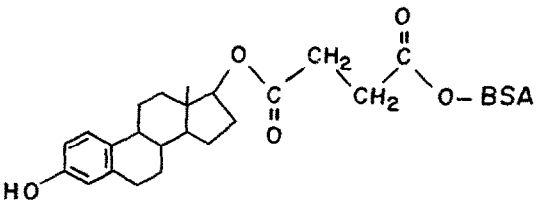
Specific antibodies to steroidal haptens can be produced by coupling the steroid to a carrier protein (1). The advent of the radioimmunoassay technique stimulated interest in attempts to produce antibodies to steroids with ever increasing specificity. The specificity of such antibodies is markedly influenced by the position on the steroid molecule used for coupling to the peptide carrier (2-4): antibodies elicited by immunizing with a steroid hormone attached to protein through one of its pre-existing functional groups usually lack specificity towards the group used for the coupling reaction and the region of the hapten in its immediate vicinity (2-4).

Consideration of this problem led this laboratory to synthesize steroids bound to protein through the 6- or 11-position of the steroid molecule, leaving the structural features that determine hormonal specificity fully exposed (2, 4). Antibodies raised against antigen I were found to be hapten specific, in contrast to antibodies elicited by II which strongly cross-reacted with estrone and partially with estriol. Antibodies produced against III were specific towards the functional groups at position 3 and 20, but did not recognize changes around the 5-position of the hapten, reacting almost equally with progesterone, 5α-pregnane-3, 20-dione and 5β-pregnane-3, 20-dione. This failure to discriminate between molecules differing but slightly in the vicinity of the 5-position was ascribed to the proximity of this position to the point of attachment of the steroid to the carrier protein. This particular "proximity effect" is eliminated by binding the steroid through position 11 to protein, and indeed antibodies raised against antigen IV (4) or a similar 11-conjugate (3) show a high degree of specificity for progesterone. However, the approach through carbon 11 is not as readily available for many hormonal



(I)

R = H or OH



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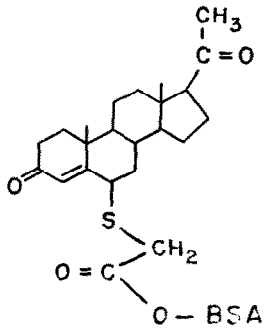

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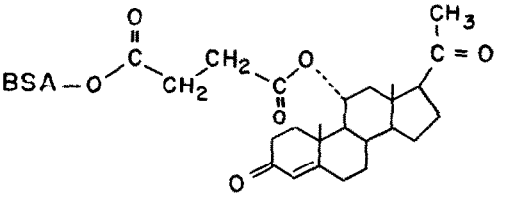


(IV)

BSA = bovine serum albumin

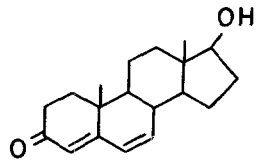
Δ^4-3-oxosteroids or their metabolites, e.g. testosterone and androstenedione (5).

This paper described another method whereby Δ^4-3-oxosteroids may be coupled to protein at a site remote from the functional groups responsible for hormonal specificity, viz. through position 7 of the steroid molecule. Its general applicability has been verified by the successful production of antigens from such diverse steroids as testosterone, androstenedione, progesterone and 17α-hydroxyprogesterone (5).

Synthesis of Antigens

Preparation of 7-substituted steroids. Entry into the 7-position of Δ^4-3-oxosteroids can be achieved by nucleophilic addition to the 4,6-dienes. Analogous reactions have been described, where the nucleophile employed was thioacetate (6a,b), mercaptide (6b,7) and cyanide anion (8). For our purpose a bifunctional reagent was required, that would possess - in addition to the nucleophilic group - a grouping suitable for coupling the steroid to protein. Thus mercaptoacetic acid and its homologue, β-mercaptopropionic acid, were chosen: the mercapto group is an efficient nucleophile, while the carboxyl group lends itself to peptide bond formation with the lysine residues of protein. β-Mercaptopropionic acid offers an opportunity to lengthen the "handle" by which the steroid is attached to the carrier. This is apt to enhance the efficiency of the hapten as an antigenic determinant.

The 6,7-dehydroderivatives of testosterone (V) (9), androstenedione (VI) (9), progesterone (VII) (6b) and 17α-hydroxyprogesterone (VIII) (10) have been described. Bromination of the parent steroid-acetylated in the case of testosterone-by N-bromosuccinimide, followed by dehydrobromination in collidine, yielded V



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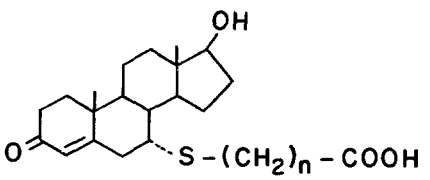

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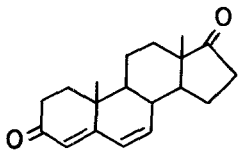

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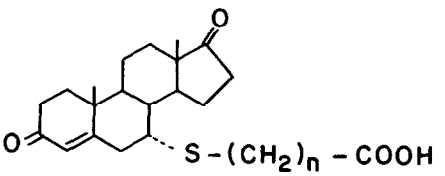
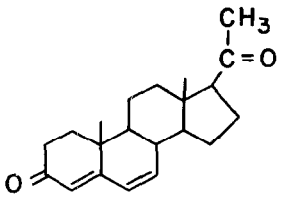


(X)

$SH-(CH_2)_n-COOH$

$OCH_3^{\ominus}$



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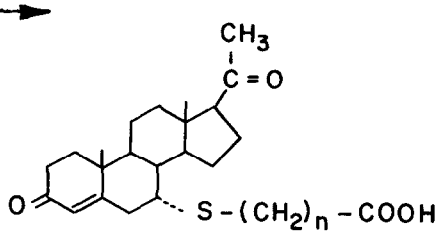

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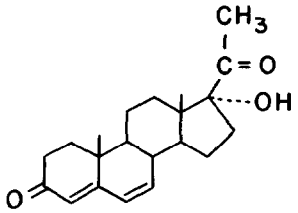

