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STEROIDS AS HAPTENS: OPTIMAL DESIGN OF ANTIGENS FOR THE FORMATION OF ANTIBODIES TO STEROID HORMONES

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SUMMARY

By judicious choice of the site of attachment of steroid haptens to a protein carrier it is possible to elicit the formation of antibodies with enhanced specificity towards selected parts of the steroid molecule. Coupling the steroid through a position remote from the functional groups responsible for its hormonal specificity preserves these substituents as antigenic determinants. This has been effected in gonadal steroids by inserting "chemical handles" (e.g. carboxymethyl oxime, carboxyethyl thioether or hemisuccinate groups) into the steroid skeleton at positions 1, 6, 7 or 11. Thus, immunization with oestrone, oestradiol-17β or oestriol coupled to protein at position 6 gave rise to antibodies discriminating between the three major oestrogenic hormones and closely related metabolites that are not distinguished by antibodies to oestrogens conjugated to protein at C-17. Likewise, antibodies to progesterone-6-, 7- or 11-conjugates recognize changes in the 17-sidechain more efficiently than antibodies to the corresponding 20-conjugate. However, the antiserum to progesterone-6 protein was least specific towards changes in the A-ring. This approach has been extended to key progesterone metabolites and to androgens. Thyroglobulin or BSA conjugates of 1-carboxyethyl thioethers of testosterone, 5α-dihydrotestosterone (DHT), androstenedione (Ad) and progesterone have been prepared. Antisera reacting preferentially with DHT, and antisera to testosterone showing minimal cross-reaction with DHT (8%) and none with Ad(<0·1%) were obtained.

DEFINITION OF THE PROBLEM AND RATIONALE OF APPROACH

The pioneering work of Lieberman and his colleagues established that steroid hormones can function as antigenic determinants when covalently linked to a protein carrier[1]. In the design of such antigenic conjugates, several variables have to be considered that may affect the properties of the antisera elicited: (i) the site on the steroid molecule through which the link is effected; (ii) the chemical nature and length of the bridge connecting hapten and carrier; (iii) the nature of the carrier chosen, and (iv) the number of steroid residues attached to each carrier molecule. The first two of these bear importantly on the specificity of the antibodies generated and will be the primary subject of this discussion; the remaining two parameters affect the intensity of the immune response, and will only be touched on lightly.

The most convenient way of achieving covalent attachment of the steroid to a macromolecule is to utilize one of the reactive groups present on the native hormone, such as a 17β-hydroxy or 20-keto group (Fig. 1, compounds **I** and **II**). Unfortunately, antigenic complexes produced in this way[1, 2] gave rise to antisera that reacted not only with the homologous hormone, but also with other naturally occurring steroids, particularly those differing from the hapten used for immunization only in the vicinity of the attachment site. For instance, progesterone conjugated through position 20 to bovine serum albumin (P-20—BSA) generates antibodies that react almost equally with progesterone, its 20-dihydro- and 17-hydroxy derivatives, testosterone and even with 11-deoxycorticosterone[1, 3].

We felt that the inadequate specificity of the traditional anti-steroidal sera was largely due to the method adopted for preparing the antigenic conjugates. For the coupling procedures used entailed chemical alteration—and possibly the masking, through proximity to the carrier,—of some of those functional groups chiefly responsible for the biological activity of the hormone. As a result, distinctive features of the hormone were apt to be lost as haptenic determinants. Furthermore, the positions on the steroid commonly chosen for attachment, such as 3, 17 and 20, are also points of attack of widely distributed steroid-metabolizing enzymes, so that potentially cross-reacting steroid metabolites abound in biological fluids. These lines of reasoning led to systematic analysis of the effect of varying the site of attachment of the steroid to the protein carrier on the specificity of the antibodies generated. Coupling was achieved in these studies by inserting reactive "handles" into the steroid molecule

