Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 283–290. Pergamon Press. Printed in Great Britain

STEREOCHEMICAL APPROACH TO INCREASE THE SPECIFICITY OF STEROID ANTIBODIES

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SUMMARY

Most of the attempts to produce steroid antibodies have utilized one of the functional groups of the steroid molecule to form the corresponding haptene. We have prepared 19-hemisuccinate derivatives of androstenedione, 5α-dihydrotestosterone and testosterone, bound to BSA via the carbodiimide reaction to obtain antibodies with high specificity. With the hemisuccinate linked at the C-19 position the disturbance of the functional groups is reduced, introducing minimal changes and yielding the highest possible resemblance between the antigenic complex and the original steroid molecule. Furthermore, with the hemisuccinate chain in the axial position, the carboxyl group is located far enough away to avoid intramolecular interactions between the protein and the free functional groups. In this form the specificity improved significantly. After 12 weeks of injecting the haptenes into rabbits, we obtained the following antisera: for androstenedione at a dilution of 1:12000, with crossreactivity to androstanedione and epiandrosterone with a displacement effect around 50% of the ^{3}H-androstenedione to dehydroepiandrosterone (DHEA) and testosterone was less than 25%; for dihydrotestosterone the antiserum titres were 1:6000 with crossreaction to testosterone, 5α-androstane 3-17-dione and androstenediol but less than 50% bound and minimal to DHEA and androstenedione; the testosterone antiserum in dilution 1:2000 revealed similar binding for dihydrotestosterone and very low for DHEA and androstenedione. We concluded: 1.—The ring D as well as the A of the steroid molecule were determinants in conferring specificity to the antibodies 2.—The coupling of the protein in the C-19 position near to the ring A together with 3-ketone group and the double bond in C-4 impeded the selective antibody production, decreasing specificity. In view of this 3.—The coupling length chain will be modified to achieve better specificity.

INTRODUCTION

Most of the studies reported in the literature describe the coupling of haptenes to obtain steroid antibodies, using one of the characteristical functional groups of the steroid molecule to conjugate it to a protein [1, 2]. In a few cases the non-characteristic functional groups were used to increase specificity, for instance, the 17α-hydroxyprogesterone antigen affords excellent specificity for progesterone [3]. Also, derivatives coupled at C-6, C-7 or C-11 yielded better results for estrogens [4–6], progestins [7–9] and androgens [10].

We prepared C-19 hemisuccinates of 19-hydroxy-androstenedione, 19-hydroxy-testosterone and 19-hydroxy-5α-dihydrotestosterone and the basic concept behind using these compounds bound to BSA is that having the hemisuccinate chain linked at the C-19 position of the steroid, avoids disturbance of the other functional groups of the original steroid molecule. The only changed atom in the whole molecule is one of the hydrogens of the C-19 methyl group by the succinate chain. This is the minimum possible change which can be made and yields the highest possible resemblance between the haptene and the original steroid molecule. Another consideration in selecting this model was that by having the hemisuccinate chain in the C-19 axial position, the chain ending with the carboxyl group would be located far enough from the steroid molecule to avoid intramolecular interactions between the albumin and the functional groups which are intended to be maintained free, until the corresponding antibodies are formed. In other words, the two factors we consider critical in obtaining antibodies with high specificity are: first, the length and type of chain and its position, and second, the resemblance of the haptene to the steroid from which specific antibodies are desired.

EXPERIMENTAL

As Fig. 1 shows, the 19-hemisuccinate of androstenedione was obtained by the reaction of the 19-alcohol (I) with succinic anhydride and pyridine. Since hemisuccinates are difficult to purify by the usual procedures, it was decided to purify them through their methyl esters; in this way, the methyl succinate (III) was prepared from (II) by treatment with diazomethane. The selective reduction of the C-17 ketone was made following the Normberski method [11] using sodium borohydride in methanol at low temperature, with 70% selectivity, the mixture of the monoalcohol (IV) and the 3,17-diol subproduct was separated by the Girard[12] derivative extraction. Furthermore, a method for the selective halo-

