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THE FORMATION OF STEROID-BSA ANTIGENS VIA A C-6β-CARBOXYMETHYL SUBSTITUENT

W. J. RILEY,* E. R. SMITH, D. M. ROBERTSON and A. E. KELLIE
The Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W1P 5PR, England

SUMMARY

Steroid haptens joined covalently to macromolecules such as bovine serum albumin may be used as antigens to raise antisera for the radioimmunoassay of steroids. Since in most applications of this technique the covalent link has been made through one or other of the steroid functional groups, it was thought that improved specificity might be achieved by effecting the union with the macromolecule at a position remote from the functional groups which appear to play a role in the determination of hormone specificity.

A new and versatile method of attachment at the C-6 position of the steroid nucleus (Ring B) has been described which leaves functional groups in Rings A & D free to act as antigenic discriminants. Synthetic routes to 6-carboxymethyltestosterone and 6-carboxymethylprogesterone have been described and these compounds have been covalently linked to bovine serum albumin. The resulting complexes have been used as antigens in rabbits to raise antisera for the radioimmunoassay of testosterone and progesterone. The sensitivity and specificity of the immune reactions of these antibodies towards the steroid hormones and cognate compounds is described.

IT IS recognized from the work of Erlanger *et al.*[1, 2] that steroids joined covalently to macromolecules such as bovine serum albumin may be used to raise antibodies which show some specificity towards the steroid hapten used. Such antibodies have been used with considerable success for the radioimmunoassay of steroids[3, 4] but in all these applications the covalent bond used to join the steroid to the macromolecule has been made by modifying one or other of the steroid functional groups. As pointed out by Midgley and Niswender[5] it may be advantageous to effect a union between the steroid and the antigenic molecule at a position in the steroid nucleus remote from the functional groups which appear to play a prominent role in the determination of hormone specificity.

A new and versatile method of attachment to the B-ring at the C-6 position of the steroid nucleus has been developed which leaves the functional groups in rings A and D free to act as antigenic discriminants. The work has been illustrated with reference to the introduction of a 6-carboxymethyl substituent but the principle has considerable latitude in that it could be applied to any carboxyalkyl substituent at any point of the steroid nucleus where an epoxide structure can be formed.

Synthetic route to 6-carboxymethyl steroids

The routes to 6β-carboxymethyltestosterone and 6β-carboxymethylprogesterone are based on standard methods for the formation of 6-methylsteroids[6, 7]. Dehydroepiandrosterone(3β-hydroxy-5-androsten-17-one) (Fig. 1, I) was reduced with borohydride to 5-androstene-3β,17β-diol and the latter was acety-

*Present address: Royal Perth Hospital, Box X2213 GPO Perth, W. Australia 6001.

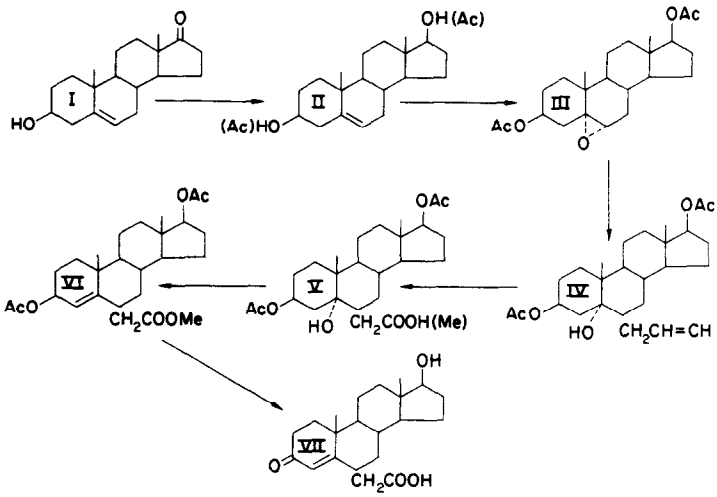


Fig. 1. Synthetic route to 6β-carboxymethyl steroids.

