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RADIOIMMUNOASSAY OF ANDROSTENEDIONE: THE STEROID MOLECULE AS A PROBE FOR ANTIBODY SPECIFICITY

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SUMMARY

Antibodies were obtained against androstenedione‡ linked either through the 1α- or 7α-position to carrier proteins. All the antibodies had high affinity for androstenedione, with greater specificity for this compound than for other steroids tested.

In the development of a radioimmunoassay for this steroid it was found that cumulative cross-reactions with plasma steroids made necessary a pre-purification step for accurate quantification.

We investigated the determinants of antibody specificity for androstenedione and found that the 3-oxo-4-ene, 17-oxo and 3-oxo functional groups on the steroid were the major sites of antibody–hapten interaction. Heterologous steroids sharing these substituents showed significant cross-reaction with the antibodies, while those lacking these determinants did not. The binding affinities were determined mainly by the individual functional groups of the steroid molecule, and appeared to increase when more than one determinant was present.

INTRODUCTION

The preparation of "specific" antibodies against steroids has been attempted by many workers [1–11]. The method generally used has been the preparation of antigens in which the steroid is linked to carrier proteins at sites remote from the biologically important functional groups. The expectancy has been that the resulting antibodies would recognize more specifically a particular steroid hormone, in contrast to antibodies obtained using antigens where the functional groups characteristic of the hormone were used for the coupling [12, 13]. Although the use of the former method has yielded, in general, more specific steroid antibodies than the latter, absolute specificity towards the entire steroid molecule has not been achieved. It is known, however, that antibodies may recognize small portions of the hapten molecule [14, 15].

To further study whether antibodies recognize particular functional groups of the steroid molecule or the overall steroid structure, we have studied antibodies prepared against androstenedione coupled *via* the 1α-position [8] to carrier proteins and compared them with antibodies obtained against androstenedione coupled *via* the 7α-position [3]. A radioimmunoassay (RIA) for androstenedione was developed and the relationship between steroid structure and the specificity of the antibody–antigen reaction was studied.

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‡ The following trivial names have been used: androstenedione, 4-androstene-3,17-dione; testosterone, 17β-hydroxy-4-androsten-3-one; 5α-dihydrostestosterone, 17β-hydroxy-5α-androstan-3-one; 17α-methyltestosterone, 17β-hydroxy-17α-methyl-4-androsten-3-one; 11β-hydroxyandrostenedione, 11β-hydroxy-4-androstene-3,17-dione; 5β-dihydrotestosterone, 17β-hydroxy-5β-androstan-3-one; epiandrosterone, 3β-hydroxy-5α-androstan-17-one; androsterone, 3α-hydroxy-5α-androstan-17-one; etiocholanolone, 3α-hydroxy-5β-androstan-17-one; 11-oxoepiandrosterone, 3β-hydroxy-5α-androstane-11,17-dione; 11β-hydroxyandrosterone, 3α,11β-dihydroxy-5α-androstan-17-one; 11-oxoetiocholanolone, 3α-hydroxy-5β-androstane-11,17-dione; 11β-hydroxyetiocholanolone, 3α,11β-dihydroxy-5β-androstan-17-one; 16α-hydroxydehydroepiandrosterone, 3β,16α-dihydroxy-5-androsten-17-one; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; estrone, 3-hydroxy-1,3,5(10)-estratrien-17-one; estradiol, 1,3,5(10)-estratriene-3,17β-diol; estriol, 1,3,5(10)-estratriene-3,16α,17β-triol; cholesterol, 5-cholesten-3β-ol; pregnenolone, 3β-hydroxy-4-androsten-3-one; 5α-dihydrostestosterone, 17β-dihydroxy-5-pregnen-20-one; progesterone, 4-pregnene-3,20-dione; 17-hydroxyprogesterone, 17-hydroxy-4-pregnene-3,20-dione; 17,21-dihydroxyprogesterone, 17,21-dihydroxy-4-pregnene-3,20-dione; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; 11β-hydroxyprogesterone, 11β-hydroxy-4-pregnene-3,20-dione; 18-hydroxyprogesterone, 18-hydroxy-4-pregnene-3,20-dione; cortisol, 11β,17,21-trihydroxy-4-pregnene-3,20-dione; cortisone, 17,21-dihydroxy-4-pregnene-3,11,20-trione; aldosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione-18-al.