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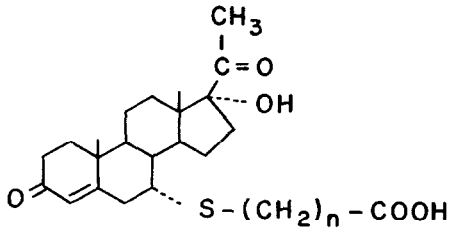

(XII)

n = 1 , IX a - XII a

n = 2 , IX b - XII b

Table 1. Yields, melting points and elementary analyses of 7-substituted steroids

Compound	Yield %	M.p. (°C)	Formula	Analyses	C	H	S
IXa	20	212-215	$C_{21}H_{30}O_4S$	Calcd.	66.44	7.99	8.46
				Found	66.59	7.89	8.67
Xa	29	215-217	$C_{21}H_{28}O_4S$	Calcd.	67.00	7.50	8.52
				Found	66.76	7.32	8.53
XIa	21	232-234	$C_{23}H_{32}O_4S$	Calcd.	68.29	7.97	7.91
				Found	68.11	7.70	8.26
XIIa	20	206-208	$C_{23}H_{32}O_5S$	Calcd.	65.69	7.67	7.61
				Found	65.39	7.38	7.87
IXb	48	210-212	$C_{22}H_{32}O_4S$	Calcd.	67.32	8.22	8.17
				Found	67.39	8.16	8.35
Xb	50	211-213	$C_{22}H_{30}O_4S$	Calcd.	67.67	7.74	8.20
				Found	67.37	8.08	8.40
XIb	42	240-242	$C_{24}H_{34}O_4S$	Calcd.	68.87	8.19	7.65
				Found	69.15	8.21	7.75
XIIb	38	223-226	$C_{24}H_{34}O_5S$	Calcd.	66.34	7.80	7.38
				Found	66.00	8.04	7.15

(after hydrolysis of the 17-acetate), VI and VII in overall yields of 40-50%, while chloranil dehydrogenation of 17α-hydroxyprogesterone by the method of Agnello and Laubach (11) afforded a 60% yield of VIII.

Reaction of the dehydroderivatives V - VIII with excess mercaptoacetic acid in the presence of sodium methoxide in dioxane gave the conjugate addition products IXa, Xa, XIa and XIIa in yields between 20-40%. A reaction time of 16 hr was sufficient for the production of the androstenedione and progesterone derivatives Xa and XIa. However, three days was found to be the optimal reaction time for the production of the testosterone and 17α-hydroxyprogesterone derivatives IXa and XIIa, no doubt due to the sparing solubility of the starting materials V and VIII in dioxane.

To make the haptenic steroid protrude farther from the carrier molecule, addition products of V - VIII with β-mercaptopropionic acid were prepared. Yields of addition products were higher in this homologous series, possibly due to greater nucleophilicity of the mercapto group in the propionic acid derivative.

Table I sets out the yields, melting points and elementary analyses for the addition products IX-XII. The microanalytical data agreed with theoretical values. Further support for the formation of the 7-addition products was obtained from the disappearance of the characteristic dienone absorption band at 280 nm and by the appearance of an absorption peak at 240 nm, establishing that the 4-en-3-one chromophore had been restored; and from mass spectrometry of the methyl esters IX-XII, which yielded parent peaks that were in agreement with the postulated molecular weights.

Stereochemistry of the 7-substituent. This was elucidated by interpretation of

the nmr spectral data. The proton attached to position 7 could readily be identified at about $\delta 3.2$. Even in the 90 megacycle spectra of the methyl esters of XIa and XIIa, the $S-CH_2$ signal partially obscured that of the proton at C-7. Nevertheless, the coupling pattern of the 7-proton resonance was clearly a quartet (J=3.5 Herz). This coupling constant establishes an equatorial conformation and hence β-configuration for the proton at C-7, it being coupled equally with the 6α, 6β and 8β-protons. In the homologous series IXb-XIIb, the 7-proton signal was well separated from any other resonance and again showed a coupling with three equivalent protons (J=3.5 Herz). Thus the thioether substituent in compounds IX-XII is α-oriented.

The assignment of an α-configuration to the 7-substituent is in accordance with the stereochemistry of other products obtained by similar reaction mechanisms. Conjugate addition of thioacetate to 4,6-diene-3-oxosteroids has yielded products for which the α-configuration has been established by molecular rotation measurements (6a). More recently, an extensive study (12) of base catalysed hydrocyanation of conjugated enones and dienones defined the conformation of the substituting cyano group as axial. Thus they observed that 1α-cyanoandrost-4-ene-3,17-dione was exclusively obtained from androst-1,4-diene-3,17-dione and 7α-cyanoandrost-4-en-3-on-17β-ol was obtained from V. The factors determining preferential formation of axial steroidal cyanides seem clearly to be operative in the production of the axial thioethers IX-XII. For although thermodynamic considerations favor equatorial substitution, because the conformational energy of the sulfur atom at position 7 is much larger than that of the cyano group (13), the reaction is kinetically controlled

due to the insolubility of the salts of IX-XII in dioxane. Hence the reaction pathway is governed by the preferred conformation of ring B in the transition state. As the preferred conformation is 1, 2-diplanar (14), an axial substituent is formed at C-7.

Mass-spectra of the steroid derivatives. The mass spectra of derivatives IX-XII each showed an abundant parent peak corresponding to the molecular weight of the respective methyl ester. In addition it was seen that elimination of the thioether sidechain from the molecular ion proceeded via a series of consecutive steps. In both the series IXa-XIIa and the homologous series IXb-XIIb, α-cleavage with respect to the sulfur atom was observed: in the series IXa-XIIa fragments of M-73 and M-105 appeared abundantly, corresponding to the elimination of radicles $\cdot CH_2\text{-}COOCH_3$ and $\cdot S\text{-}CH_2\text{-}COOCH_3$; in the series IXb-XIIb the homologous fragments M-87 and M-119 appeared, corresponding to elimination of $\cdot CH_2\text{-}CH_2\text{-}COOCH_3$ and $\cdot S\text{-}CH_2\text{-}CH_2\text{-}COOCH_3$. Fragments M-106 in series IXa-XIIa and M-120 in the homologous series were also detected, attributable to the splitting out of the thioether sidechain plus extraction of hydrogen from the charged moiety. The main difference between the two series was the cleaving out of the radicles $\cdot COOCH_3$ (M=59) in series IXa-XIIa, contrasting with the elimination of methyl formate (M=60) in greater abundance in the b-series. This differential fragmentation pattern is characteristic for homologous fatty acids.