lated (II). Treatment with 3-chloroperbenzoic acid gave 5α,6α-epoxyandrostane-3β,17β-diol diacetate (III), which on reaction with allyl magnesium bromide gave 6β-allylandrostane-3β,5α,17β-triol-3β,17β-diacetate (IV). Permanganate oxidation of the diacetate gave the 6-carboxymethyl compound which was isolated as the methyl ester (V) after treatment with diazomethane. The ester (V) was converted to 6β-carbomethoxy-4-androstene-3β,17β-diol diacetate methyl ester (VI) by treatment with thionyl chloride in pyridine. This methyl ester was saponified to give the diol acid which on oxidation with manganese dioxide in chloroform yielded 6β-carboxymethyltestosterone, m.p. 158–160°C (VII). By analogous reactions starting with pregnenolone(3β-hydroxy-5-pregnen-20-one) and carrying out the final oxidation with chromium trioxide in pyridine (Sarett's reagent), 6β-carboxymethylprogesterone, m.p. 215–217°C, has been prepared; practical details will be published elsewhere.

Formation of 6-carboxymethylsteroid-BSA antigens

6-Carboxymethyltestosterone (16·7 mg) dissolved in dioxan (1 ml) containing tributylamine (0·012 ml) was kept at 12°C and a dioxan solution (0·1 ml) of isobutyl chlorocarbonate (0·0064 ml) added; the mixture stood at room temperature for 20 min. Bovine serum albumin (66 mg) was dissolved in 0·032 M-NaOH (2·07 ml), dioxan (1·4 ml) was added and the mixture cooled to 0°C. The steroid solution was added with stirring and M-NaOH (0·06 ml) used to adjust the solution to pH 8. The precipitate formed initially dissolved within 5 min. After stirring at 0°C for 4 h the mixture was kept overnight at 4°C. It was filtered through Celite 535 and separated by gel filtration through a Sephadex G-25 column (50 cm × 2 cm internal dia.), developing the column with water. Fractions (~4 ml) were collected and after suitable dilution the U.V. absorbance of each was recorded with a Unicam SP 800 spectrometer. On the assumption that the extinction of bovine serum albumin (278 nm) and that of 6-carboxymethyltestosterone (249 nm) are not affected by covalent linkage the composition of each serial fraction was calculated. The protein (27·6 mg calc.) and 6β-carboxymethyltestosterone (2·7 mg calc.) were eluted together in fractions F 16–18, and unreacted 6β-carboxymethyltestosterone

(5·8 mg calc.) was obtained in fractions F 27–60. On the assumption of 70,000 for the molecular weight of bovine serum albumin the protein-complex eluted contained 17 mol 6-carboxymethyltestosterone per mol of bovine serum albumin. Approximately 61 terminal NH_2 groups are present in the bovine serum albumin molecule, but owing to folding they may not all be accessible (c.f. Erlanger *et al.* [1]). The 6-carboxymethylprogesterone-BSA complex formed in an analogous manner in dimethylformamide contained, on average, 35 mol 6β-carboxymethylprogesterone per mol of bovine serum albumin. With each hapten the ratio of protein/steroid eluted in consecutive fractions from the Sephadex column was fairly constant and the most concentrated fractions, e.g. 17 ml containing 30 mg of BSA-6β-carboxymethyltestosterone, were used for injection into three New Zealand white rabbits at a dose level of 2 mg of protein per animal on three occasions at 14 day intervals. The dose was distributed between subcutaneous and intramuscular sites and blood was collected from the rabbits 10 days after the last injection; a booster dose (3 mg) was given and the animals were finally bled after three weeks.