EXPERIMENTAL

Solvents, reagents, and glassware

Anhydrous ethyl ether, boric acid, and ammonium sulfate were analytical reagents, while isooctane (2,2,4-trimethylpentane) and ethyl acetate were nanograde (Mallinckrodt Chemical Works). Methanol and ethanol were distilled twice before use. Dioxane was

dried over Linde's molecular sieve Type 4Å 1/16 in. pellets (Allied Chemical) and filtered before use. Tributylamine and isobutyl chloroformate were obtained from Eastman Organic Chemicals. Bovine serum albumin (Fraction V) was purchased from Armour Pharmaceutical Company; bovine gamma-globulins BGII (Cohn Fraction II), bovine thyroglobulin Type I, and 1-ethyl-3-(3-dimethylaminopropylcarbodiimide) were obtained from the Sigma Chemical Company. Ethylene glycol (chromatoquality) and celite analytical filter-aid were obtained from Matheson, Coleman & Bell. The celite was fired overnight in a muffle furnace at 800°C. Androstenedione and the other steroids used in the studies of specificity were obtained from Steraloids, Inc., and from Ikapharm. [1,2,6,7-^{3}H]-Testosterone (S.A. ~ 100 Ci/mmol) was obtained from New England Nuclear. Disposable culture tubes (12 × 75 mm) were cleaned in a self-cleaning oven. Kimble 5 ml disposable serological pipets were used for preparation of chromatography mini-columns [16].

Physical measurements. Melting points were determined with a Koefler microstage melting point apparatus and are reported uncorrected. Ultraviolet spectra were obtained using a Hitachi–Perkin–Elmer Coleman 124 spectrophotometer; the infra-red spectra were obtained with a Perkin–Elmer spectrophotometer Model 237 using potassium bromide pellets. The mass spectrum was measured in an Atlas CH-7 spectrometer, and nuclear magnetic resonance (n.m.r.) spectra were obtained with a Varian A-60A spectrometer, using deuterium chloroform as solvent and tetramethylsilane as internal standard.

Synthesis of [1,2,6,7-^{3}H]-androstenedione. [1,2,6,7-^{3}H]-Testosterone (0·25 mCi, 0·0008 mg) in 0·25 ml benzene–ethanol (9:1 v/v) was transferred to a 16 × 125 mm tube (Teflon cap) and dried with nitrogen at 40°C. One ml of acetone was added followed by 50 µl of Jones reagent [17] (chromium trioxide 1·03 g, concentrated sulfuric acid 0·87 ml, and water 3 ml). After stirring and leaving at room temperature for 10 min, 2 ml of water was added and this was followed by 3 extractions with 7 ml of ethyl ether. The organic layer was backwashed twice with 1 ml of water, dried with nitrogen and the product purified by chromatography on a celite mini-column as described below. The minimum yield of [1,2,6,7-^{3}H]-androstenedione obtained in several experiments was 86%. The purified product was dried with nitrogen at 40°C, dissolved in 10 ml of ethanol and kept at 4°C.

Radioimmunoassay buffer. Borate buffer 0·05 M, pH 7·9, contained 0·5% BSA, 0·1% gamma globulin and [1,2,6,7-^{3}H]-androstenedione (~8000 c.p.m./ml). This buffer was stored at 4°C.