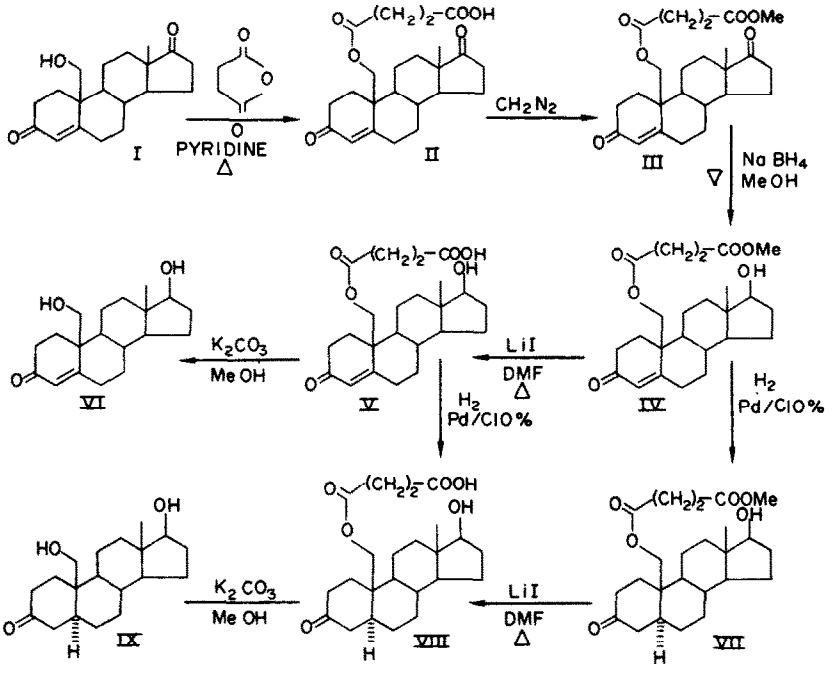

Fig. 1. Synthesis of the 19-hemisuccinate derivatives of androstenedione (II), testosterone (V) and 5α-dihydrotestosterone (VIII).

genolysis of the succinate methyl ester without involvement of the whole chain was applied following the Tachner and Liberech [13] method using an excess of lithium iodide in dimethyl formamide at reflux temperature yielding testosterone-19-hemisuccinate (V). The catalytic hydrogenation of (IV) in methanol with palladium over charcoal (10%) yielded 5α-dihydro-methyl-succinate (VII), which by halogenolysis affords 5α-dihydrotestosterone 19-hemisuccinate. The hemisuccinate (V) and (VII) are oily products and in order to identify these compounds, we saponified them with potasium carbonate in methanol, obtaining their conversion to the 19-alcohols (VI) and (IX); these compounds correspond in all their properties to the reported materials.

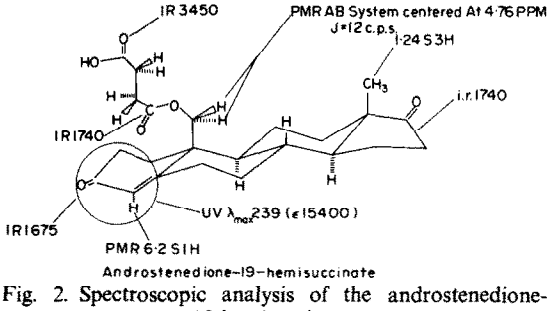

Fig. 2. Spectroscopic analysis of the androstenedione-19-hemisuccinate.

In Fig. 2, we show the spectroscopic data for the androstenedione 19-hemisuccinate. In the U.V., there is a maximum at 239 nm with 15,400, according with its structure; the infrared showed the characteristic band of the carboxy group (3450, very wide), the carbonyl bands of the ester with the 5 membered ketone group at 1740 cm.$^{-1}$ and the carbonyl of the enone at 1675 cm.$^{-1}$.

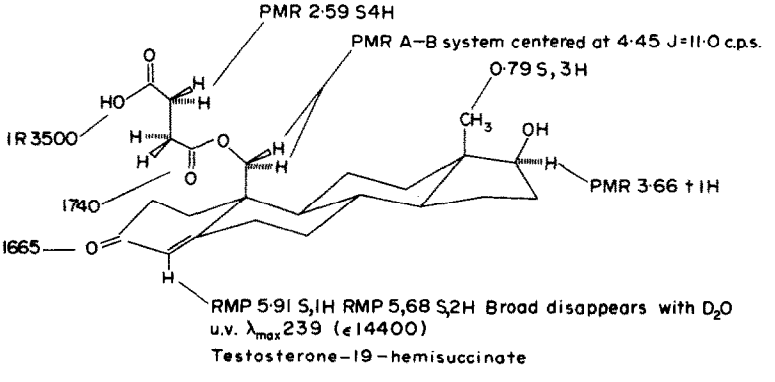

Fig. 3. Spectroscopic analysis of the testosterone-19-hemisuccinate.

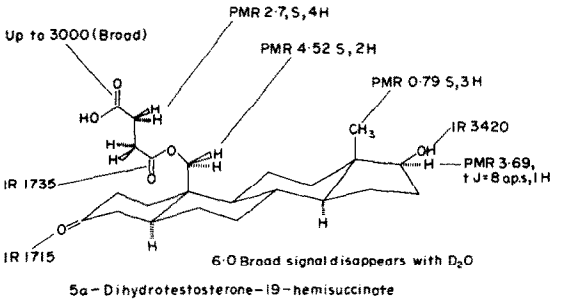

Fig. 4. Spectroscopic analysis of the 5α-dihydrotestosterone-19-hemisuccinate.