Reactions of the antisera with radioactive ligands

Anti-6β-carboxymethyltestosterone serum. Incubation of the anti-6β-carboxymethyltestosterone serum in serial dilution with [1,2-^{3}H]testosterone at 30°C for 30 min (or 4°C for 16 h) in (1) the absence of, and (2) the presence of non-radioactive testosterone indicated (1) that the percentage binding of the radioactive ligand was dependent on protein dilution (Fig. 2) and (2) that the addition of non-radioactive testosterone progressively lowered the percentage of the total

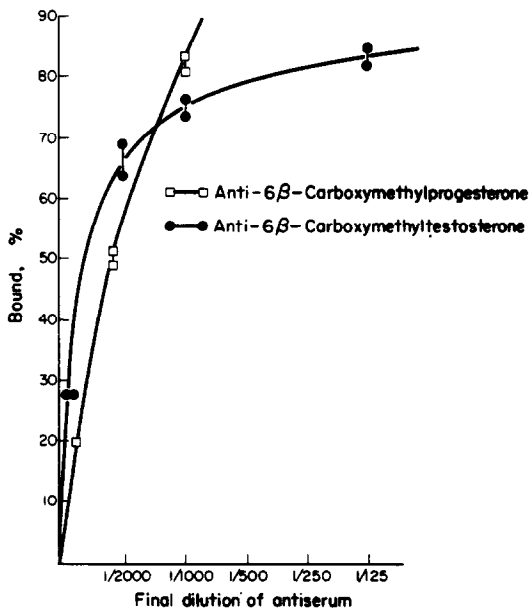


Fig. 2. Protein dilution curves of anti-6β-carboxymethyl steroid sera. Diluted antiserum incubated with radioactive ligand at 30°C for 30 min with buffer (pH 7·2) in the presence of 0·9 per cent NaCl and 0·1 per cent gelatine. Total volume 200 μl. [^{3}H]Testosterone/anti-6β-carboxymethyltestosterone serum ●——●; [^{3}H]progesterone/anti-6β-carboxymethylprogesterone serum □——□.

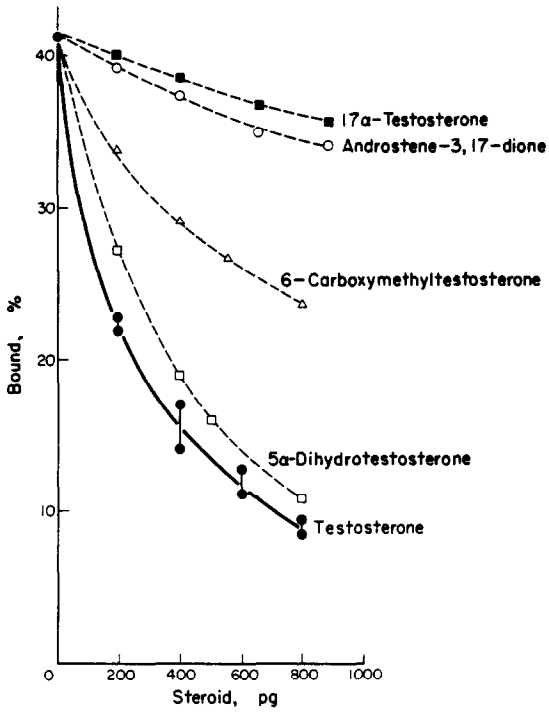


Fig. 3. Radioimmunoassay calibration graph of non-radioactive testosterone ●——●. The figure also illustrates the effect of some cognate steroids on the percentage binding of [^{3}H]testosterone by anti-6β-carboxymethyltestosterone serum. (Final dilution 1/4,000).

radioactivity bound (Fig. 3). The equilibrium data presented as a Scatchard plot [8] gave a linear graph corresponding to a dissociation constant (K_d) anti-6β-carboxymethyltestosterone serum/testosterone 8×10^{-10} M (30°C). For radioimmunoassay purposes the antiserum at a final dilution of 1/4000 gave 45 per cent binding of the radioactive ligand, and a 50 per cent decrease in the percentage binding was produced by 267 pg of non-radioactive testosterone.