Haptens: (A) *Androstenedione-1α-carboxyethyl-thioether.* The title compound was synthesized by the reaction of 1,4-androstadiene-3,17-dione (Steraloids, Inc.) with β-mercaptopropionic acid (Eastman Organic Chemicals) in the presence of base, as follows: 1,4-Androstadiene-3,17-dione (1·0 g) was dissolved in 20 ml of dry dioxane, and 2·8 ml of β-mercaptopropionic acid was added. Linde's molecular sieve Type 4Å (2·0 g) was added and this was followed by sodium methoxide (2·6 g) (Matheson, Coleman & Bell). The mixture was stirred for 1 h and left for 72 h at room temperature where a gel was formed. Dioxane (20 ml) was added to disperse the gel, followed by 120 ml of ice-cold water. The resulting solution was extracted 3 times with 20 ml ethyl acetate and the remaining aqueous phase was acidified with a solution of 7 ml concentrated hydrochloric acid in 150 ml of water. Crystals began to separate, and the mixture was left at 4°C for 15 h. The precipitate was collected by filtration, washed extensively with water and air dried. Thin-layer chromatography (t.l.c.) of the product, which was carried out on silica gel GF_{254} using the solvent system ethyl acetate–benzene–acetic acid (20:4:0·25 by vol.), indicated that no starting material was present. However, a minor contaminant was observed which migrated ahead of androstenedione-1α-carboxyethyl–thioether. The product (400 mg) was purified using a modified Bush B-3 system (hexane–benzene–methanol–water 6:4:8:2 by vol.) on a celite column. The fractions containing the steroid derivative, on slight evaporation at room temperature, produced crystals of pure androstenedione-1α-carboxyethyl–thioether. The crystals were filtered and recrystallized twice from acetone to give analytical material m.p. 177–183°C; λ_{max} (ethanol) 240 nm (ε = 13,800); I.R./cm., (KBr) 3450 (carboxyl OH), 1744 ($C_{17}C$ = 0), 1725 (carboxyl C = 0), 1642 (conjugated C = 0), 1620 (C = C); n.m.r. ($CDCl_3$) δ, 0·92 (18-CH_3), 1·37 (19-CH_3), 2·70 (—S—CH_2—CH_2—COOH,m), 3·25 (1β-H,t,J = 3·5 Hz), 5·77 ppm (4-H,s). The carboxylic acid hydrogen appeared superimposed upon the 4-H signal at 5·77 ppm. Addition of one drop of deuterium oxide shifted part of that signal corresponding to 1 hydrogen, to 6·63 ppm leaving the signal corresponding to the 4-H at 5·77 ppm. The mass spectrum of the methyl ester derivative (obtained by reaction of androstenedione-1α-carboxyethyl–thioether with diazomethane in ethyl ether) showed a small molecular ion peak at *m/e* 404.

The assignment of the 1α-orientation of the thioether substituent is based on the coupling constants of 3·5 Hz observed with the C-1 proton, which indicates coupling of the 1β-H with both the 2α-equatorial and 2β-axial protons [18].

(B) *Androstenedione-7α-carboxymethyl–thioether.* The title compound was prepared as described by Weinstein *et al.*[3] and showed the following physical characteristics: m.p. 211–216°C; λ_{max} (ethanol) 238 nm (ε = 13,000); I.R./cm., (KBr) 1740 ($C_{17}C$ = 0), 1725 (carboxyl C = 0), 1640 (conjugated C = 0); n.m.r. ($CDCl_3$) δ, 0·90 (18-CH_3), 1·25 (19-CH_3), 3·27 (—S—CH_2—COOH,s), 5·83 (4-H,s).

Antigens. The protein–steroid conjugate used to immunize rabbits M86A and M86B (Table 1) was prepared according to the method reported by Erlanger *et al.*[12]. The number of steroid molecules incorporated per molecule of protein are shown in Table 1.

Table 1

Antigens injected into rabbits showing the number of hapten molecules coupled per molecule of protein[a], titers[b], and sensitivities[c] of the antibodies obtained in this study.

Rabbit		Antigen	Molecules Hapten per Molecule Protein	Antibody Titers	Sensitivity (pg)
M43	A	Androstenedione-7α-	7.2[e]	4,500	5
	B	carboxymethyl-thioether		2,400	5
	C	bovine serum albumin		2,600	6
M45	A	Androstenedione-7α-	129[e]	560	5
	B	carboxymethyl-thioether		9,200	5
	C	thyroglobulin		8,200	5
M47	A	Androstenedione-1α-	22.3[e]	600	6
	B	carboxyethyl-thioether		620	5
	C	bovine serum albumin		1,300	5
M86	A	Androstenedione-1α-	285[f]	9,200	5
	B	carboxyethyl-thioether thyroglobulin[d]		8,200	5

a) The number of steroid molecules conjugated per molecule of protein were calculated by differential ultra-violet spectroscopy as described by Erlanger et al. (12).
b) The initial antibody dilutions at which 50% binding to androstenedione-1,2,6,7-^{3}H were obtained, are reported as titers.
c) The sensitivity of the standard curve is defined as the 95% confidence limit of the zero dose (19).
d) Two rabbits were immunized with this antigen.
e) Incorporated using water soluble 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide as coupling reagent.
f) Incorporated using the mixed anhydride technique (12).