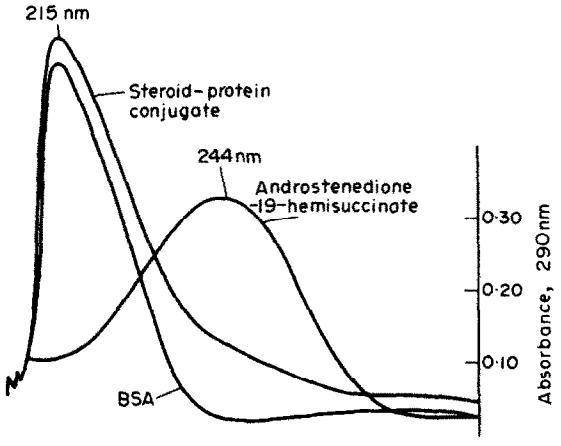

Fig. 5. Ultraviolet spectrum for the 19-hydroxyandrostenedione hemisuccinate steroid residues calculation.

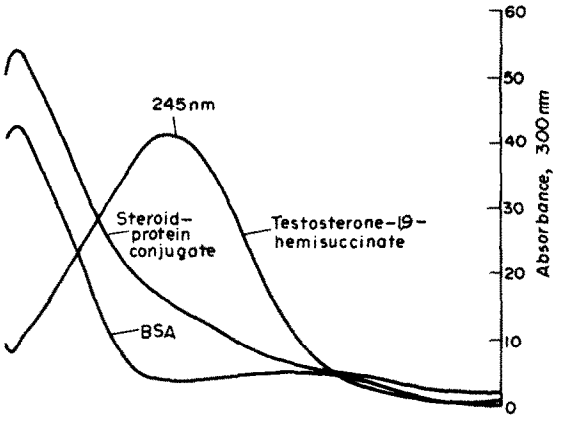

Fig. 6. Ultraviolet spectrum for the 19-hydroxytestosterone hemisuccinate steroid residues calculation.

In the proton magnetic resonance spectrum, we can see the system AB centered at 4·76 ppm with $J = 12$ c.p.s., characteristic of the C-19 methylenes, when rotation between C-10 and C-19 carbons is hindered; we have also the C-18 methyl group at 1·24 ppm and the vynilic proton in C-14 at 6·2 ppm. This spectrum was run in dimethyl sulfoxide-d-6. For this reason, the signals show a little displacement.

In Fig. 3, we have the identification data for the testosterone-19-hemisuccinate which had a U.V. maximum at 239 nm (ε 14,400); the infrared spectrum showed a wide band at 3500 cm.$^{-1}$ characteristic of the carboxylic acids with the hydroxyl at C-17, at 1740 cm.$^{-1}$ the corresponding succinic ester band and at 1665 cm.$^{-1}$ the enone signal at position 3.

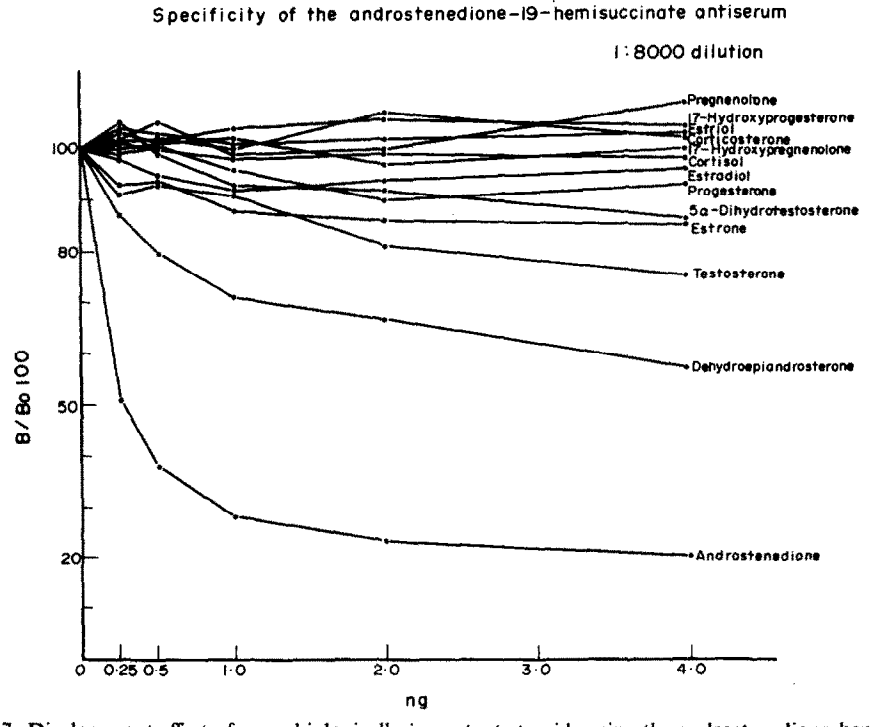

Fig. 7. Displacement effect of some biologically important steroids using the androstenedione hemisuccinate antiserum.