Anti-6β-carboxymethylprogesterone serum. Similar relationships were established with anti-6β-carboxymethylprogesterone serum and [^{3}H]progesterone in the absence and presence of non-radioactive progesterone. Figure 2 shows the dependence of percentage binding on the dilution of the antiserum, and Fig. 4 illustrates that the addition of non-radioactive progesterone progressively lowered the percentage binding of the radioactive ligand. The Scatchard plot was linear corresponding to a dissociation constant (K_d) anti-6β-carboxymethylprogesterone serum/progesterone of 9×10^{-10} M at 30°C. At a final dilution of 1/3,000 the antiserum gave 45 per cent binding of radioactive progesterone, and a 50 per cent decrease in binding was produced by 150 pg of non-radioactive progesterone.

Cross-reaction against cognate steroids

The testing of the antisera for cross-reaction against cognate compounds was carried out by the normal calibration procedure in the range 0–1000 pg expressed in terms of the steroid content of the 200 μl reaction mixture.

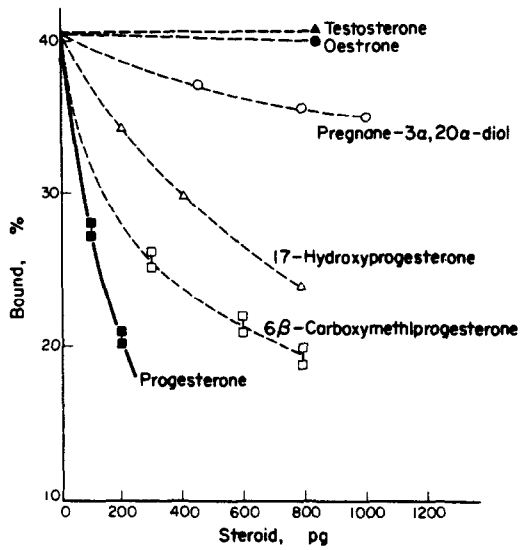


Fig. 4. Radioimmunoassay calibration graph of non-radioactive progesterone ■——■. The figure also illustrates the effect of some cognate steroids on the percentage binding of [^{3}H]progesterone by anti-6β-carboxymethylprogesterone serum (Final dilution 1/3,000).

Cross-reactions to anti-6β-carboxymethyltestosterone serum. The binding of [^{3}H]testosterone to the diluted anti-6β-carboxymethyltestosterone serum was affected by several related compounds and the absolute specificity hoped for by exposing both functional groups (at C-3 and C-17) was not attained. The immunological response of the anti-6β-carboxymethyltestosterone serum was more marked towards testosterone (*S* 12) than towards the hapten 6β-carboxymethyltestosterone (*S* 15). Figure 5 shows the effect of cognate compounds on the binding of radioactive testosterone. It is noteworthy that 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) (*S* 16) showed a marked cross-reaction whereas the 5β-orientated compounds (5β-androstane-3α,17β-diol (*S* 22); 5β-androstane-3β,17β-diol (*S* 24); 17β-hydroxy-5β-androstan-3-one (*S* 25) did not.

Cross-reactions to anti-6β-carboxymethylprogesterone serum. In a similar manner the binding of [^{3}H]progesterone to the diluted anti-6β-carboxymethylprogesterone serum was studied, and here also some degree of cross-reaction with cognate steroids was observed (Fig. 6). The immunological response of the antiserum to progesterone was more marked than to the hapten 6β-carboxymethylprogesterone (*S* 14). Of the related compounds tested, the chief competitor was pregnenolone (3β-hydroxy-5-pregnen-20-one, *S* 18) but pregnane-3,20-dione (*S* 6) and 20β-hydroxy-4-pregnen-3-one (*S* 8) also lowered the percentage of radioactive progesterone bound. These related derivatives may be present in biological fluids and could interfere with the determination of progesterone by radioimmunoassay.