When comparing the thioethers IX-XII among themselves, the differences between their mass spectra could be accounted for by the known differences in

fragmentation pattern between androstane and pregnane derivatives (15).

The hormone derivatives IX-XII were bound to bovine serum albumin (BSA) by use of the reagent 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (4) to yield steroid-protein conjugates with an average of 10 steroid residues per molecule of BSA.

Immunological Studies

Characterization of antisera. Immunization of rabbits with IXa-BSA or IXb-BSA gave rise within 5 weeks to antisera able to bind 60% of tritiated testosterone (20 pg) at 1:4000 dilution; one month later, the titer of the antiserum had increased to 1:10 000. Addition of cold testosterone over the range of 10 to 200-300 pg per assay tube inhibited this binding to a degree linearly related to log mass of added hormone (Fig. 1). Similarly, immunization of rabbits with Xb-BSA yielded after 5 weeks antisera directed against androstenedione, with titers of about 1:3000 (Fig. 2). The affinity constant (Ka) of the antiserum to IXa-BSA for testosterone, derived from a Scatchard plot (16), was 9.4×10^9 l/mol and its binding capacity for testosterone was 0.86 μ mol/l.

The antiserum to Xb had a Ka-value for androstenedione of 1.04×10^{10} l/mol and its binding capacity was 0.16 μ mol/l. The affinity for testosterone of the antisera to IX-BSA was thus about 50 times higher than that reported for the testosterone-binding globulin of normal plasma (17), and hence the serum affords higher sensitivity when used in a competitive protein binding assay. The natural binding globulin has no appreciable affinity for androstenedione (18).

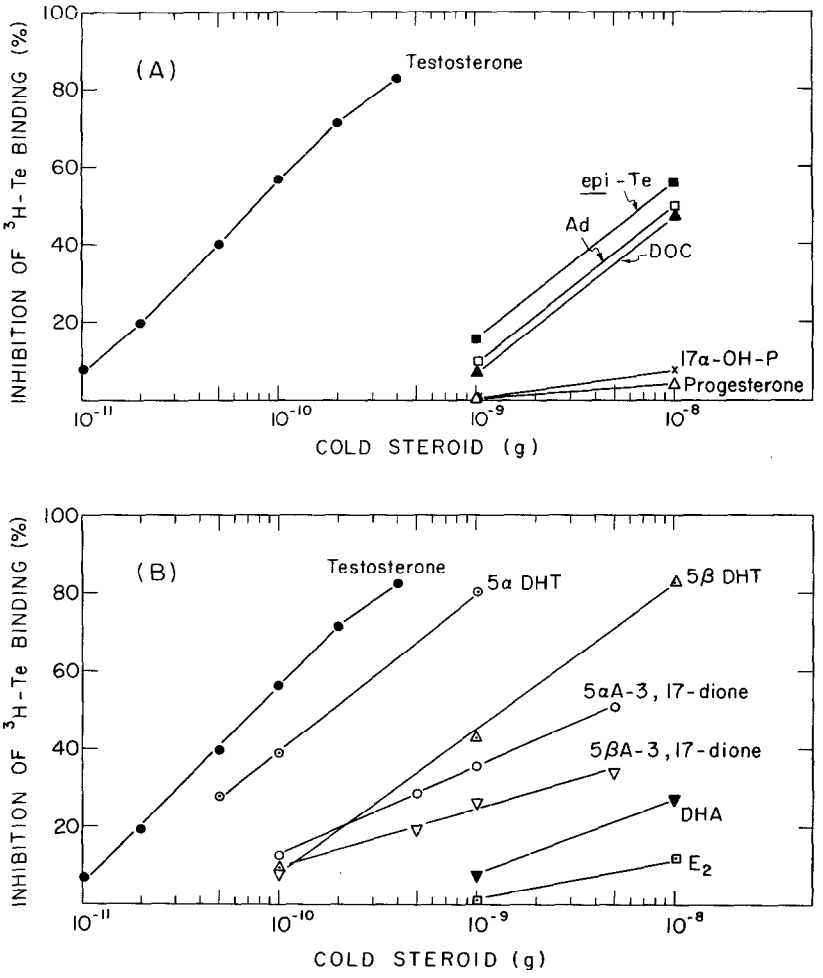

Fig. 1. (A & B) Effect of various steroids on the binding of tritiated testosterone (20 pg) by rabbit antiserum (diluted 1:4000) to 7-conjugate of testosterone (IXa-BSA). Abbreviations: Ad = androstenedione; epi-Te = 17α-epitestosterone; 5αDHT = 5α-dihydrotestosterone; 5βDHT = 5β-dihydrotestosterone; 5αA-3, 17-dione = 5α-androstane-3, 17-dione; 5βA-3, 17-dione = 5β-androstane-3, 17-dione; DHA = dehydroepiandrosterone; DOC = 11-deoxycorticosterone; 17α-OH-P = 17α-hydroxyprogesterone; E_2 = estradiol-17β.