Antigens used with rabbits M43, M45, and M47 (Table 1) were prepared by the carbodiimide method, and were dialyzed and lyophilized essentially as described by Weinstein *et al.*[3]. The number of steroid molecules incorporated per molecule of protein are indicated in Table 1.

Antibodies. The antibodies to androstenedione were produced in New Zealand 4-month-old male rabbits. Conjugates of androstenedione-1α-carboxyethyl–thioether with BSA and with Tg, and of androstenedione-7α-carboxymethyl–thioether with the same protein carriers were used for immunization: 2 mg of each antigen was emulsified with 1 ml of Freund's complete adjuvant and with enough saline solution to bring the final vol. to 2 ml. Immunizations were by the multiple intradermal technique or by subcutaneous injection in four sites: three rabbits were used per antigen with the exception of M86 where only two were injected. Three weeks after immunization, a booster injection containing 1 mg of antigen was given subcutaneously. Two weeks after the first booster injection the rabbits were bled and a booster injection was given. Bleedings were repeated at 2-week intervals and each one was followed by additional injections of antigen. Blood cells were removed by centrifugation and the plasma was stored at −20°C.

Extraction of androstenedione from human plasma. Two ml of male or female human plasma was used for extraction. To each sample 25 μl of an ethanolic solution containing [1,2,6,7-^{3}H]-androstenedione (approximately 2000 c.p.m., 8 pg) was added as internal recovery standard. After stirring the plasma was extracted once with 7 ml of ethyl ether. The ethyl ether was evaporated to dryness with nitrogen and the residue was chromatographed on celite mini-columns.

Chromatography on "mini-columns". Separation of androstenedione and isopolar compounds from other plasma steroids was carried out by using the celite mini-column method reported by Abraham *et al.*[16], slightly modified. The column was prepared by packing a mixture of 1·0 g of celite mixed with 0·75 ml of ethylene glycol into a serological pipet and then conditioned with 5·5 ml of 15% ethyl acetate in isooctane followed by 5·5 ml of isooctane. The ethyl ether extract was transferred to the column with 0·5 ml of 5% ethyl acetate in isooctane, and the tube was rinsed with 0·5 ml of isooctane which was transferred to the column (nitrogen pressure was used throughout). Elution of androstenedione was carried out with 5·5 ml isooctane. The bulk of the androstenedione peak (2·5 ml), as determined by using labeled steroid in developmental experiments, was collected and used for RIA.

Radioimmunoassay. A one-half ml aliquot of the isooctane fraction containing the androstenedione was transferred to a scintillation vial to determine the recovery and 200 μl aliquots in triplicate were transferred to 12 × 75 mm glass tubes. Triplicate 0·1 ml ethanolic aliquots containing 0, 10, 25, 50, 100, 250 and 500 pg of androstenedione were used for the standard curve. Each delivery of an aliquot was followed by a rinse with 0·1 ml of methanol containing 2% propylene glycol (Automatic Pipette, Micromedic Systems, Inc.). After drying the solvent mixture *in vacuo* at room temperature, 0·5 ml of RIA buffer containing the appropriate antibody dilution was added to each tube. In addition, one-half ml of RIA buffer without the antibody was added to 6 tubes for calculation of total radioactivity. After incubation for 18 h at 4°C, the bound steroid was precipitated with 0·5 ml of cold saturated ammonium sulfate solution. After shaking and centrifugation for 20 min at 4°C, 0·5 ml aliquots of the supernatants were transferred to minivials (Rochester Scientific Co., Inc.) and to these 4 ml of scintillation fluid [3·8 l. toluene, 15·0 g Omnifluor (New England Nuclear) and 76 ml methanol] was added. The vials were capped and shaken thoroughly to effect partition of the radioactive steroid into the organic phase. After phase separation, which was

Table 2

Cross reactions with androstane derivatives of four antibodies against androstenedione.