DISCUSSION

There can be no doubt that attachment of these steroid haptens to BSA via the 6β-carboxymethyl substituent has failed to give antisera of high specificity such as might be used for the determination of testosterone and progesterone in

Compound ref. no.	Name
S12	Testosterone
S16	5α-Dihydrotestosterone
S15	6β-Carboxymethyltestosterone
S19	17α-Testosterone
S20	Androstene-3,17-dione
S17	Dehydroepiandrosterone
S18	Pregnenolone
S13	Progesterone
S14	6β-Carboxymethylprogesterone
S22	5β-Androstane-3α,17β-diol
S25	17β-Hydroxy-5β-androstan-3-one
S24	3β-Hydroxy-5β-androstan-17β-ol.

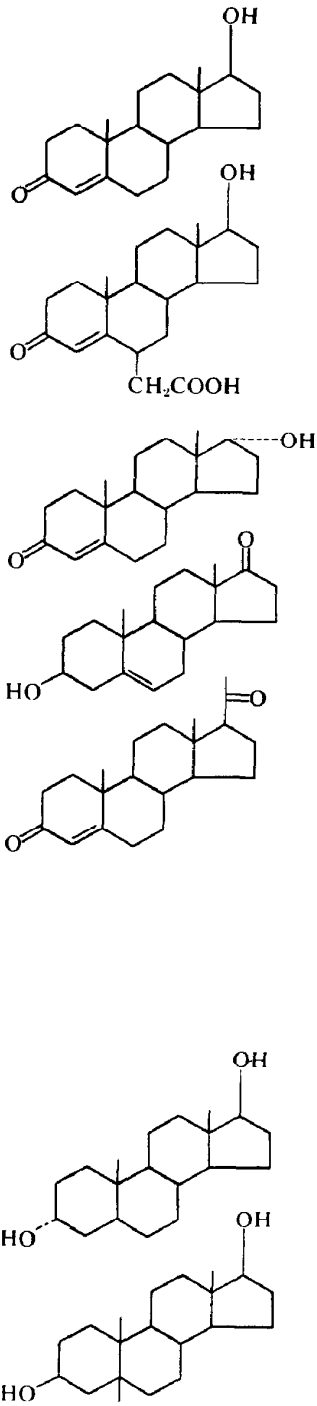
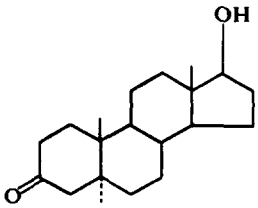
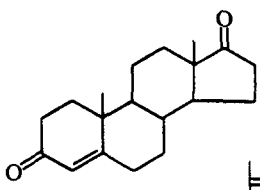
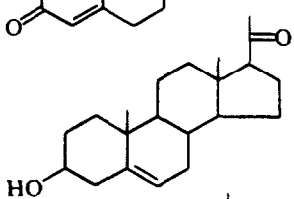
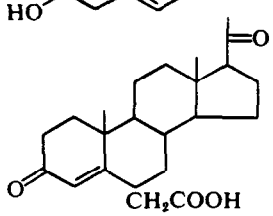
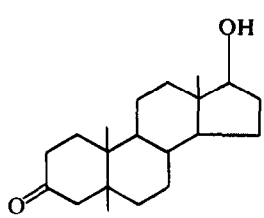
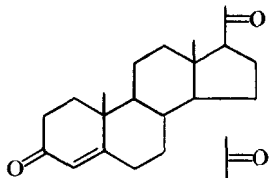
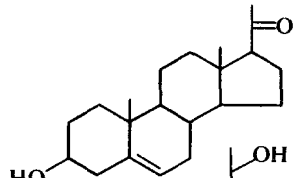
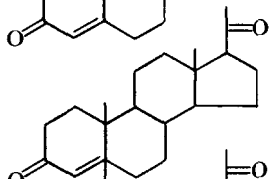
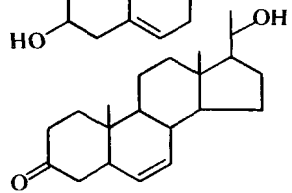
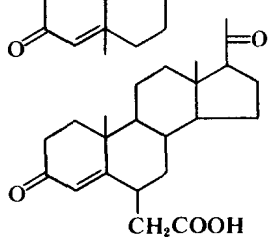
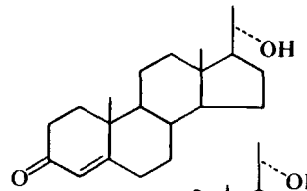
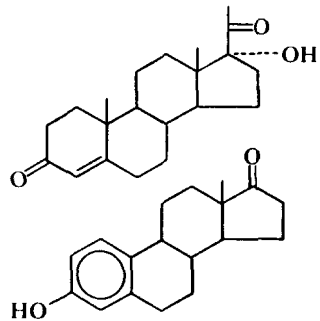
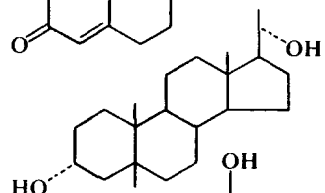
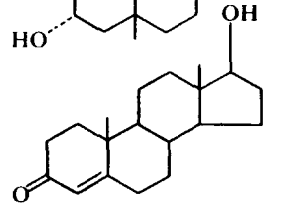
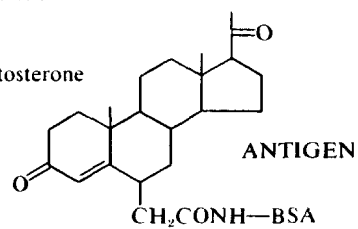