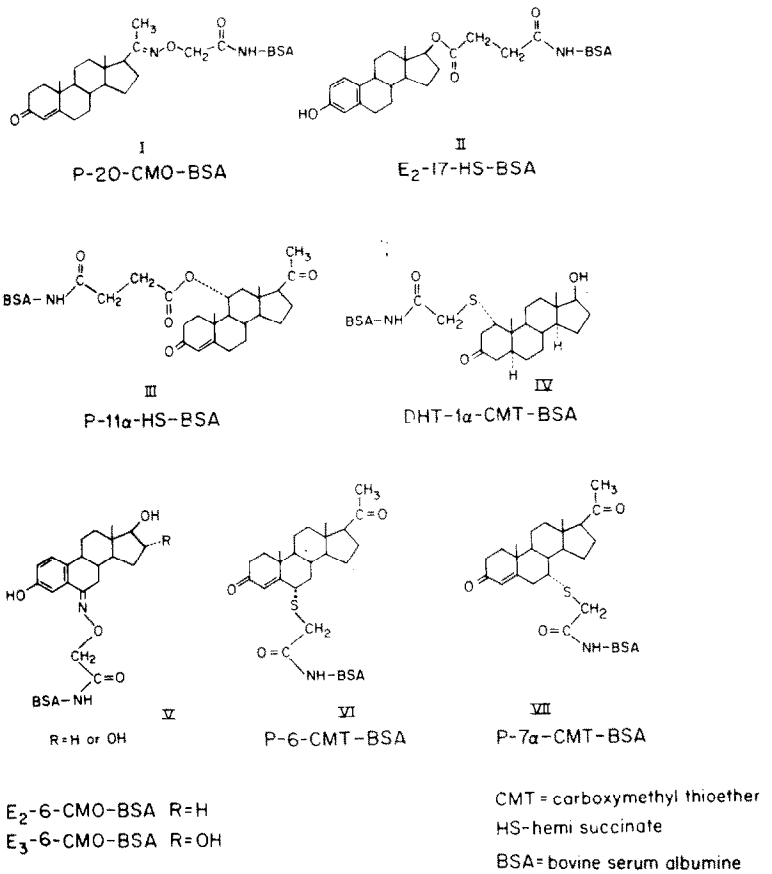

Fig. 1. Some antigenic steroid–bovine serum albumin conjugates prepared by coupling through pre-existing functional groups of the steroid (**I–II**) or coupling through chemical "handles" introduced elsewhere into the steroid skeleton (**III–VII**).

(Fig. 1) at positions distant from the functional groups that determine its hormonal specificity[3–5].

In this presentation we will review briefly the attempts undertaken in our laboratory to design antigenic steroid-protein conjugates that give rise to antisera better able to discriminate between the major gonadal hormones and some of their common metabolites.

GENERAL METHODS

The steroid derivatives were conjugated to protein carriers—bovine serum albumin (BSA) or thyroglobulin (TG)—by use of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide as the coupling reagent [6]. The use of thyroglobulin was recently explored, as it was shown to be a particularly effective carrier [7]. The number of steroid residues covalently attached to each molecule of carrier was determined, after exhaustive dialysis of the conjugate, by one of the following methods: (i) spectrophotometrically, provided the hapten carries a chromophore whose absorption peak(s) can be resolved from that of the carrier (8); (ii) by determining the number of lysine residues in the conjugate that carry covalently attached steroid, using the deamination procedure of Anfinsen, Sela and Cooke [9]; (iii) by determining the specific activity (s.a.) of the antigenic complex, provided a radioactively labelled steroid of known s.a. was used for preparing it [10]. The steroid–protein conjugates usually contained 15–30 steroid residues, on the average, per molecule of BSA and 140–160 residues per molecule of TG. A more detailed account of the synthetic methods used for preparing reactive steroid derivatives and steroid–protein conjugates in this study is given by Kohen *et al.* [11].

Rabbits were immunized with antigens incorporated in complete Freund's adjuvant by multiple intradermal injections, followed by monthly booster injections [12]. The antisera obtained were characterized by measuring their capacity to bind the homologous tritiated steroid and the ability of various unlabelled steroids to compete with the labelled hapten for binding sites. Dextran-coated charcoal was used for separating bound and unbound steroid [13]. The degree of cross-reaction was expressed as defined by Thorneycroft [14]. Association constants of the antisera for their homologous steroids were derived from Scatchard plots [15, 16] using data obtained by incubating a constant amount of antiserum with increasing amounts of tritiated steroid at 4°C overnight.