	Cross-reaction (%)* Antibody (Antigen)			
	M43B (And-7α-BSA)	M45B (And-7α Tg)	M47C (And-1α-BSA)	M86A (And-1α-Tg)
4-Androstene-3,17-dione	100	100	100	100
11β-Hydroxy-4-androstene-3,17-dione	18.6	1.1	3.2	3.4
5α-Androstane-3,17-dione	55	4.2	52	36.4
3β-Hydroxy-5α-androstan-17-one	2.2	5.0	23.6	11.0
3β-Hydroxy-5α-androstane-11,17-dione	< 0.1	0.2	< 0.1	< 0.1
3α-Hydroxy-5α-androstan-17-one	5.6	< 0.1	7.4	7.8
3α,11β-Dihydroxy-5α-androstan-17-one	0.5	< 0.1	0.2	0.1
17β-Hydroxy-4-androsten-3-one	3.9	1.2	2.6	4.5
17β-Hydroxy-17α-methyl-4-androsten-3-one	< 0.1	< 0.1	0.5	0.4
17β-Hydroxy-5α-androstan-3-one	1.8	< 0.1	0.9	0.8
4-Androstene-3β,17β-diol	< 0.1	< 0.1	< 0.1	< 0.1
5α-Androstane-3α,17β-diol	< 0.1	< 0.1	< 0.1	< 0.1
5β-Androstane-3,17-dione	3.4	0.7	3.9	4.6
3α-Hydroxy-5β-androstan-17-one	1.0	0.4	1.1	2.6
3α-Hydroxy-5β-androstane-11,17-dione	< 0.1	< 0.1	< 0.1	< 0.1
3α,11β-Dihydroxy-5β-androstan-17-one	< 0.1	< 0.1	< 0.1	< 0.1
17β-Hydroxy-5β-androstan-3-one	0.1	< 0.1	< 0.1	0.1
3β-Hydroxy-5-androsten-17-one	1.3	2.3	14.5	14.8
3β,16α-Dihydroxy-5-androsten-17-one	0.2	1.6	< 0.1	< 0.1

*Calculated as $\frac{\text{pg androstenedione at 50\% binding}}{\text{pg heterologous steroid at 50\% binding}} \times 100$, as defined by Thorneycroft, et al. (19).

allowed to take place in the counter (Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3330), the samples were counted to a precision of $\pm 2\%$.

For all standard curves, an initial antibody dilution corresponding to about 70% bound [1,2,6,7-^{3}H]-androstenedione was selected.

Specificity. The relative affinity of heterologous steroids for the antibodies was determined by measuring the displacement of [1,2,6,7-^{3}H]-androstenedione from the antibody, as proposed by Thorneycroft *et al.*[19] (Tables 2, 3 and 4).

RESULTS

Initial antibody dilutions with which 50% of [1,2,6,7-^{3}H]-androstenedione was bound are reported in Table 1. These titers were determined approximately 3 months after immunization. Although no direct correlation was obtained between the number of molecules of androstenedione incorporated per molecule of carrier protein and the titers of the elicited antibodies, it appears that titers were higher with Tg than with BSA as the carrier protein (Table 1 and unpublished observations). The sensitivity [20] of the antibodies obtained in the present study are approximately 6 pg (Table 1).

The specificity of the antibodies [4] was examined with [1,2,6,7-^{3}H]-androstenedione in competition with each of 35 other steroids. Each antibody studied had greater specificity for androstenedione than for any of the other steroids. Of particular interest, antibody M45B showed the lowest cumulative cross-reaction, particularly with the 5α-reduced C_{19}-steroids (Tables 2 and 3). To determine if specificity was effected by the antigen used (androstenedione-7α-carboxymethylthioether-Tg) or by the response of the rabbit itself, a comparative study of cross-reactions with 5α-reduced steroids was carried out with antibodies M45A and M45C, obtained from the two other rabbits immunized at the same time. The results, shown in Table 4, indicate that the high specificity was conferred by the immune response of rabbit M45B rather than by the nature of the antigen.

Thermodynamic parameters

The available thermodynamic information describing the antibody–androstenedione complex reaction is summarized in Table 5. Association constants for

Table 3

Cross reactions with pregnane, cholestane, and estrane derivatives of four antibodies against androstenedione.