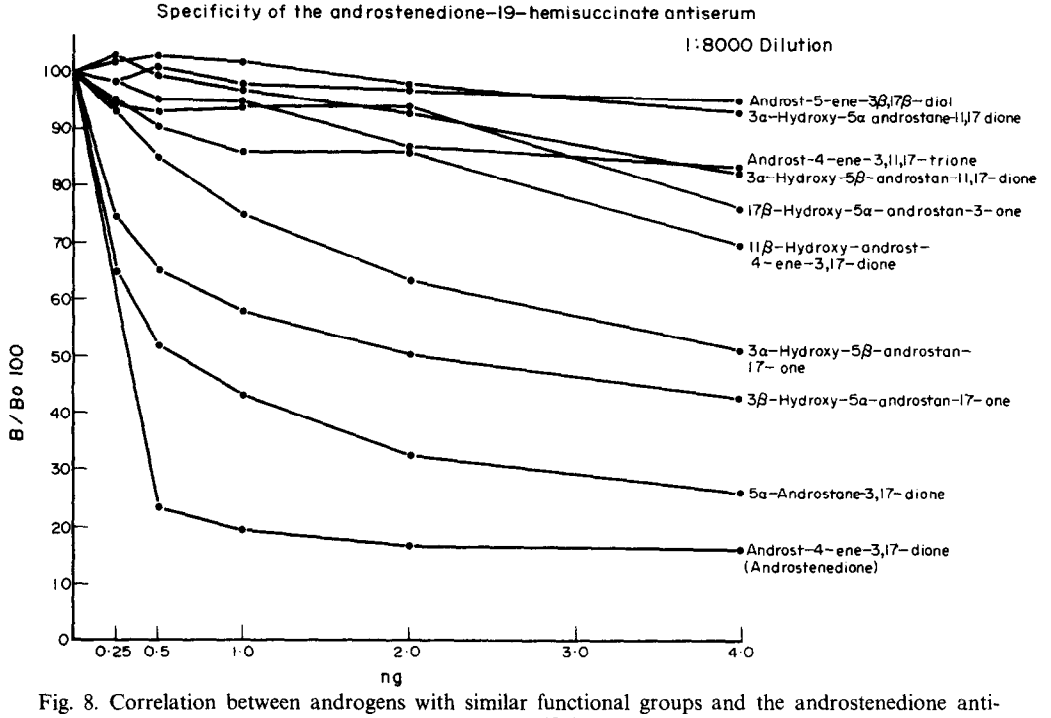

Fig. 8. Correlation between androgens with similar functional groups and the androstenedione antiserum specificity.

The compound shows proton magnetic resonance at 0·79 ppm (18 methyl group), at 2·59 a singlet corresponding to the methylenes of the hemisuccinate chain, at 3·66 a triplet with $J = 8$ c.p.s. characteristic of the base proton of the 17β alcohols; AB system centered at 4·45 with $J = 11$ c.p.s. assigned to the 19-methylene group, at 5·91 a singlet for the vynilic proton at C_3 and at 5·68 there was a broad signal that integrates for two hydrogens and disappears with deuterated water and was assigned to the hydrogens of the hydroxyl and carboxyl groups at C-17 and in the chain respectively. Its position within the field could be explained because of dimerization or intramolecular association.