PROPERTIES OF ANTISERA TO GONADAL STEROIDS IN RELATION TO STRUCTURE OF THE ANTIGENIC CONJUGATE

Antisera to androgens

Antisera to testosterone were first generated by Lieberman *et al.*[1] by immunization with a testosterone-17-hemisuccinate–protein conjugate. Sera of this type, however, were found not to distinguish between testosterone and androstenedione, and in addition they cross-react with progesterone (55%) and 11-deoxycorticosterone (55%)[3].

In the quest for antigens yielding more specific antisera, general methods were devised for attaching 4-ene-3-ketosteroids to protein through carbon 7[13] or through carbon 1. 7-Carboxymethylene thioethers (7-CMT) of various steroids, including testosterone and androstenedione (Fig. 2, top), were prepared from the corresponding 6, 7-dehydroderivatives. This was done by use of an ambidentate reagent which carries an SH group for nucleophilic attack on carbon 7 of the steroid on one end and a carboxyl group for formation of a peptide bond with lysine residues of the carrier on the other. This method of coupling leaves both the important 4-ene-3-keto function and the characteristic substituent at C-17 of these steroids fully exposed to function as antigenic determinants. The number of carbon atoms intercalated between the steroid and carrier can be varied at will by using mercaptoacetic, mercaptopropionic or a higher mercaptoalkanoic acid for forming the link. More recently, we have used an analogous technique to attach these androgens, as well as 5α-dihydrotestosterone, to protein through position 1 (Fig. 2, bottom). This position, too, is well removed from the characteristic functions of the androgens and neither position 1 nor 7 is a site of major peripheral metabolism of androgens.

Immunization of rabbits with the 1- or 7-conjugates of testosterone (T-1—BSA and T-7—TG, respectively) or with the 1-conjugate of 5α-dihydrotestosterone (5α-DHT-1—BSA) gave rise to antisera binding only 11-deoxy C_{19}-steroids that carry a 17β-hydroxy group: none of these sera reacted to a significant extent with androstenedione and 5α-androstane-3, 17-dione (<1%), or 17α-epitestosterone (≤1%) (Table 1). Conversely, the 1- and 7-conjugates of androstenedione (Ad-1—BSA and Ad-7—BSA), yielded antisera that required the presence of a 17-keto group for high-affinity binding (Table 1), and showed little cross-reaction with testosterone (<0·5%) or 17α-epitestosterone (~2%).

The antiserum to 5α-DHT—BSA reacted preferentially with 17β-hydroxysteroids having a 5α-androstane configuration: there was only 3% cross-reaction with 5β-DHT and 10% with testosterone. On the other hand, antisera to T-7—BSA and Ad-7—BSA showed preference, but not absolute specificity,

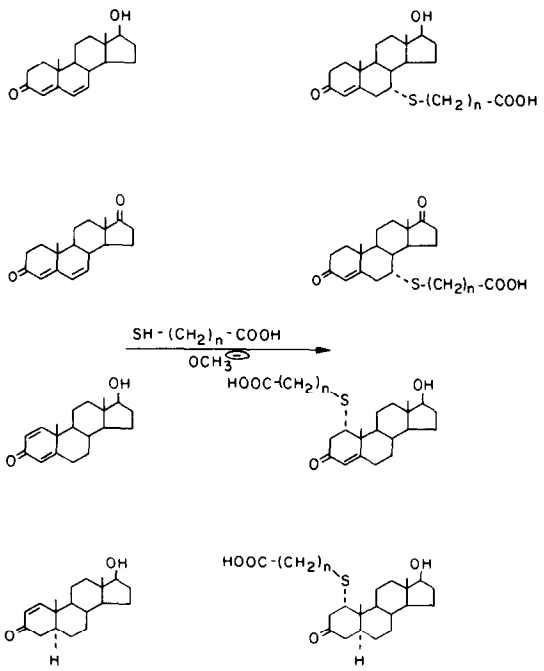

Fig. 2. Preparation of carboxyl derivatives of androgens through linking groups at positions 1 and 7. Top to bottom: alkanoic acid thioethers of testosterone and androstenedione (position 7); testosterone and 5α-dihydrotestosterone (position 1).