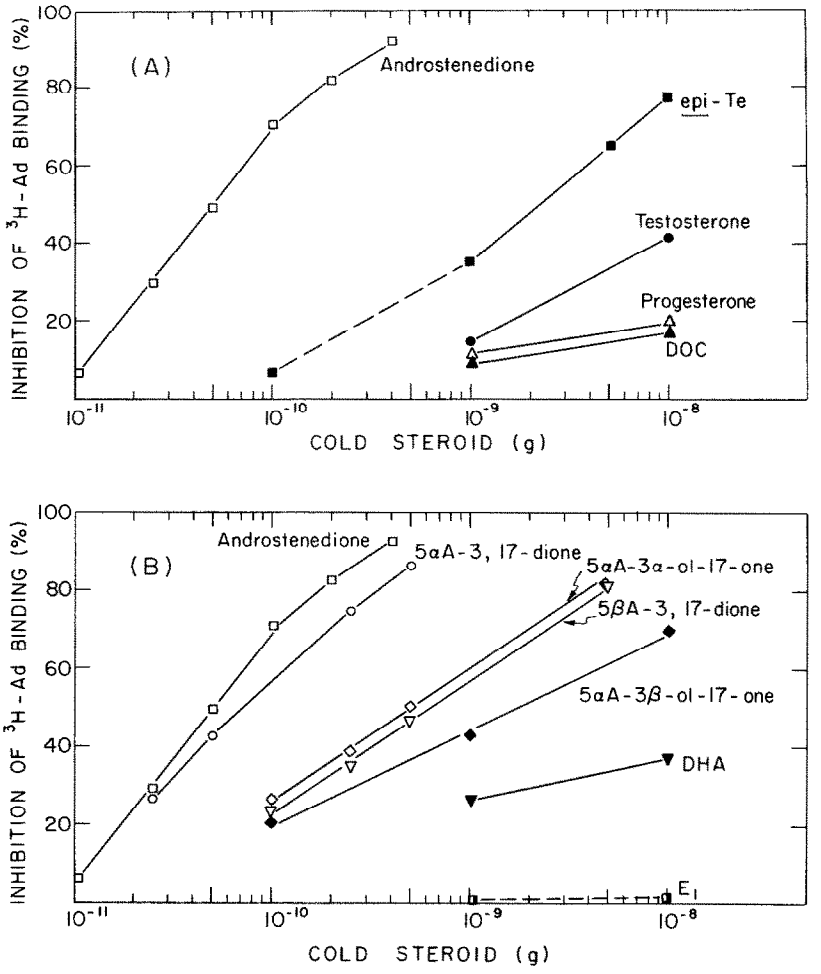

Fig. 2. (A & B) Effect of various steroids on the binding of tritiated androstenedione (20 pg) by rabbit antiserum (diluted 1:3000) to 7-conjugate of androstenedione (Xa-BSA). Abbreviations: 5α-3α-ol-17-one = 3α-hydroxy-5α-androstan-17-one; 5αA-3β-ol-17-one = 3β-hydroxy-5α-androstan-17-one; E_1 = estrone; for other compounds see legend to Fig. 1.

The anti-IXa-BSA and anti IXb-BSA sera showed only negligible cross-reaction (< 1%) with androstenedione (Fig. 1 and Table 2); conversely, the anti-Xb-BSA serum showed little if any cross-reaction (< 0.5%) with testosterone (Fig. 2 and Table 2). None of these sera reacted significantly with the closely related steroids 17α-epitestosterone (1-2%) or dehydroepiandrosterone (< 0.5), or with 11-deoxycorticosterone (<0.5%), progesterone, 17α-hydroxyprogesterone, estrone and estradiol-17β (< 0.1%). However, the anti-testosterone sera cross-reacted markedly with 5α-dihydrotestosterone (40-50%) and 17β-hydroxyandrost-4,6-dien-3-one (100%). These compounds have an overall skeletal shape similar to that of testosterone (see Fig. 3, V & XIII). On the other hand, there was only moderate cross-reaction with 5β-dihydrotestosterone ($\simeq$ 5%; cf. Fig. 3, XIV) and with 5α-androstane-3,17β-diols (7-17%), and almost none with 17-oxoandrostanes ($\leq$ 0.5%; Table 2). The antiserum to androstenedione showed minimal cross-reaction with 5α- or 5β-dihydrotestosterone (1% and < 0.1%, respectively), but did react markedly with 5α-androstane-3,17-dione (71%), and moderately with the other 17-oxo-androstane derivatives tested (4-10%; Table 2).

The ability of the anti-IX sera to discriminate between testosterone, androstenedione and epitestosterone contrasts with the lack of specificity of antisera raised to 17β-hemisuccinyl conjugates of testosterone towards steroids differing only in substitution at C-17 (3): the latter antisera cross-react markedly even with progesterone (55%) and 11-deoxycorticosterone (55%). However, the specificity of the new sera described in this paper with respect to changes about the D-ring is matched by antisera to 3-conjugates of testosterone (3, 20, 21).

Table 2. Specificity of antisera raised in rabbits with 7-conjugates of different androgens

Compound	Cross Reaction (%)* Anti-Testosterone (anti-IXa-BSA)	Anti-Testosterone (anti-IXb-BSA)	Anti-Androstenedione (anti-Xb-BSA)
Testosterone	100	100	<0.5
Androstenedione	<1	<1	100
17α-epitestosterone	1	1	2
5α-Dihydrotestosterone	42	55	1
5β-Dihydrotestosterone	5	4	<0.1
5α-Androstane-3,17-dione	0.5		71
5β-Androstane-3,17-dione	<0.5		8
3α-Hydroxy-5α-androstan-17-one (androsterone)	<0.5		10
3β-Hydroxy-5α-androstan-17-one (epiandrosterone)	<0.5		4
5α-Androstane-3α,17β-diol	17	8	
5α-Androstane-3β,17β-diol	7		
Dehydroepiandrosterone	<0.5	<0.5	<0.5
11-Deoxycorticosterone	<0.5	<0.1	<0.1
Progesterone	<0.1	<0.1	<0.1
17α-Hydroxyprogesterone	<0.1		
Estradiol-17β	<0.1		
Estrone			<0.1

* Defined (19) as $\frac{100x}{y}$, where x is the mass of unlabeled homologous steroid and y the mass of the heterologous compound required to produce 50% inhibition of the binding of tritiated testosterone or androstenedione by antibody.