Fig. 5. Cross-reactions of cognate compounds in the system

	For 50% fall in % Bdg. pg	Relative cross reaction
	267	1·0
	335	0·80
	570	0·47
	12,800	0·02
	15,000	0·02
	No cross reaction	0·00
	No cross reaction	0·00
	No cross reaction	0·00
	No cross reaction	0·00
	No cross reaction	0·00
	No cross reaction	0·00
	No cross reaction	0·00

[^{3}H]testosterone/anti-6β-carboxymethyltestosterone serum.

Compound ref. no.	Name		pg for 50% fall in % Bdg.	Relative cross reaction
S13	Progesterone		150	1
S18	Pregnenolone		220	0·68
S 6	Pregnanedione		289	0·52
S 8	20β-Hydroxy-4-pregnen-3-one		407	0·37
S14	6-Carboxymethylprogesterone		550	0·27
S 7	20α-Hydroxy-4-pregnen-3-one		1900	0·08
S10	17-Hydroxyprogesterone		No cross reaction	0
S 9	5β-Pregnane-3α,20α-diol		No cross reaction	0
S11	Oestrone		No cross reaction	0
S12	Testosterone		No cross reaction	0

biological fluids without purification. With anti-6β-carboxymethyltestosterone serum three of the four C_{19} compounds tested which gave a positive cross-reaction had a 4-en-3-one structure in the A-ring, and this structure appears to be more effective as a discriminant than the 17β-hydroxyl group on the D-ring, since compounds *S* 22, *S* 24 and *S* 25 (Fig. 5) failed to cross-react. The cross reaction given by 5α-dihydrotestosterone was in sharp contrast to the absence of reaction displayed by these 5β-derivatives. Table 1, which compares briefly the results of the

Table 1. Cross-reactions to cognate steroids by sera produced by different types of testosterone haptens

Steroid	6β-Carboxymethyl-testosterone-BSA	Testosterone--3-BSA*	Testosterone--17-BSA*
Testosterone	1·00	1·00	1·00
5α-Dihydro-testosterone	0·80	—	—
6β-Carboxymethyl-testosterone	0·47	—	—
5-Androstene-3,17-diol	—	0·01	0·00
4-Androstene-3,17-dione	0·02	0·00	0·31
DHA	0·00	0·00	0·00
Pregnenolone	0·00	0·00	0·00
Progesterone	0·00	0·00	0·55

*Midgley and Niswender[5].

present study with those of Midgley and Niswender[5], also illustrates the disadvantage of leaving the 4-en-3-one structure exposed since the antibodies raised against testosterone-17β-BSA complexes cross-react with 4-androstene-3,17-dione and progesterone whereas those raised against the testosterone-3-BSA complex do not.