Table 1. Specificity of antisera raised in rabbits with different androgen conjugates

Compound	Cross reaction (%) Antisera to Ad-7—BSA*	T-7—BSA*	T-1—TG	5α-DHT-1—BSA
Testosterone	<0·5	100	100	10
5α-Dihydrotestosterone	1	42	8–15	100
5β-Dihydrotestosterone	<0·1	5	5	3
17α-*Epi*testosterone	2	1	<0·5	<0·1
Androstenedione	100	0·7	<0·1	<0·5
5α-Androstane-3, 17-dione	33	0·5	0·9	
5β-Androstane-3, 17-dione	5	<0·5	<0·1	
Androsterone	10	<0·5	0·5	<0·5
*Epi*androsterone	4	<0·5	<0·1	<0·5
Dehydro*epi*androsterone	<0·5	<0·5	<0·5	<0·5
5α-Androstane-3α, 17β-diol		3	6	16
5α-Androstane-3β, 17β-diol		2	1	1
11-Oxotestosterone		3		
11β-Hydroxytestosterone		0·2		
Progesterone	<0·1	<0·1	<0·1	
Oestradiol-17β		<0·1	<0·1	0·1

* Includes data from Weinstein, Lindner, Friedlander and Bauminger[13].
Ad, androstenedione; T, testosterone; 5α-DHT, 5α-dihydrotestosterone.

for the 4-ene-3-keto group. These sera cross-reacted to the extent of 33–42% with the corresponding 3-keto-5α-androstane and 3–10% with the 3α-hydroxy-5α-androstane compounds, provided the appropriate substituent at C-17 was present. With respect to testosterone, the cross-reaction with the structurally closely related 5α-DHT was significantly reduced (to 8%) when the 1-conjugate, rather than the 7-conjugate of testosterone was used for immunization (Table 1).

In contrast to sera generated with 17-conjugates of testosterone, the antisera to 1- or 7-conjugates of androgens did not bind C_{21}-steroids, such as progesterone, 17α-hydroxyprogesterone or 11-deoxycorticosterone (Table 1 and [13]).

The antisera to androstenedione and to 5α-DHT described here are the only ones hitherto prepared that preferentially bind these two androgen metabolites. The antiserum to T-1—TG appears to be the most specific one available at present for testosterone, judged by testing it against steroids likely to be encountered in significant amounts in the circulation. However, promising results were also obtained using antisera generated with the 3-conjugate[17–20] or 11-conjugate [20] of testosterone. Both were reported to show good resolution of steroids differing in substitution at C-17. However, the T-3—BSA derived sera, like those generated with T-7—BSA, showed marked cross-reaction with 5α-DHT (42–100%). The T-11—BSA derived serum cross-reacted to a lesser extent with this metabolite (15%;[20]), but considerably with 11-oxotestosterone (17%) and 11β-hydroxytestosterone (25%; [20]). 11-Oxygenated androgens are released in significant amounts by the adrenal in several mammalian species and hence may be expected to interfere with testosterone assays when using this serum. Anti T-7—BSA and anti T-1—BSA sera do not react significantly with 11-oxygenated testosterone metabolites.

Anti T-1—BSA or T-7—BSA sera have been used for radioimmunoassay (RIA) of testosterone in unfractionated lipid extracts from peripheral plasma of bulls[5], goats (unpublished data) or normal human males (unpublished data). The results were in reasonable agreement with those obtained by RIA applied after chromatographic separation of the hormone; however, when applying this RIA to plasma of normal women, a similar comparison revealed that omission of the chromatographic step often resulted in overestimation of the testosterone concentration.

Sera derived from different rabbits immunized with the same antigen differed only slightly in the spectrum and magnitude of cross-reactions. Site of conjugation clearly outweighed individual variation of response in determining antibody specificity. Specificity tended to increase with time after the first immunizing injection. For instance, cross-reaction of the anti Ad-7—BSA serum with 5α-dihydroandrostenedione was reduced from 71 to 33% with successive bleedings of the same rabbit between the 2nd and the 8th month after primary immunization.