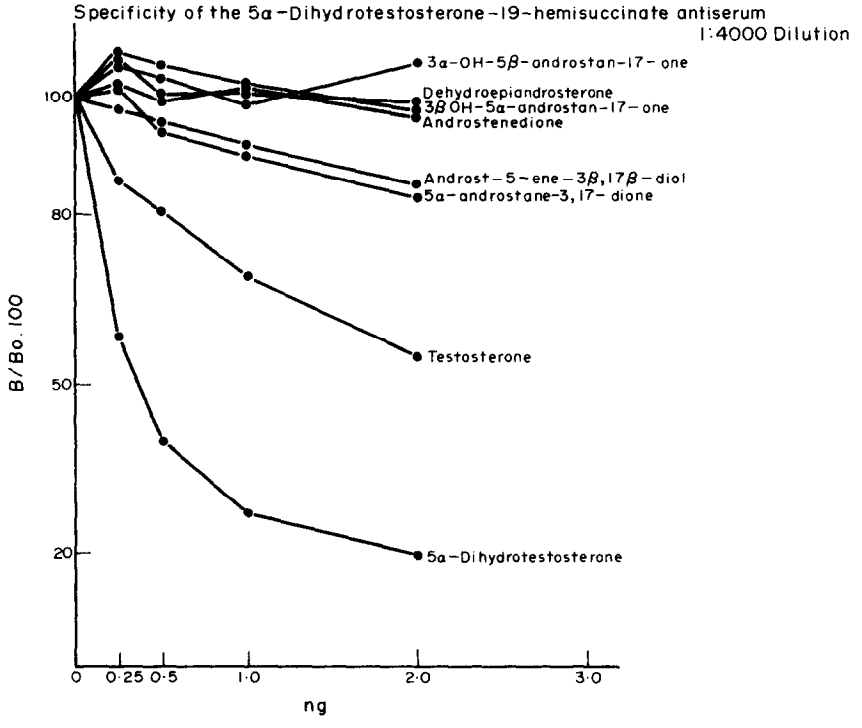

Fig. 9. Displacement effect of some androgens with the 5α-dihydrotestosterone antiserum.

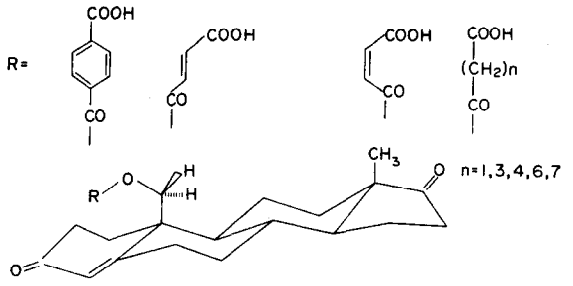

Fig. 10. Various types of chains for coupling the steroid molecule to bovine serum albumin.

The spectroscopic analysis of the 5α-dihydrotestosterone-19-hemisuccinate shows (Fig. 4) no bands in the U.V. spectrum above 200 nm; the I.R. spectrum revealed the wide band of the carboxyl group above 3000 cm^{-1}, with the signal of the 17-hydroxyl group at 3420 cm^{-1}, the ester carbonyl group at 1735 cm^{-1}, the 6 membered ring ketone at 1715 cm^{-1}; in the n.m.r. spectrum we observe the 18 methyl group at 0·79 ppm, the methylene signal of the hemisuccinate chain at 2·7 ppm, the base proton of the 17β-alcohol as a triplet $J = 8$ c.p.s. at 3·69 ppm, and the 19-methylene signal as a singlet at 4·52 ppm. Contrasting with the A–B system observed in the previous compounds, it appears that the hydrogenation of the C-4 double bond gives more conformational mobility to ring A, permitting the free rotation of the C-10–C-19 bond.

The mass spectra of all of these compounds were in agreement with their structures.

The coupling of the 19-hemisuccinates to BSA was performed via the carbodiimide reaction, using potasium phosphate buffer, 0·05 M, at 5°C with agitation during 30 days; the crude extracts were purified by filtration with diaflo membrane PM-30 or dialysis against water and then lyophylized. To determine the number of haptenes incorporated to the BSA, we used Lieberman's method, measuring U.V. absorption at 240 nm, revealing 14 steroid residues for androstenedione. Figure 5 shows the U.V. spectra for the conjugate, androstenedione hemisuccinate and BSA. In the case of testosterone we found 13 steroid residues and the U.V. spectra for BSA, the conjugate and the testosterone-19-hemisuccinate are shown in Fig. 6. Since there is no commercially available radioactive 19-hydroxy-5α-dihydrotestosterone, we did not determine the number of steroid residues attached to BSA.

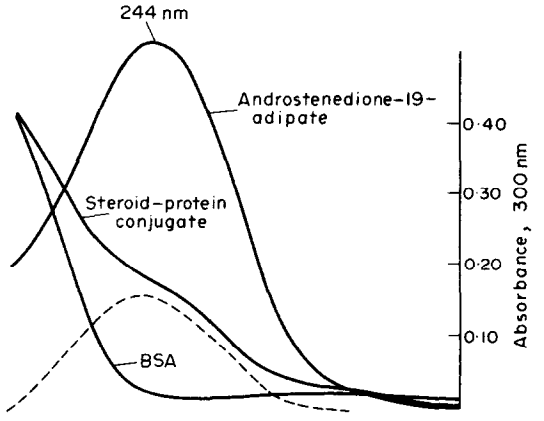

Fig. 12. Ultraviolet spectrum for the 19-hydroxyandrostenedione adipate steroid residues calculation.

The haptenes were injected subcutaneously in male rabbits in a mixture containing 1 ml of the conjugate (3·5 mg) and 1 ml of complete Freund's adjuvant. (The injections were weekly for the first 5 weeks and later on, once or twice a month).

RESULTS

After 6–8 weeks, we obtained a reasonably good immunological response, with titres up to 1:12000 for androstenedione. The antiserum specificity was examined by the displacement effect of ^{3}H-androstenedione by several steroids of biological importance. In Fig. 7, we have graphed in the abscissa the dose in ng and in the ordinate the percentage binding, with zero dose as 100%. None of them displaced 50% of the radioactivity at the maximal dose of 4 ng. Notice that DHEA gave the greater displacement effect.