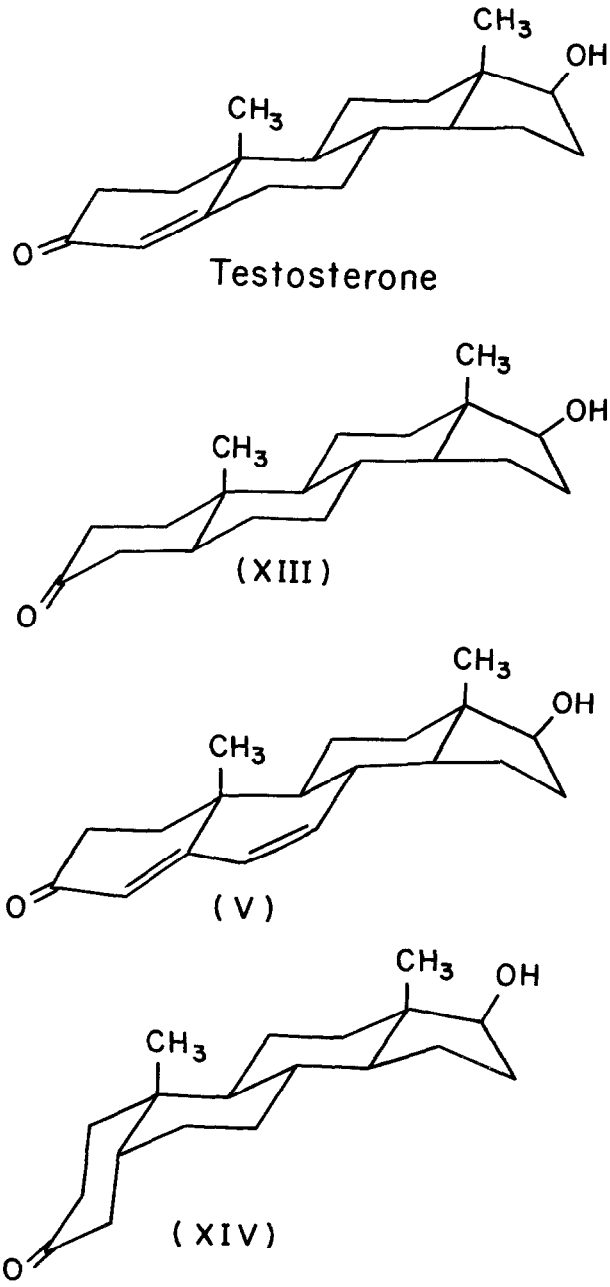

Fig. 3. Comparison of structure of testosterone with that of steroids showing extensive (V, XIII) or minor (XIV) cross-reaction with anti-IX-BSA sera.

Surprisingly, the latter sera were also able to distinguish between 3-oxo and 3-hydroxy-androstane derivatives, although the 3-oxo group is lost during formation of the 3-oxime conjugate used as antigen. Both types of sera (to 3- and 7-conjugates) cross-react significantly with 5α and 5β-dihydrotestosterone. However, the concentration of these steroids in human peripheral plasma appears to be exceedingly low (22).

The antisera to androstenedione are thus far unique in being able to recognize this steroid specifically and to distinguish it clearly from testosterone.

The production and properties of antisera to 7-conjugates of progesterone and 17α-hydroxyprogesterone will be the subject of a later publication.

Radioimmunoassay. Further support for the specificity of the new anti-testosterone serum (anti-IXa-BSA) derives from preliminary data obtained by their use in radioimmunoassay of testosterone in human plasma extracts prepared without prior chromatography. Unlabeled testosterone (0.5 to 2.5 ng/ml) added to this serum was recovered with an efficiency of 93%±3.8 S.E. (6 samples; coefficient of variation 10%). No testosterone was detected in serum from a $3\frac{1}{2}$-year-old girl. Sera from normal non-pregnant women aged 25-35 years collected at random during the menstrual cycle at 8-10 a.m. contained 0.41 ng/ml ± 0.045 S.E. testosterone (9 subjects), and sera from normal 30 to 40-year-old men contained 5.2 ng/ml±0.43 S.E. testosterone (12 subjects). These data are in reasonable agreement with those obtained by fluorimetry (23), double isotope dilution assay (24), gas-liquid chromatography (25), competitive protein binding assay using the testosterone-binding globulin (26) and

by different types of radioimmunoassay with and without prior chromatographic purification of the steroid (21, 22, 27, 28). This agreement suggests that the concentration of steroid hormones or metabolites cross-reacting with the new antiserum to testosterone in normal human serum must be low.

EXPERIMENTAL

General. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Spectra were obtained with a Perkin Elmer Model 237 Grating Spectrophotometer in KBr pellets in the infrared range and Model 402 in the ultraviolet range. Nmr spectra were scanned on a Varian A-60 spectrometer or a Bruker Scientific High Resolution HFX-10 NMR Instrument with a Fourier Fast Transform analyser, using tetramethylsilane as internal standard; spectra of methyl esters of IX-XII were obtained on the latter machine. Mass spectra were determined with an Atlas CH-4 spectrometer.

Ether was washed with aqueous ferrous sulphate (29) and distilled immediately before use.

Mercaptoacetic acid was purchased from British Drug Houses (Poole, U. K.); β-mercaptopropionic acid from K & K Laboratories, Inc. (Plainview, N. Y.); 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride from Ott Chemical Co. (Muskegon, Michigan); crystalline bovine serum albumin from Mann Research Laboratories Inc. (New York, N. Y.).

[1,2-^{3}H]testosterone (45 Ci/mmol) and [1,2-^{3}H]androstenedione (48 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, Mass.). Radioactivity was measured with a Liquid Scintillation Spectrometer (Packard Instrument Co., Model 3310), using Instagel (Packard Instrument Co.) as the scintillation fluid.