The affinity of the anti-androgen sera for the homologous steroids was high. Typical association constants (Ka) were $9{\cdot}4 \times 10^9$ (anti T-7—BSA), $1{\cdot}04 \times 10^{10}$ (anti Ad-7—BSA) and $1{\cdot}4 \times 10^9$ (anti DHT-1—BSA) l/mol.

Antisera to progestagens

Antisera raised with a 20-conjugate of progesterone (see Introduction and Fig. 1) proved unable to discriminate between progesterone and many other steroids (e.g. testosterone, deoxycorticosterone) that do not differ from it in the chemistry of the A, B and C rings[1, 3]. We therefore prepared a series of progesterone-protein conjugates in which the sidechain at C-17 is not modified by the coupling procedure and available for recognition by the antibody-forming system. In this series, positions 6, 7 and 11 of the steroid were utilized as attachment sites.

The 6-carboxymethyl thioether of progesterone (Fig. 3, top), prepared by action of thioacetic acid on 6-bromoprogesterone, was coupled to BSA (P-6—BSA [10]). This antigen yielded sera that recognized changes in substitution of the D-ring, but failed to distinguish progesterone efficiently from its 5-dihydro derivatives (especially 5α-pregnane-3, 20-dione) and also cross-reacted with pregnenolone (Table 2). Lack of adequate specificity towards the A-ring was also observed when antisera were raised with 6-conjugates of progesterone prepared in different ways by other authors[21, 24].

On shifting the site of attachment to C-7 (as described in the section dealing with androgens; see also Fig. 3 and [13]) or to C-11 (via the 11α-hemisuccinate, Fig. 1 and [4, 10]; or via the 11-chlorocarbonate,[21]), antisera were obtained in which specificity towards the sidechain at C-17 was essentially retained, and cross-reaction with pregnane compounds and pregnenolone was markedly reduced (Table 2). Coupling through the 11 position introduces cross-reactions with related 11-oxygenated steroids, e.g. 11β-hydroxyprogesterone (8%) and with 11α-hydroxyprogesterone (44%); the latter steroid, however, is not known to occur naturally in body fluids.

Immunization with a 7-conjugate of 17α-hydroxyprogesterone (Fig. 3) yielded antisera specific for the pattern of substitution at C-17 characteristic of this steroid (Table 3); these sera showed only minimal cross-reaction with progesterone (2%).

Progesterone (21) and progesterone metabolites, such as 17α-hydroxyprogesterone (22) or 20α-dihydroprogesterone (Fig. 3; see also [23]), have also been coupled to protein via position 3, using the O-carboxymethyl oxime of the steroids. These antigens