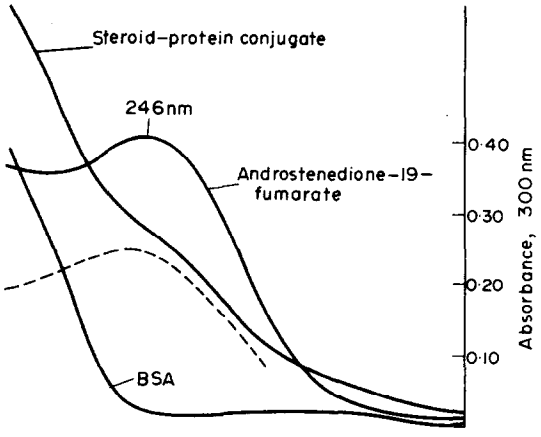

Fig. 11. Ultraviolet spectrum of the 19-hydroxyandrostenedione fumarate for steroid residues calculation.

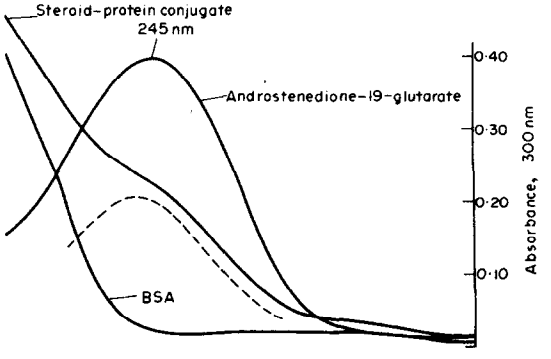

Fig. 13. Ultraviolet spectrum for the 19-hydroxyandrostenedione glutarate steroid residues calculation.

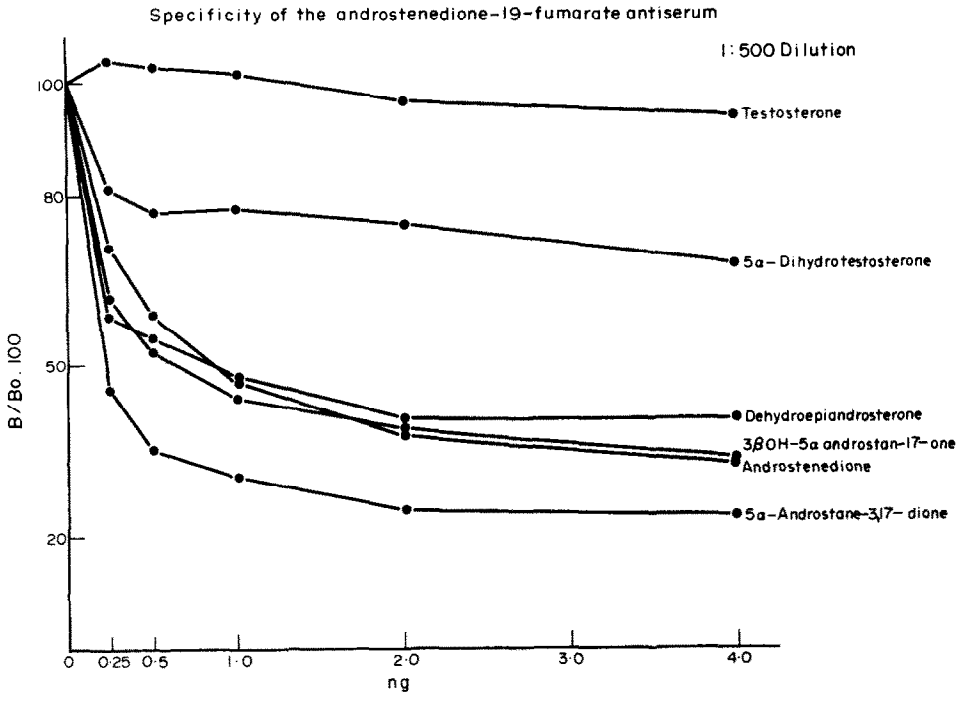

Fig. 14. Cross-reactivity with other androgens of the androstenedione-19-fumarate antiserum.

The correlation between the functional groups and the antiserum specificity shown in Fig. 8, confirmed the importance of ring D with its carbonyl group in C-17, since we found androstanedione and the 3β-hydroxy-5α-androstan-17-one (epi-androsterone) to give the greatest displacement effect; the double bond at C-4 and the 3-ketone group showed less affinity [10].

With the antiserum against 5α-dihydrotestosterone at a dilution of 1:4000 we found in the cross-reaction study, no displacement effect at 50% of any of the steroids examined including testosterone, (Fig. 9).

