Journal of Steroid Biochemistry, 1974, Vol. 5, pp. 749–756. Pergamon Press. Printed in Great Britain.

SOME ASPECTS OF THE USE OF ^{125}I-LABELLED LIGANDS FOR STEROID RADIOIMMUNOASSAY

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SUMMARY

Steroid-[^{125}I]-iodohistamine radioligands were synthesized and used to investigate a number of potential radioimmunoassay systems for the assay of oestradiol, progesterone, testosterone, norethisterone, norethisterone acetate, *D*-norgestrel, testosterone-17β-glucuronoside, androsterone-3α-glucuronoside and aetiocholanolone-3α-glucuronoside. Viable systems were predicted for example using anti-oestradiol-6-(O-carboxymethyl)imino–BSA with either oestradiol-3-hemisuccinyl-[^{125}I]-iodohistamine or oestradiol-17β-hemisuccinyl-[^{125}I]-iodohistamine radioligands. Similarly, the free 4-en-3-oxosteroids could be assayed using anti-steroid-3-(O-carboxymethyl)imino–BSA sera with a steroid-3-(O-carboxymethyl)-imino-[^{125}I]-iodohistamine (steroid-3-^{125}I) radioligand (homologous system), or antisteroid-11α-hemisuccinyl–BSA sera with a steroid-3-^{125}I radioligand (heterologous system). All such systems are less specific than those employing a ^{3}H-labelled tracer but certain homologous systems using rare antisera are more specific still and therefore retain the advantages of using a radio-iodine label. The radio-iodine labelled ligands may also be used to investigate the nature, size and shape of antibody binding sites. For example, the occurrence of binding of a particular ^{125}I-labelled radioligand to an antiserum indicates that the serum cannot "see" that part of the molecule bearing the radio-iodine labelled side-chain. Absence of binding conversely may indicate that the position of attachment of the label is involved in the binding of the unlabelled steroid.

INTRODUCTION

The preparation and use of radio-iodine labelled tyrosyl methyl ester derivatives for the radioimmunoassay of steroids has been reported previously[1, 2]. In our hands, the techniques described proved to be unsatisfactory, mainly due to solubility problems particularly with nonpolar steroids. Nars and Hunter[3] recently described a procedure in which oestradiol-6-(O-carboxymethyl) imino-[^{125}I] iodohistamine (E_2-6-^{125}I) was prepared by preliminary iodination of histamine followed by its coupling to the oestradiol derivative using a mixed anhydride synthesis[4]. The method is simple to operate, and has the merit of producing a pure radioligand free from non-labelled material which might interfere with the binding and to date we have successfully used the procedure to synthesize upwards of twenty different ^{125}I-labelled steroid ligands for the study of potential radioimmunoassay systems[5, 6, 7].

The report which follows describes some of our most recent findings in this field and attempts to draw up some of the "ground rules" which seem to apply in the use of steroid-[^{125}I]iodohistamine radioligands.

EXPERIMENTAL

All common reagents and solutions were as described previously[6, 8]. Labelled steroids[1,2,6,7-$^{3}H_4$]testosterone, [1,2,6,7-$^{3}H_4$]progesterone, [2,4,6,7-$^{3}H_4$]oestradiol (all of S.A. 100 Ci/mmol) and [1,2-$^{3}H_2$]androsterone (S.A. 50 Ci/mmol) together with Na^{125}I (S.A. >14 mCi/μg of carrier-free iodide) were purchased from the Radiochemical Centre, Amersham, Bucks, U.K.

[^{3}H-G]Norethisterone (S.A. 20 Ci/mmol) and [1,2-$^{3}H_2$] aetiocholanolone (S.A. 50 Ci/mmol) were obtained from NEN Gmbh, Frankfurt am Main, Germany. The non-radioactive synthetic steroids norethisterone and *D*-norgestrel and their derivatives were very kindly supplied by Schering AG, Berlin. Testosterone-11α-hemisuccinate and part of the progesterone-11α-hemisuccinate were generously donated

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by Dr. G. F. Woods, Organon Laboratories Ltd., Newhouse, Lanarkshire, Scotland.

Antigens and antisera

Steroid-3-(O-carboxymethyl)oximes were prepared by the method of Arnold and James[9], and hemisuccinates by refluxing the free steroid with succinic anhydride in pyridine. Antisera to steroids were raised in New Zealand White rabbits as described previously [10]. ^{125}I-Labelled steroid radioligands were prepared by the method of Nars and Hunter[3] and purified by thin layer chromatography on silica gel or alumina precoated plastic sheets in the solvent systems shown in Table 1. After scanning the chromatograms on a Berthold radiochromatogram scanner, the required material was eluted with methanol[10].