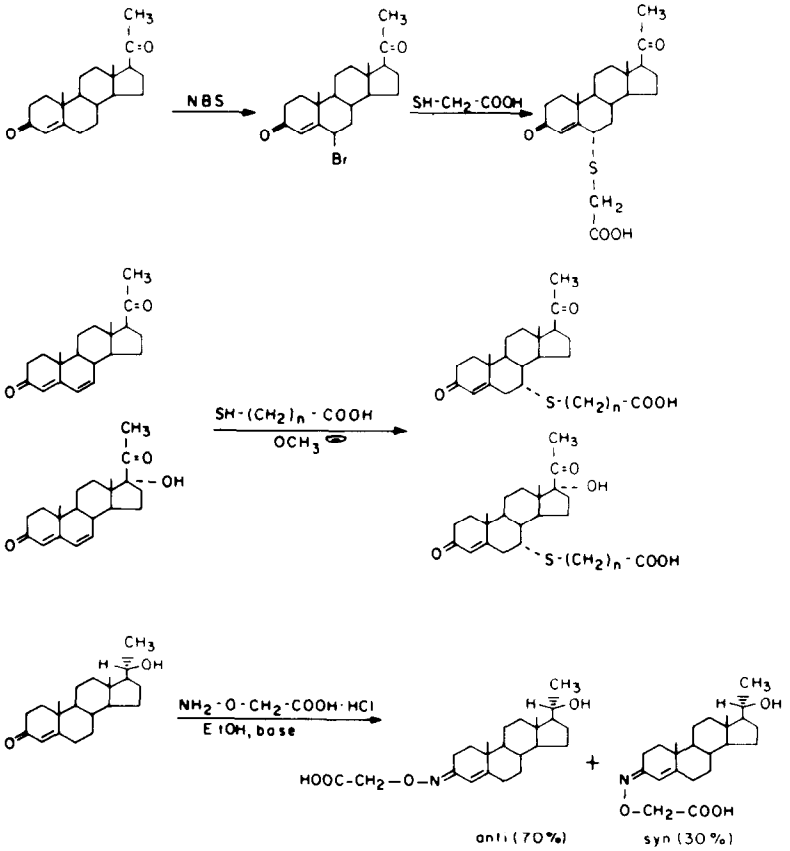

Fig. 3. Preparation of carboxyl derivatives of progesterone and progesterone metabolites (17-hydroxy- and 20-dihydroprogesterone) through linking groups at positions 6, 7 or 3.

Table 2. Specificity of antisera raised in rabbits with different progesterone conjugates

Compound	Cross reaction (%) Antisera to P-6—BSA*	P-7—BSA†	P-11—BSA*
Progesterone	100	100	100
5α-Pregnane-3, 20-dione	38	11	3
5β-Pregnane-3, 20-dione	15	2	
3β-Hydroxy-5-pregnen-20-one	8–14	4	<0·3
3β, 17-Dihydroxy-5-pregnen-20-one	3		
17-Hydroxyprogesterone	4	5	2
20α-Hydroxy-4-pregnen-3-one	1–2	1	0·1
20β-Hydroxy-4-pregnen-3-one	1–2	2	1
11β-Hydroxyprogesterone			8
Corticosterone	0·3		0·4
Testosterone	<0·3	<0·1	
Oestradiol-17β	<0·3	<0·1	<0·3

* Includes data from Lindner, Perel, Friedlander and Zeitlin[10].
† From Bauminger, Lindner and Weinstein[12].

generated sera that efficiently recognize the specific 17-sidechain of each steroid (Table 3). This is not surprising, since this site of attachment is remote from the D-ring. However, cross-reaction with the corresponding pregnane compounds would be expected, and is indeed observed[21].

A radioimmunoassay (RIA) procedure for progesterone was applied directly to crude plasma lipid extracts from women, using antisera to either P-6—BSA[5, 25] or P-11—BSA[26]. These simple assays clearly differentiated between the luteal and follicular phase of the menstrual cycle and gave results closely correlated with data obtained by RIA applied after chromatographic separation of the steroid. This agreement would suggest that the 5α-pregnane compounds with which the anti P-6—BSA cross reacts do not occur in high concentrations in peripheral plasma of cycling women. During pregnancy, however, RIA without chromatography slightly overestimated plasma progesterone. Use of the anti P-11—BSA serum also permitted rapid assay of the rate of progesterone release into the medium of cultured follicles[27]; results were closely related ($r = 0{\cdot}99$) with those obtained when progesterone was separated by paper-chromatography and subsequently determined by RIA.

Antisera to oestrogens

Oestradiol-17β hemisuccinate linked to BSA (E_2-17—BSA)[3] yields antibodies that are absolutely specific towards steroids with a phenolic A-ring; but like the corresponding 17-conjugate of testosterone (see above) fail to distinguish between steroids differing

Table 3. Specificity of antisera raised in rabbits with conjugates of progesterone metabolites

Compound	Cross reaction (%) Antisera to 17-OH-P-7—BSA*	20α-H_2-P-3—TG
17-Hydroxyprogesterone	100	<0·5
20α-Hydroxy-4-pregnen-3-one	<0·1	100
20β-Hydroxy-4-pregnen-3-one	<0·1	8
Progesterone	2	<0·5
3β-Hydroxy-5-pregnen-20-one	2	<0·5
3β-17-Dihydroxy-5-pregnen-20-one	13	<0·5
5-Pregnene-3β, 20α-diol		8
5-Pregnene-3β, 20β-diol		<0·5
5α-Pregnane-3, 20-dione	2	<0·5
5β-Pregnane-3, 20-dione	2	<0·5
Oestradiol-17β	<0·1	<0·1
Testosterone	<0·1	<0·1
Cortisone	0·6	<0·1

* From Bauminger, Lindner and Weinstein[12].
17-OH-P, 17-hydroxyprogesterone; 20α-H_2P, 20α-dihydroprogesterone.