17β-Hydroxyandrost-4,6-dien-3-one (V) was prepared according to the method of Djerassi et al. (9) by bromination of testosterone acetate to yield 6-bromotestosterone acetate, m.p. 144-146°; λ_{max}^{EtOH} 249 nm (ε 18 500), ν_{max} 1730, 1675, 1610 cm^{-1}; nmr ($CDCl_3$) 0.90 (18-CH_3), 1.56 (19-CH_3), 2.05 (acetate methyl), 5.00 (6-H), 5.92 (4-H) δ. The bromo compound was dehydrobrominated in refluxing collidine for 15 min to yield 17β-acetoxyandrost-4,6-dien-3-one, m.p. 147-148°; λ_{max}^{EtOH} 284 nm (ε 26 500; ν_{max} 1740, 1665, 1620 and 1580 cm^{-1}; nmr ($CDCl_3$) 0.85 (18-CH_3), 1.10 (19-CH_3), 2.00 (acetyl methyl), 4.65 (17α-H), 5.58 (4-H), 6.05 (6- and 7-H). Hydrolysis of the 17β-acetoxy group in 20% methanolic KOH for 18 hr yielded 17β-hydroxyandrost-4,6-dien-3-one, m.p. 204-205°; λ_{max}^{EtOH} 287 nm (ε 21 000); ν_{max} 3450, 3320, 3210 (OH), 1665, 1650 (conjugated C=O), 1620, 1585 (C=C) cm^{-1}; nmr 0.85 (18-CH_3), 1.14 (19-CH_3), 3.67 (17α-H), 5.72 (4-H), 6.14 (7-H) δ. Lit. values (9) for 6-bromotestosterone acetate: m.p. 140-142°; λ_{max}^{EtOH} 248 nm (ε 15 500);

for 17β-acetoxyandrost-4-6-dien-3-one: m.p. 143-144°; λ_{max}^{EtOH} 284 nm (ε 33 880); for 17β-hydroxyandrost-4,6-dien-3-one: m.p. 204-205°, λ_{max}^{EtOH} 284 nm (ε 26 920).

Androst-4,6-diene-3,17-dione (VI) was prepared as reported in the literature (9) by dehydrobromination of 6-bromoandrost-4-ene-3,17-dione, m.p. 174°, λ_{max}^{EtOH} 244 nm (ε 12 400); ν_{max} 1735, 1680, 1610 cm^{-1}; nmr ($CDCl_3$) 1.02 (18-CH_3), 1.58 (19-CH_3), 5.05 (6-H), 5.93 (4-H) δ. VI was obtained as yellowish needles m.p. 168-169°; λ_{max}^{EtOH} 285 nm (ε 25 200); ν_{max} 1740, 1655, 1615 and 1580 cm^{-1}; nmr ($CDCl_3$) 0.95 (18-CH_3), 1.16 (19-CH_3), 5.73 (4-H), 6.22 (6- and 7-H) δ; literature values (9) for 6-bromoandrost-4-ene-3,17-dione: m.p. 175-177°; λ_{max}^{EtOH} 240 nm (ε 17 000); for androst-4,6-dione-3,17-dione: m.p. 168-170°; λ_{max}^{EtOH} 282 nm (ε 33 110).

Pregn-4,6-diene-3,20-dione (VII) was prepared according to the literature (6b) from 6-bromoprogesterone m.p. 144-146°; λ_{max}^{EtOH} 246 nm (ε 14 200); ν_{max} 1695, 1665, 1616 cm^{-1}; nmr ($CDCl_3$) 0.73 (18-CH_3), 1.54 (19-CH_3), 2.14 (21-CH_3), 4.97 (6-H), 5.94 (4-H) δ. Refluxing of the bromo compound for 15 min in collidine yielded pregn-4,6-diene-3,20-dione m.p. 134-136°; λ_{max}^{EtOH} 286 nm (ε 26 000); ν_{max} 1700, 1665, 1620, 1590 cm^{-1}; nmr ($CDCl_3$) 0.73 (18-CH_3), 1.14 (19-CH_3), 2.15 (21-CH_3), 5.73 (4-H), 6.17 (6- and 7-H) δ; literature values for 6-bromoprogesterone (30): m.p. 139-141°, λ_{max}^{EtOH} 283 nm (ε 24 800); for VII (6b): m.p. 143-145°; λ_{max}^{EtOH} 248 nm (ε 16 600).

17α-Hydroxypregn-4,6-diene-3,20-dione (VIII) was prepared according to the method of Agnello and Laubach (11). 17α-Hydroxypregn-4-ene-3,20-dione (5 g) in freshly distilled tert-butanol (350 ml) containing acetic acid (5.6 ml) was refluxed with chloranil for 3 hr. The excess chloranil was filtered off and the filtrate evaporated to dryness. The residue was dissolved in chloroform (300 ml) and the solution washed repeatedly with water (1/10 vol.), sodium hydroxide solution (5%; 1/10 vol.) and again with water (1/10 vol.). After drying, the chloroform solution yielded tan-colored crystals which on recrystallisation from acetone/petroleum ether yielded 2.6 g of off-white crystals m.p. 254-256°; λ_{max}^{EtOH} 287 nm (ε 24 800); ν_{max} 3400 (OH), 1695 (C=O), 1640 (conjugated C=O, 1615 and 1580 (C=C) cm^{-1}; these constants were identical with those of an authentic sample kindly made available by Syntex Corp., Palo Alto. Nmr ($CDCl_3$) 0.79 (18-CH_3), 1.10 (19-CH_3), 2.25 (21-CH_3), 5.70 (4-H), 6.12 (6- and 7-H) δ.

Preparation of 7α-thioethers. 6,7-Dehydrosteroid (2 mmol) was stirred for 1-3 days in the presence of the mercaptoacid (15 mmol) and sodium methoxide (20 mmol) in dioxane (10 ml). Water and ice were than added (75 ml) and the resultant mixture extracted with ethyl acetate (2 x 20 ml). The aqueous phase was acidified with hydrochloric acid (10%). If the precipitate so formed was oily (viz. IXa and XIIa), the acidified mixture was extracted with ethyl acetate (2 x 30 ml), the organic phase extracted with saturated sodium hydrogen carbonate solution (2 x 15 ml) which was then carefully acidified with concentrated hydrochloric acid. The acidified mixture was left overnight at 4° and the resultant crystals filtered, washed well with water, and dried in a desiccator. Recrystallisation of the resultant 7-thioether derivatives was carried out in acetone/

petroleum ether.

Compounds IX-XII dissolved in a minimum of methanol were methylated by treatment with an ethereal solution of diazomethane. After evaporation of the solvent under N_2 the methyl esters were recrystallised from acetone/petroleum ether.