	Cross reaction (%)* Antibody (Antigen)			
	M43B (And-7α-BSA)	M45B (And-7α Tg)	M47C (And-1α-BSA)	M86A (And-1α-Tg)
4-Pregnene-3,20-dione	<0.1	3.6	2.1	1.5
17-Hydroxy-4-pregnene-3,20-dione	<0.1	1.2	0.4	0.2
17,21-Dihydroxy-4-pregnene-3,20-dione	<0.1	3.8	<0.1	0.4
21-Hydroxy-4-pregnene-3,20-dione	<0.1	0.8	<0.1	0.4
11β-Hydroxy-4-pregnene-3,20-dione	<0.1	<0.1	0.3	0.4
11β,21-Dihydroxy-4-pregnene-3,20-dione	<0.1	<0.1	<0.1	<0.1
11β,17,21-Trihydroxy-4-pregnene-3,20-dione	<0.1	<0.1	<0.1	<0.1
17,21-Dihydroxy-4-pregnene-3,11,20-trione	<0.1	<0.1	<0.1	<0.1
11β,21-Dihydroxy-4-pregnene-3,18,20-trione	----**	<0.1	<0.1	<0.1
18-Hydroxy-4-pregnene-3,20-dione	<0.1	0.1	<0.1	<0.1
3β-Hydroxy-5-pregnen-20-one	<0.1	<0.1	0.3	0.1
3β,17-Dihydroxy-5-pregnen-20-one	<0.1	<0.1	<0.1	<0.1
5-Cholesten-3β-ol	<0.1	<0.1	<0.1	<0.1
3-Hydroxy-1,3,5(10)-estratrien-17-one	<0.1	<0.1	<0.1	<0.1
1,3,5(10)-estratriene-3,17β-diol	<0.1	<0.1	<0.1	<0.1
1,3,5(10)-estratriene-3,16α,17β-triol	<0.1	<0.1	<0.1	<0.1

*Calculated as described in Table II.
**Not measured.

Table 4

Cross-reactions with selected 5α-reduced steroids of three antibodies against androstenedione, obtained by immunization of rabbits with the androstenedione-7α-carboxymethyl-thioether-throglobulin complex.

Steroid	Cross-reaction* (%) Antibody M45A	M45B	M45C
Androstenedione	100	100	100
17β-Hydroxy-5α-androstan-3-one	0.8	< 0.1	0.4
5α-Androstane-3, 17-dione	47	4.2	55
3α-Hydroxy-5α-androstan-17-one	0.2	< 0.1	3.9

*Calculated as described in Table II.

androstenedione indicate that high-affinity antibodies were obtained with all four antigens in each of the immunized rabbits. These high affinities do not appear to be related to the structure of the protein carrier (BSA or Tg), to the site of steroid conjugation (1α- or 7α-positions), to the method used for coupling [(N,N-dimethyl-3-propyl)-ethyl-carbodiimide or the mixed anhydride technique] or to the difference in immunization procedure (intradermal or subcutaneous). The high entropy values obtained most likely account for the observed standard free energy of the specific reaction [21, 22].

Radioimmunoassay of androstenedione in peripheral plasma

The antibody obtained from rabbit M45B was selected for development and use in routine laboratory analysis of androstenedione. The blank of the assay was approximately 5 pg (Table 1). The overall recovery of androstenedione, after mini-column chromatography of extracts of 2 ml of blood plasma samples, was 78·3% ± 11·3 (mean ± S.D., n = 24). Discarded male blood bank plasma was used to examine accuracy throughout the range of 0–2·0 ng of androstenedione. Figure 1 shows the relation of androstenedione added to androstenedione measured; no systematic error was observed. Intra-assay precision was determined in plasma pools obtained from male and female subjects as well as in discarded blood bank male and female plasma pools. The coefficient of variation for these assays varied from 1·53 to 10·03% (Table 6). Inter-assay precision was examined by analysis of the androstenedione concentration in a blood bank male plasma pool which was measured

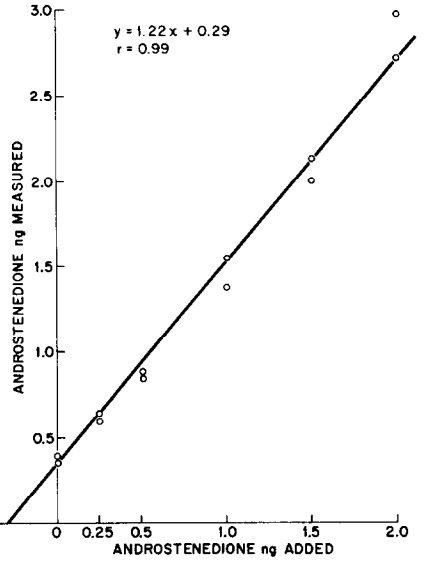

Fig. 1. Androstenedione measured using antibody M45B, plotted against androstenedione added to male blood bank plasma.

throughout a period of 7 months. The mean was 0·41 ng, the standard deviation 0·08 ng (n = 11) and the coefficient of variation 19·7%. The intra-assay precision results are satisfactory for physiological studies.