For the testosterone antiserum at 1:2000 dilution, the crossreaction with 5α-dihydrotestosterone was high, with no difference at 50% bound.

With these results we thought that coupling of the haptene to the protein in the C-19 position near to ring A, together with the 3-ketone group and the

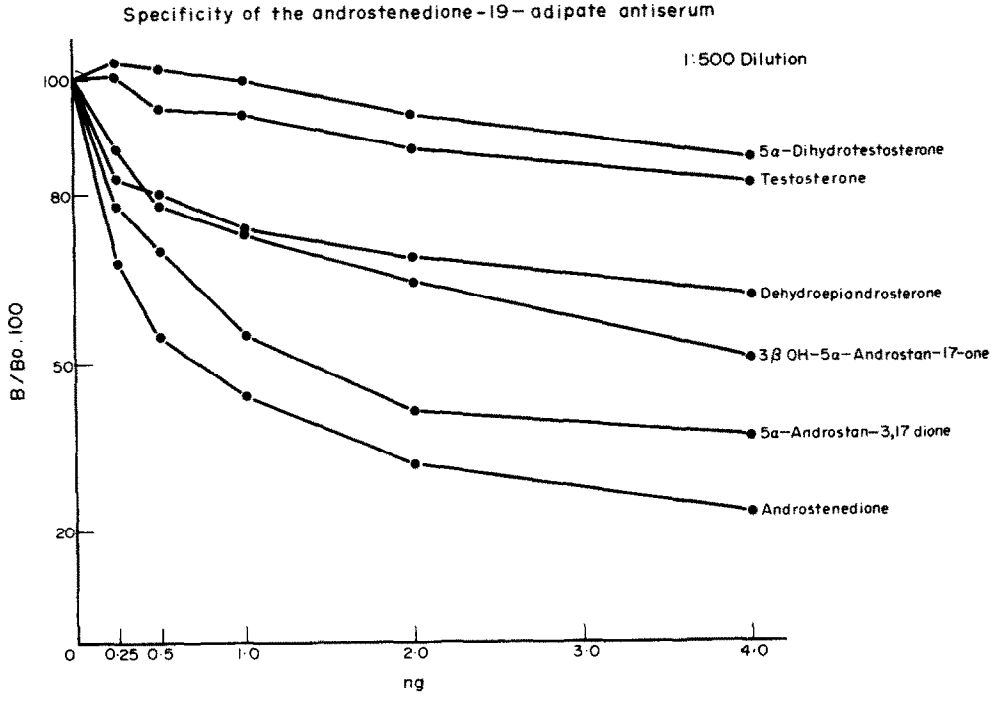

Fig. 15. Cross-reactivity with other androgens of the androstenedione-19-adipate antiserum.

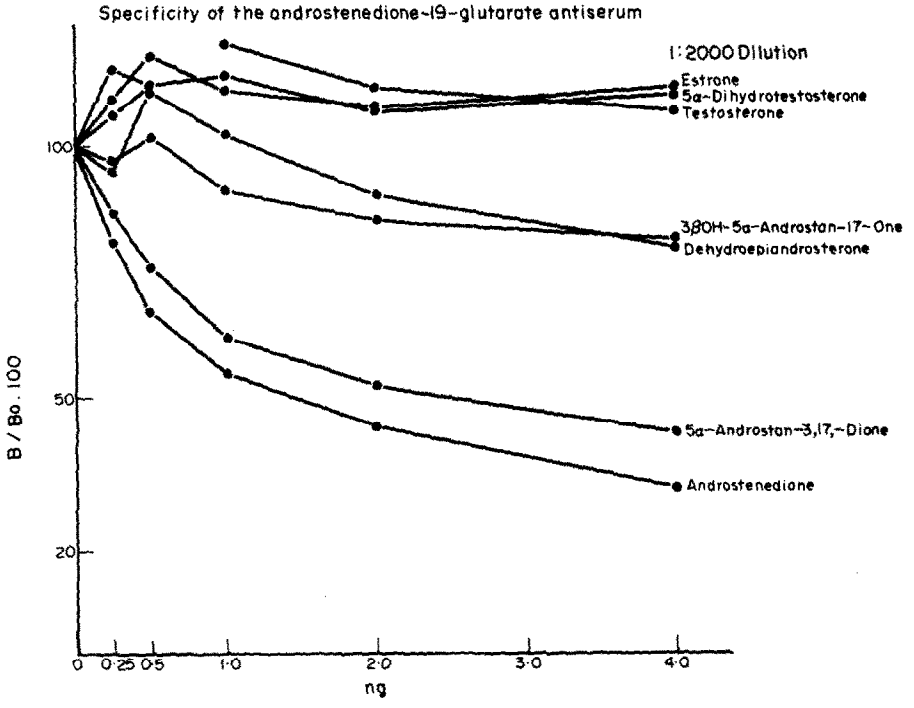

Fig. 16. Cross-reactivity with other androgens of the androstenedione-19-glutarate antiserum.

double bond at C-4 impeded selective antibody production; for this reason, we developed haptenes with different types of chains to couple to BSA. Figure 10 shows rigid structures terephthalate, fumarate and maleate (with 4 carbons, trans and cis configuration), and linear chains with a certain amount of flexibility: malonate (3c), glutarate (5c) and adipate (6c).