17β-Hydroxyandrost-4-en-3-one-7α-carboxymethyl thioether (IXa): λ_{max}^{EtOH} 243 nm (ε 17 900); ν_{max} 3265 (OH), 2610 (carboxyl OH), 1710 (carboxyl C=O), 1665 (conjugated C=O), 1615 (C=C) cm^{-1}; nmr [$(CD_3)_2SO$] 0.67 (18-CH_3), 1.19 (19-CH_3), 3.24 (S-CH_2), broad band between 3.35 and 3.80 (17α-H and 7β-H), 5.73 (4-H) δ .

The methyl ester had m.p. 195-196°; λ_{max}^{EtOH} 243 nm (ε 12 700); ν_{max} 3440 (OH), 1725 (ester C=O), 1665 (conjugated C=O), 1620 (C=C) cm^{-1}; nmr ($CDCl_3$) 0.81 (18-CH_3), 1.24 (19-CH_3), 3.22 (S-CH_2), 3.29 (quartet, J=3.5 Herz; 7β-H), 3.77 (ester methyl and 17α-H), 5.79 (4-H) δ ; M^+ at m/e 392.

The methyl ester was acetylated with acetic anhydride in pyridine overnight and yielded white needles m.p. 149-150°; λ_{max}^{EtOH} 243 nm (ε 13 600); ν_{max} 1740 (acetyl C=O), 1730 (ester C=O), 1665 (conjugated C=O), 1620 (C=C) cm^{-1}; nmr ($CDCl_3$) 0.86 (18-CH_3), 1.23 (19-CH_3), 2.06 (acetyl methyl), 3.20 (S-CH_2), 3.28 (quartet, J=3.5 Herz; 7β-H), 3.74 (ester methyl), 4.64 (17α-H), 5.79 (4-H) δ ; M^+ at m/e 434.

Androst-4-ene-3,17-dione-7α-carboxymethyl thioether (Xa): λ_{max}^{EtOH} 242 nm (ε 13 300); ν_{max} 2600 (carboxyl OH), 1730 (carboxyl C=O and 17-one), 1640 (conjugated C=O) cm^{-1}; nmr [$(CD_3)_2SO$] 0.82 (18-CH_3), 1.19 (19-CH_3), 3.26 (S-CH_2), 3.39 (7β-H), 5.73 (4-H) δ.

The methyl ester had m.p. 141-142°; λ_{max}^{EtOH} 242 nm (ε 14 500); ν_{max} 1743 (C=O), 1730 (ester C=O), 1670 (conjugated C=O), 1620 (C=C) cm^{-1}; nmr ($CDCl_3$) 0.93 (18-CH_3), 1.26 (19-CH_3), 3.23 (S-CH_2), 3.40 (quartet, J=3.5 Herz; 7β-H), 3.76 (ester methyl), 5.80 (4-H) δ ; M^+ at m/e 390.

Pregn-4-ene-3,20-dione-7α-carboxymethyl thioether (XIa): λ_{max}^{EtOH} 242 nm (ε 13 100); ν_{max} 2610 (carboxyl OH), 1730 (carboxyl C=O), 1700 (C=O), 1645 (conjugated C=O), 1610 (shoulder, C=C) cm^{-1}; nmr [$(CD_3)_2$ SO] 0.59 (18-CH_3), 1.18 (19-CH_3), 2.08 (21-CH_3), 3.21 (S-CH_2 superimposed on 7β-H), 5.71 (4-H) δ

The methyl ester had m.p. 165-167°; λ_{max}^{EtOH} 243 nm (ε 13 200); ν_{max} 1740 (ester C=O), 1695 (C=O), 1670 (conjugated C=O), 1610 (C=C) cm^{-1}; nmr ($CDCl_3$) 0.69 (18-CH_3), 1.22 (19-CH_3), 2.14 (21-CH_3), 3.22 (S-CH_2), 3.26 (quartet, J=3.5 Herz; 7β-H), 3.76 (ester methyl), 5.78 (4-H) δ ; M^+ at m/e 418.

17α-Hydroxypregn-4-ene-3,20-dione-7α-carboxymethyl thioether (XIIa): λ_{max}^{EtOH} 244 nm (ε 13 500); ν_{max} 3500 (OH), 2610 (carboxyl OH), 1727 (carboxyl C=O), 1700 (C=O), 1645 (conjugated C=O) cm^{-1}; nmr [$(CD_3)_2SO$] 0.57 (18-CH_3), 1.19 (19-CH_3), 2.10 (21-CH_3), 3.23 (S-CH_2 superimposed on 7β-H), 5.72 (4-H) δ

The methyl ester had m.p. 138-139°; λ_{max}^{EtOH} 242 nm (ε 13 200); ν_{max} 3380 (OH), 1730 (ester C=O), 1700 (C=O), 1660 (conjugated C=O), 1620 (C=C) cm^{-1}; nmr ($CDCl_3$) 0.73 (18-CH_3), 1.21 (19-CH_3), 2.24 (21-CH_3), 3.16 (S-CH_2), 3.24 (quartet, J=3.5 Herz; 7β-H), 5.70 (4-H) δ ; M^+ at m/e 434.

17β-Hydroxyandrost-4-en-3-one-7α-carboxyethyl thioether (IXb): λ_{max}^{EtOH} 244 nm (ε 14400); ν_{max} 3530 (OH), 2630 (carboxyl OH), 1730, 1710 (carboxyl C=O), 1660, 1640 (conjugated C=O), 1620 (C=C) cm^{-1}; nmr (C_5D_5N) 0.98 (18-CH_3), 1.12 (19-CH_3), 3.20 (7β-H), 3.85 (17α-H), 6.07 (4-H) δ.

The methyl ester had m.p. 182-184°; λ_{max}^{EtOH} 243 nm (ε 14000); ν_{max} 3450 (OH), 1725 (ester C=O), 1655 (conjugated C=O), 1615 (C=C) cm^{-1}; nmr ($CDCl_3$) 0.77 (18-CH_3), 1.21 (19-CH_3), 3.06 (quartet, J=3.5 Herz; 7β-H), 3.66 (ester methyl superimposed on 17α-H), 5.73 (4-H) δ ; M^+ at m/e 406.