Quantitation with and without chromatographic separation. The specificity studies (Tables 2 and 3) indicated that the *cumulative cross reactions* of plasma steroids might interfere with direct analysis of androstenedione in either unextracted human plasma or in a solvent extract of it. Data presented in Table 7 indicate that overestimations are obtained when the assays are performed directly on either isooctane or ethyl ether extracts of plasma as opposed to chromatographic separation on a standardized celite mini-column.

DISCUSSION

Steroid antigens may be prepared by conjugation to proteins *via* normal functional groups of steroid hormones [12] or to positions which are removed from these groups [1–11].

Table 5

Thermodynamic functions for the formation of the antibody-androstenedione complexes.

Antibody		K_A (4°C)* (liter/mole)	K_A (25°C)* (liter/mole)	ΔH° (kcal/mole)	ΔF° (4°C) (kcal/mole)	ΔF° (25°C) (kcal/mole)	ΔS° (at 4°C and 25°C) (e.u./mole)
M43	A	7.6×10^9					
	B	6.9×10^9	4.9×10^9	-2.7	-12.5	-13.2	35.4
	C	1.5×10^9					
M45	A	6.8×10^9					
	B	3.8×10^9	2.4×10^9	-3.6	-12.1	-12.8	30.8
	C	2.1×10^9					
M47	A	1.7×10^9					
	B	8.0×10^8					
	C	4.8×10^9	3.1×10^9	-3.4	-12.3	-12.9	32.0
M86	A	3.5×10^9	2.4×10^9	-3.0	-12.1	-12.8	33.0
	B	4.5×10^9					

* Association constants were calculated from a Scatchard plot (26) as modified by Sandberg et al. (27)

Table 6

Intra-assay measurements of androstenedione using antibody M45B.

Plasma Pool	Androstenedione ng/ml (Mean ± S.D., n=5)	Coefficient of variation (%)
Male	1.12 ± 0.112	9.85
Female	1.35 ± 0.136	10.03
Male (blood bank)	0.31 ± 0.017	5.67
Female (blood bank)	0.33 ± 0.005	1.53

We have investigated the conjugation of androstenedione through the 1α-position because this position is relatively remote from the distinctive steroid determinants (3-oxo-4-ene and 17-oxo groups), and because the preparation of the hapten derivative at this position is easy. For this purpose, advantage was taken of the greater reactivity towards nucleophilic reagents shown by the 1,2-double bond of 1,4-androstadiene-3,17-dione [8, 23–25] to synthesize androstenedione-1α-carboxyethyl-thioether.

The radioimmunoassay of androstenedione in plasma required a pre-purification step due to cumulative cross-reactions with other steroids. Celite chromatography separated androstenedione from the more polar steroids eluting in later fractions. The isopolar steroid pregnenolone did not cross-react significantly.

Specificities, as shown by the studies of Landsteiner with benzene–hapten derivatives [14], and subsequently confirmed by other workers [15], are directed towards determinants within the haptenic molecule. This is also the case with the antibodies against androstenedione (Tables 2, 3 and 4). In the specificity study reported here, using the 4 different antibodies against androstenedione, we show that they recognize small regions of the steroid molecule in the context of the whole steroid backbone. The binding of heterologous steroids by these antibodies was blocked, partially or totally, by additional substituents in the steroid molecule.