With the anti-6β-carboxymethylprogesterone serum cross-reactions were given by 5 compounds related to progesterone, and of these, 3 contained the 4-en-3-one structure (Fig. 6). The lack of specificity shown by the cross-reaction with pregnenolone (*S* 18) and 5β-pregnanedione (*S* 6) was unexpected. The analogue of dihydrotestosterone, 5α-pregnanedione, was not available for testing. Table 2 compares the results of the present study using the 6β-carboxymethylprogesterone-BSA complex with other antigens[5, 9]. Comparing the cross-reactions of the progesterone-3-BSA antiserum with those of the progesterone-20-BSA antiserum, the advantage of masking the 4-en-3-one structure of the hormone is again apparent since the latter antiserum, unlike the former, cross-reacts with 17-hydroxyprogesterone, 20α- and 20β-hydroxy-4-pregnen-3-one and testosterone.

The antiserum raised against 'progesterone-11-BSA' shows higher specificity than other antisera cited by Midgley and Niswender[5]. The authors do not specify the nature of the linkage employed, but presumably the covalent link was effected through the 11α-valency bond (cf. [10]). If this is indeed the case, this antigen, unlike other steroid-BSA complexes, may expose the β-side of the steroid molecule. By analogy BSA complexes formed with 6β-carboxymethyl steroids would expose the α-side of the steroid nucleus and the antibodies raised

Table 2. Cross-reactions to cognate steroids by sera produced by different types of progesterone haptens

Steroid	6β-Carboxymethyl-progesterone-BSA	Progesterone -3-BSA*	Progesterone -11α-BSA*	Progesterone -20-BSA*	21-Hydroxy-progesterone-21-BSA†
Progesterone	1·00	1·00	1·00	1·00	1·00
17-Hydroxy-progesterone	0·00	0·05	0·01	0·98	0·90
20α-Dihydro-progesterone	0·08	0·00	0·00	0·34	0·00
20β-Dihydro-progesterone	0·37	0·00	0·00	0·97	—
Pregnenolone	0·68	0·13	0·00	0·01	0·00
Pregnanediol	0·00	0·00	0·00	0·00	0·00
Testosterone	0·00	0·00	0·00	0·95	0·01

*Midgley and Niswender [5].

†Abraham, Swerdloff, Tulchinsky and Odell [9].

may be less specific for this reason. In both the C_{19} and C_{21} series the 6α-methyl configuration is more stable than the 6β-methyl form and compounds of the latter type are readily convertible to the more stable 6α-configuration. It is proposed to prepare the 6α-carboxymethyl derivatives of testosterone and progesterone and to study the effect of the changed configuration on the specificity of antisera raised by BSA complexes of these haptens.

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DISCUSSION

Munck: Can these reactions be applied to corticosteroids as well as to the steroids you've tried?

Smith: I think they may be a little rough for such compounds, but they might possibly be made to work.

Exley: Can I point out two features of this work which are rather surprising. You've gone to a lot of trouble to conjugate BSA at the C-6 position, to get away from functional groups, and yet you obtain anti-sera which cross-react with dihydrotestosterone more strongly than if you'd conjugated at the C-3 position.

Smith: Yes, exactly.

Exley: This is rather surprising. The second point is that tomorrow I'll be talking about using the carboxymethyloxime derivative at C-6, which for oestrogens is very good.