Androst-4-ene-3,17-dione-7α-carboxyethyl thioether (Xb): λ_{max}^{EtOH} 244 nm (ε 14700); ν_{max} 2630 (carboxyl OH), 1735 (carboxyl C=O and 17-one), 1645 (conjugated C=O), 1610 (C=C) cm^{-1}; nmr (C_5D_5N) 0.84 (18-CH_3), 1.08 (19-CH_3), 3.29 (7β-H), 6.08 (4-H) δ.

The methyl ester had m.p. 145-146°; λ_{max}^{EtOH} 241 nm (ε 16000); ν_{max} 1735 (17-one), 1730 (shoulder, ester carbonyl), 1670 (conjugated C=O), 1612 (C=C) cm^{-1}; nmr ($CDCl_3$) 0.91 (18-CH_3), 1.22 (19-CH_3), 3.16 (quartet, J=3.5 Herz; 7β-H), 3.66 (ester methyl), 5.72 (4-H) δ ; M^+ at m/e 404.

Pregn-4-ene-3,20-dione-7α-carboxyethyl thioether (XIb): λ_{max}^{EtOH} 244 nm (ε14600); ν_{max} 2640 (carboxyl OH), 1725 (carboxyl C=O), 1700 (C=O), 1640 (conjugated C=O), 1610 (C=C) cm^{-1}; nmr (C_5D_5N) 0.66 (18-CH_3), 1.07 (19-CH_3), 2.08 (21-CH_3), 3.19 (7β-H), 3.66 (ester C=O), 5.71 (4-H) δ ; M^+ at m/e 432.

The methyl ester had m.p. 122-123°; λ_{max}^{EtOH} 242 nm (ε 15100); ν_{max} 1730 (ester C=O), 1690 (C=O), 1665 (conjugated C=O), 1615 (C=C) cm^{-1}; nmr ($CDCl_3$) 0.68 (18-CH_3), 1.20 (19-CH_3), 2.11 (21-CH_3), 3.04 (quartet, J=3.5 Herz; 7β-H), 3.66 (ester methyl), 5.71 (4-H) δ ; M^+ at m/e 432.

17α-Hydroxypregn-4-ene-3,20-dione -7α-carboxyethyl thioether (XIIb): λ_{max}^{EtOH} 244 nm (ε 15000); ν_{max} 3590 (OH), 2640 (carboxyl OH), 1730 (carboxyl C=O), 1695 (C=O) cm^{-1}; nmr (C_5D_5N) 0.77 (18-CH_3), 1.11 (19-CH_3), 2.42 (21-CH_3), 3.25 (7β-H), 6.08 (4-H) δ.

The methyl ester had m.p. 148-149°; λ_{max}^{EtOH} 244 nm (ε 14700); ν_{max} 3370 (OH), 1735 (ester C=O), 1695 (C=O), 1660 (conjugated C=O), 1610 (C=C) cm^{-1}; nmr ($CDCl_3$) 0.74 (18-CH_3), 1.21 (19-CH_3), 2.26 (21-CH_3), 3.08 (quartet, J=3.5 Herz; 7β-H), 3.66 (ester methyl), 5.73 (4-H) δ ; M^+ at m/e 448.

Preparation of IX-BSA - XII-BSA: Steroid derivative IX-XII (50 mg) was stirred in a mixture of dimethylformamide (5 ml) and water (2 ml) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (50 mg) for thirty minutes. To this was added BSA (100 mg) dissolved in phosphate buffer (0.02M, pH 7.8, 10 ml) and the solution stirred for two days. Dialysis of this solution overnight against sodium hydrogen carbonate solution (0.05M) and against frequent changes of water for two days followed by lyophilization yielded the steroid-protein conjugate as a fluffy white powder. The amount of hapten in each preparation was calculated from the absorbancy at 248 nm in 0.1 N-NaOH. The steroid-protein conjugates contained an average of 10 hapten residues per molecule of BSA.

Immunization procedure: Antigen (steroid-BSA conjugate, 2 mg per animal) was dissolved in 2 ml saline and emulsified with an equal volume of complete Freund's adjuvant (CFA). Four-month-old female rabbits were injected once with this emulsion into multiple intradermal sites. The rabbits were bled from

the marginal ear-vein five weeks after injection, and again one month later. However, only the earlier sera were fully characterized.

Characterization of sera was performed as previously described (4), except that equilibration of the antiserum with the tritiated steroid was done at 37°C for 2 hr. Association constants and binding capacity were derived from a Scatchard plot (16) of data obtained by incubating a constant amount of antiserum with increasing amounts of labeled steroid at 37°C for 3 hr.

Radioimmunoassay of testosterone. Serum samples (0.1 ml from normal adult men or 0.4 ml. from normal non-pregnant women), to which [^{3}H]testosterone (2000 dpm), 0.05 M Tris-HCl buffer (pH 8.0, containing 0.1 M NaCl and 0.1% w/v NaN_3) to a total volume of 0.5 ml, and 0.1 ml of conc. ammonia solution had been added, were extracted with 4 ml ether. An aliquot (1 ml) of the organic phase was taken to dryness and used to determine the percentage recovery of the added tritiated testosterone by liquid scintillation counting. The mean recovery was 98% ± 4.4 SEM (10 samples). Another aliquot (1 ml from the ether extract of female sera and 0.5 ml. from those of male sera) was withdrawn into disposable glass tubes (10 x 75 mm), evaporated to dryness and redissolved in 0.1 ml of Tris buffer containing 1.5% (v/v) serum from normal female rabbits. Antiserum to testosterone (0.4 ml), appropriately diluted to bind 40-50% of the labeled steroid (15-20 pg) in the absence of cold hormone, was added. After standing for 30 min at room temperature, 0.1 ml of a solution of [^{3}H]testosterone (15-20 pg) in the Tris buffer was added, and the samples were incubated for 2 hr at 37°C. Separation of bound from unbound steroid was effected by use of dextran-coated charcoal as previously described (4).

When applied to ether-extracts from 0.4 ml Tris-buffer ('water blank'), the radioimmunoassay yielded no detectable value.

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