The representative antibody obtained against androstenedione-1α-Tg (M86A) recognized the 3-oxo-4-ene determinant (Tables 2 and 3) in androstenedione (100%), testosterone (4·5%) and progesterone (1·5%) as well as in 17α-methyltestosterone (0·4%), 17-hydroxyprogesterone (0·2%), 17,21-dihydroxyprogesterone (0·4%), and deoxycorticosterone (0·4%). The decrease in cross-reactions observed when the 17α-methyl, 17α-hydroxy and 21-hydroxy substituents were present indicated that binding by the antibody population which recognized the 3-oxo-4-ene function is only slightly hindered by these substituents. In addition, steroids containing the 3-oxo-4-ene group having a 11β-hydroxy or 11-oxo substituent, showed decreased binding when compared with the corresponding unsubstituted steroids, i.e. 11β-hydroxyandrostenedione (3·4%), 11β-hydroxyprogesterone (0·4%), corticosterone (<0·1%), cortisol (<0·1%), cortisone (<0·1%) and aldosterone (<0·1%). Substituents at C-18 also blocked the binding of the antibody to the 3-oxo-4-ene determinant, as shown with 18-hydroxyprogesterone (<0·1%).

Table 7

Measurement of androstenedione* in male and female plasma pools, directly in solvent extracts and following chromatographic separation.

Solvent	Prepurification	Androstenedione ng/ml (Mean ± S.D., n=3) Male pool	Female pool
Isooctane	none	1.88 ± 0.10	2.31 ± 0.17
Ethyl ether	none	1.77 ± 0.06	2.69 ± 0.10
Ethyl ether	chromatography	1.05 ± 0.12	1.41 ± 0.01

*Antibody M45B was used in these determinations

The isolated 3-oxo function was readily recognized in the 5α-steroid series, as shown with 5α-dihydrotestosterone (0·8%), but very slightly in the 5β-series, i.e. 5β-dihydrotestosterone (0·1%).

The 17-oxo determinant was recognized in the androstane series, in both 5α- and 5β-reduced as well as in 5-ene steroids, as seen with epiandrosterone (11%), androsterone (7·8%), 5β-androstane-3,17-dione (4·6%), etiocholanolone (2·6%), and dehydroepiandrosterone (14·8%). Substituents at the 11-position (11β-hydroxy and 11-oxo groups) appeared to interfere with antibody binding to the 17-oxo function, i.e. 11-oxoepiandrosterone (<0·1%), 11β-hydroxyandrosterone (0·1%), 11-oxoetiocholanolone (<0·1%), 11β-hydroxyetiocholanolone (<0·1%). Substituents at the 16α-position also interfered with binding to the 17-oxo determinant as shown with 16α-hydroxydehydroepiandrosterone (<0·1%). The data suggested that the 17-oxo determinant was recognized by an antibody population requiring a spatial fit involving at least the β-phases or rings C and D, and the α-phase of ring D of the steroid nucleus. The data also indicate that the antibody has a higher binding affinity for 5α-reduced and 5-ene-steroids than for the corresponding 5β-reduced steroids.

Binding with heterologous steroids appears to be increased when more than one recognizable determinant is present on the steroid nucleus, as shown by comparing the binding of 5α-androstane-3,17-dione (36·4%) with 5α-dihydrotestosterone (0·8%), epiandrosterone (11%) and androsterone (7·8%).

Steroids such as 5α-androstane-3α,17β-diol, cholesterol, estradiol and estriol, which do not share a single common functional group with androstenedione were not recognized by the antibody. Estrone, however, which has a 17-oxo group was not bound by the antibody, suggesting either that the C-10 methyl group was required for recognition, or that the aromatic A ring of estrone interfered sterically with the binding.

Similar studies carried out with antibodies obtained against androstenedione-1α-BSA (M47C) and androstenedione-7α-BSA (M43B) confirm the above findings (Tables 2 and 3).

Antibody M45B, which was obtained against androstenedione-7α-Tg, was found to have specificities markedly different from that of the other antibodies obtained in this study, particularly regarding cross-reactions with 5α-reduced C-19 steroids (Tables

2, 3 and 4). It recognized the 3-oxo-4-ene determinant in both C_{19}- and C_{21}-steroids, and this binding was inhibited by a 11β-hydroxy or 11-oxo substituent. Antiserum M45B reacted to the combined determinants of the entire 4-androstene-3,17-dione molecule to a higher degree than the other antibodies studied. Therefore, it was used to develop a radioimmunoassay for androstenedione.

We have shown that antigens obtained by conjugation of androstenedione to BSA or Tg, either through the 1α- or the 7α-positions, elicit antibodies which have similar cross-reactivities.

Data presented in Tables 2, 3 and 4 indicate that the antibodies recognize individual determinants of androstenedione in the framework of the whole steroid structure, thus explaining the observed lack of absolute hapten specificity in cross-reaction studies.

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