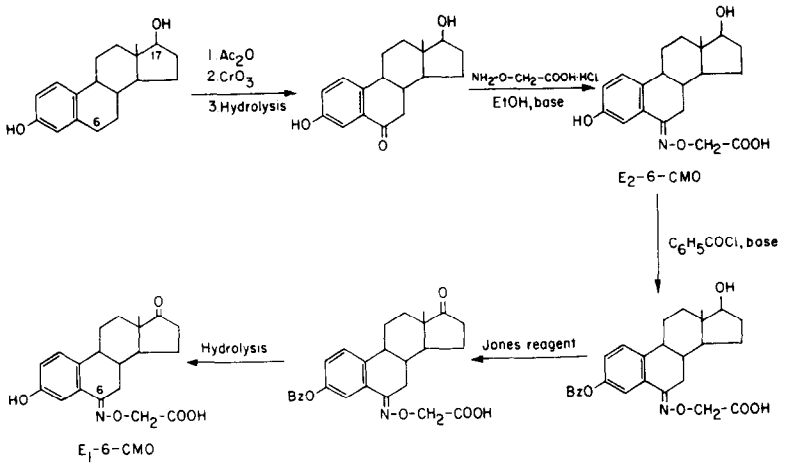

Fig. 4. Synthesis of oestradiol- and oestrone-6-(O-carboxymethyl)-oximes.

in substitution at C-17 (reviewed in [5]). It was therefore proposed to attach oestradiol to protein through position 6, and antisera to conjugates of 6-amino oestradiol and of the 6-(O-carboxymethyl)-oxime of oestradiol were raised[4]. The 6 position was chosen in order to leave the two important hydroxyl groups of the oestrogen untouched, and because its benzylic nature facilitates selective oxidation to the 6-oxo-derivative. By use of this derivative and the C-carboxymethoxylamine hemihydrochloride reagent introduced by Erlanger *et al.*[28], the 6-conjugates of the three primary oestrogens are readily prepared and have been attached to protein (Fig. 1 and 4; and [4, 10,29–32]). These conjugates generate antisera that are highly specific for the phenolic steroids, and in addition show little cross-reaction with oestrogens differing from the homologous hapten in substitution of the D-ring (Table 4; cf. [10, 30, 32–36]). Thus an antiserum to oestrone-6—TG showed only minimal cross-reaction with oestradiol-17β, oestradiol-17α and with oestriol (<0·5%; Table 4). However, these sera cross-react strongly with the corresponding 6-oxygenated oestrogens (Table 4; cf. [36]). Since both the 6-oxo and 6α-hydroxy derivatives of oestrogens showed this cross-reaction, it does not seem to be attributable to the presence of the C=N double bond at C-6 in the steroid conjugates used for immunization, but rather to masking of this region of the hapten, or else a change in the conformation of the steroid skeleton introduced by the substitution at C-6.

Table 4. Specificity of antisera raised in rabbits with different oestrogen conjugates

Compound	Cross reaction (%) Antisera to E_1-6—TG	E_2-6—BSA*	E_3-6—BSA
Oestrone	100	1–4	1
Oestradiol-17β	0·4	100	1
Oestriol	<0·1	0·4–1·5	100
Oestradiol-17α	0·2	1–2	0·7†
Oestriol-16α, 17α			<1
Oestriol-16β, 17α			<1
6α-Hydroxy-oestradiol-17β		⩾100	
6α-Hydroxy-oestriol			100
6-Keto-oestradiol-17β		80	
6-Keto-oestriol			68†
17α-Ethynyl-oestradiol-17β	<0·1	0·2	
Equilin	1	<0·1	<0·1
Equilenin	5	<0·1	<0·1
Testosterone	<0·1		<0·5
Progesterone	<0·1	<0·2	<0·5

* Includes data from Lindner, Perel, Friedlander and Zeitlin [10]
† From Wright, Collins and Preedy[36].
E, oestrone; E_2, oestradiol-17β; E_3, oestriol.