The product identification was carried out in a similar way by their spectroscopic characteristics and coupling to BSA was achieved by the carbodiimide reaction, with the exception of the fumarate in which we used Erlanger's method [1] (mixed-anhydride with isobutyl chloroformate and tributylamine).

We have some preliminary results for androstenedione-19-fumarate, in which we obtained 34 steroid residues coupled to BSA, glutarate with 27 residues and 23 for the adipate, all determined by Lieberman's method and shown in Figs. 11, 12 and 13, respectively.

The cross-reaction study for the androstenedione-19-fumarate (1:500 dilution) revealed poor specificity

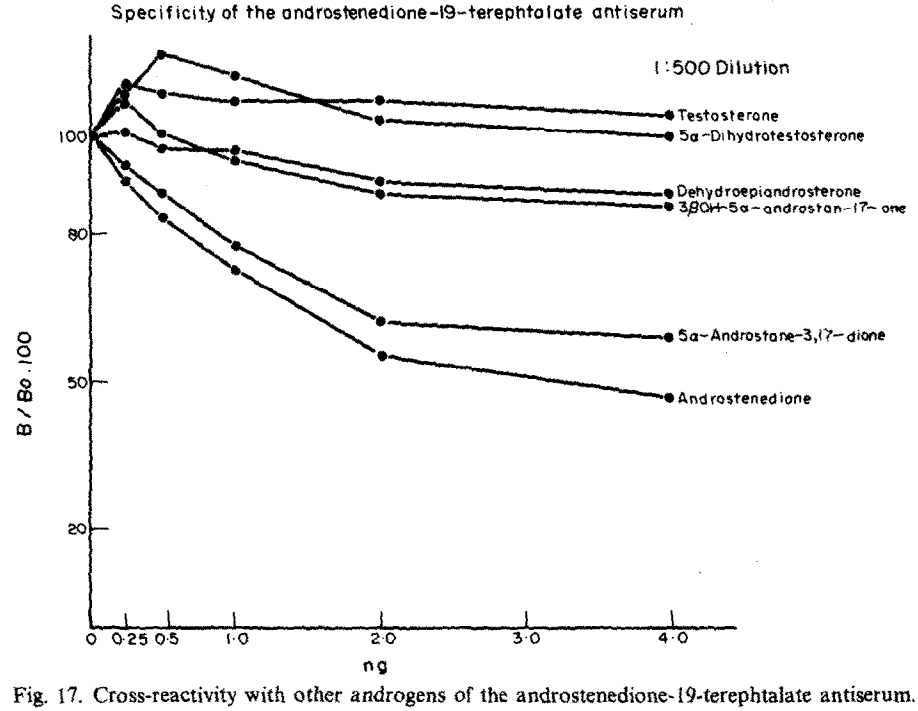

Fig. 17. Cross-reactivity with other androgens of the androstenedione-19-terephtalate antiserum.

with large displacement for all the steroids tested; however, it was less with testosterone and dihydrotestosterone (Fig. 14).

The antiserum for the androstenedione-19-adipate at 1:500 dilution showed a different pattern displacement (Fig. 15). The arrangement is by pairs of steroids with very similar structures.

With the androstenedione-19-glutarate antiserum (Fig. 16) the previous findings were reproduced but with less crossing-over among the steroids (1:2000 dilution).

Finally, in Fig. 17, we have represented the results of the cross-reaction study with the androstenedione-19-terephthalate antiserum, revealing a good discrimination. Androstenedione and its reduced form showed a similar displacement effect.

DISCUSSION

It seems that with these preliminary results our working hypothesis was confirmed, since the adipate with 6-carbon and the glutarate with 5-carbon chains yielded a slightly better specificity of the antibodies obtained; furthermore, the androstenedione-19-terephthalate (a rigid chain) antiserum showed similar specificity, having the highest cross-reaction for 5α-androstane-3-17-dione, 76% (considering as 100% the displacement effect of androstenedione at 4 ng).

These findings suggested the existence of at least two populations of antibodies, one for the original compound and the other for its metabolites. A reasonable explanation for this, could be the catabolism of the antigen within the rabbit tissue, eliminating the double bond at carbon 4 and producing another antigenic structure yielding a different set of antibodies.

Acknowledgements—To Miss Susana Ortiz de Elguea and Miss Raquel Salas for preparing the different types of chains.

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