Antigenic oestrogen-protein complexes have recently also been prepared by conjugation through C-11. The resultant antisera did not cross-react with 6-oxygenated oestrogens[37], but were reported to be less specific towards the D-ring (38): the latter authors[38] observed 10–22% cross reaction of their anti E_2-11α—BSA serum with oestrone, compared with 1·8% for the Rehovot anti E_2-6—BSA serum. Likewise, their serum to oestrone-11α—BSA cross-reacts markedly with oestradiol-17β (9–27%).

Our antiserum to E_2-6—BSA has been used for direct RIA of oestradiol in maternal peripheral and umbilical blood[39], in peripheral blood during the menstrual cycle (Dr. D. T. Baird, M.R.C., Edinburgh, personal communication), and in cultures of Graafian follicles[5], and were found to give values that are in good agreement with data obtained by sequential chromatography and RIA. Similar agreement was reported between oestriol values in maternal and cord blood measured by RIA before and after celite chromatography[39].

CONCLUDING REMARKS

We have now at our disposal a variety of methods for attaching steroids to a protein carrier in such a way that the characteristic functional groups of the hormone or metabolite are preserved as antigenic determinants. By judicious choice of the site of attachment it is possible to elicit the formation of antibodies with enhanced specificity towards selected portions of the steroid molecule: absolute specificity is probably not attainable, but we may direct the attention of the lymphocyte, as it were, to those features of the steroid that we consider most important for our purpose.

With sera obtained by use of antigens conjugated through positions 1, 6, 7 or 11, it proved possible to develop radioimmunoassay procedures for the direct determination of the primary gonadal hormones (testosterone, progesterone and oestradiol) in unfractionated lipid extracts of plasma in a number of defined physiological conditions, and in culture media, with reasonable accuracy. By a similar approach, antisera have now been developed that will preferentially bind important metabolites of gonadal steroids, such as oestrone and oestriol, androstenedione and 5α-dihydrotestosterone, 17-hydroxyprogesterone and 20α-dihydroprogesterone.

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DISCUSSION

Kellie:

Dr. Lindner, you have done an excellent job in showing the important positions in the steroid nucleus at which to couple. Could I ask you whether you would agree that not only should the steroid be considered to be a hapten but also the steroid and the "bridge". Can you give us any information from your work whether the length of the bridge is important? Finally, I would very much like to see the slide showing the proportion of the cis and trans oximes.

Lindner:

I would agree that in many instances the "bridge" used to link the steroid to protein carrier may function as an additional antigenic determinant, and in this sense may properly be regarded as part of the hapten. Indeed, Dr. E. H. D. Cameron at the Tenovus Institute, Cardiff, has shown that the steroid-plus-bridge component of the antigenic complex is usually bound by the antiserum with greater affinity than the native steroid on its own. We found that our antisera to oestrone, oestradiol and oestriol raised with the 6-carboxymethyl oxime conjugates all show marked cross-reaction with the corresponding 6-keto oestrogen. This may be due to one of two reasons: either the presence in the antigenic conjugate of the C=N double bond at the 6-position, or else simply to masking by the coupling group of that particular region of the steroid. We are now trying to sort this out. This problem is really the reason why we are testing further sites of attachment in oestrogens that may be metabolically less active than the 6-position, since 6-hydroxy and 6-methoxy metabolites are widely distributed, as Dr. Breuer and others have shown. Regarding your second question, we have not yet seen a decisive advantage in lengthening the "bridge" in our alkanoic acid thio-ether series of conjugates, but this point has not been exhaustively analyzed. As to the last question,—we haven't resolved the cis and anti forms of the 3-carboxymethyl oximes of 20α-dihydroprogesterone. We know from the NMR data that we get those two isomers in about the proportion 70:30, but we immunized with the mixture. This would be the next step, to see if we get different specificities with the antibody if we use the purified cis or anti-isomer.