Steroid glucuronosides

Methyl (2,3,4-tri-O-acetyl-1α-bromo-1-deoxy-*D*-glucopyranosid)-uronate was prepared from β-*D*-glucuronolactone by standard procedures[11] and stored dry at −20°C. This reagent was used to prepare testosterone-17β-glucuronoside, androsterone-3α-glucuronoside and aetiocholanolone-3α-glucuronoside [12, 13]. The steroid glucuronosides were coupled to BSA by a mixed anhydride synthesis[4] and the conjugates used to raise antisera in New Zealand White rabbits in the normal way[10]. [1,2-$^3H_2$]Testosterone-β-*D*-glucuronoside (S.A. 50 Ci/mmol) was purchased from NEN Gmbh and [6,7-$^3H_2$]aetiocholanolone-glucuronoside (S.A. 9·9 Ci/mmol) was kindly supplied by Prof. A. E. Kellie, Courtauld Institute of Biochemistry, Middlesex Hospital, London. The ^{125}I-labelled glucuronoside ligands were prepared as described above.

Radioimmunoassay and allied procedures

These techniques have been adequately described previously[6, 8]. Antisera titrations were invariably performed with and without the addition of 500 pg of the respective steroid to assess the degree of inhibition of radioligand binding. Cross-reactions were determined as described by Abraham[14].

RESULTS

Oestradiol-17β

The successful use of the E_2-6-^{125}I ligand for oestradiol radioimmunoassay[3] appeared to be limited to one particular antiserum[15] which had originally been supplied by Dr. D. Exley, Queen Elizabeth College, London. All anti-oestradiol-6-(O-carboxymethyl)imino-BSA (anti-E_2-6-BSA) sera raised in our laboratories failed to show inhibition of radioligand

Table 1. Solvent systems for the purification of steroid ^{125}I-labelled radioligands by thin layer chromatography on silica gel or alumina precoated plastic sheets

Radioligand	t.l.c. (medium)	Solvent system (by vol.)
T-3-^{125}I*	S†	chloroform–methanol–acetic acid (90:10:1)
T-11α-^{125}I	S	benzene–acetone–methanol–acetic acid (50:50:20:2)
P-3-^{125}I	S	chloroform–methanol–acetic acid (90:10:1)
P-11α-^{125}I	S	benzene–acetone–methanol–acetic acid (50:50:20:2)
NE-3-^{125}I	A	benzene–ethanol–acetic acid (75:24:1)
NE-11α-^{125}I	A	ethyl acetate–acetic acid (70:2·5)
NEA-3-^{125}I	A	benzene–ethyl acetate–acetic acid (60:40:10)
NG-3-^{125}I	A	benzene–ethanol–acetic acid (75:20:5)
T-17β-gluc-^{125}I	S	ethyl acetate–hexane–ethanol–acetic acid (70:20:5:3)
aetio-3α-gluc-^{125}I	S	ethyl acetate–hexane–ethanol–acetic acid (70:20:5:3)
andro-3α-gluc-^{125}I	S	ethyl acetate–hexane–ethanol–acetic acid (70:20:5:3)

* T = testosterone; P = progesterone; NE = norethisterone; NEA = norethisterone acetate; NG = *D*-norgestrel.

† S = silica gel; A = alumina.

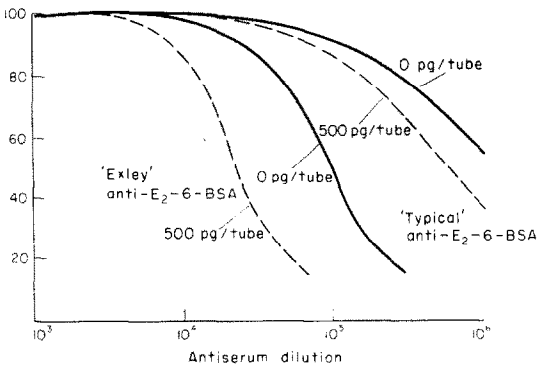

Fig. 1. Dilution curves for a 'typical' anti-E_2-6-BSA serum and the "Exley" anti-E_2-6-BSA serum using the E_2-6-^{125}I radioligand and performed with and without inhibition of ligand binding by 500 pg non-radioactive oestradiol per tube. (anti-E_2-6-BSA = anti-oestradiol-6-(O-carboxymethyl)-imino-BSA; E_2-6-^{125}I = oestradiol-6-(O-carboxymethyl)-imino-[^{125}I]iodohistamine).

binding following addition of non-radioactive oestradiol (Fig. 1). Note that inhibition of ligand binding by the addition of non-radioactive oestradiol predicts a useful assay system. *Heterologous* systems (position of attachment of ^{125}I and the original antigenic hapten different)[5] were now tried using the same type of antisera but using oestradiol-3-hemisuccinyl-[^{125}I]-iodohistamine (E_2-3-^{125}I) and oestradiol-17β-hemisuccinyl-[^{125}I]iodohistamine (E_2-17β-^{125}I) radioligands. An anti-E_2-17β-BSA serum (kindly supplied by Dr. Vernon C. Stevens, Dept. of Obstetrics and Gynecology, The Ohio State University Hospital, 410, West 10th St., Columbus, Ohio, U.S.A.) was also investigated for comparative purposes. The results obtained are shown in Fig. 2 and, bearing in mind that the data applies to most such sera, it can be seen that the *homologous* systems (position of attachment of ^{125}I and the original antigenic protein identical)[5] are not generally useful. The affinity of the antiserum for ligand greatly exceeds that of oestradiol and usually

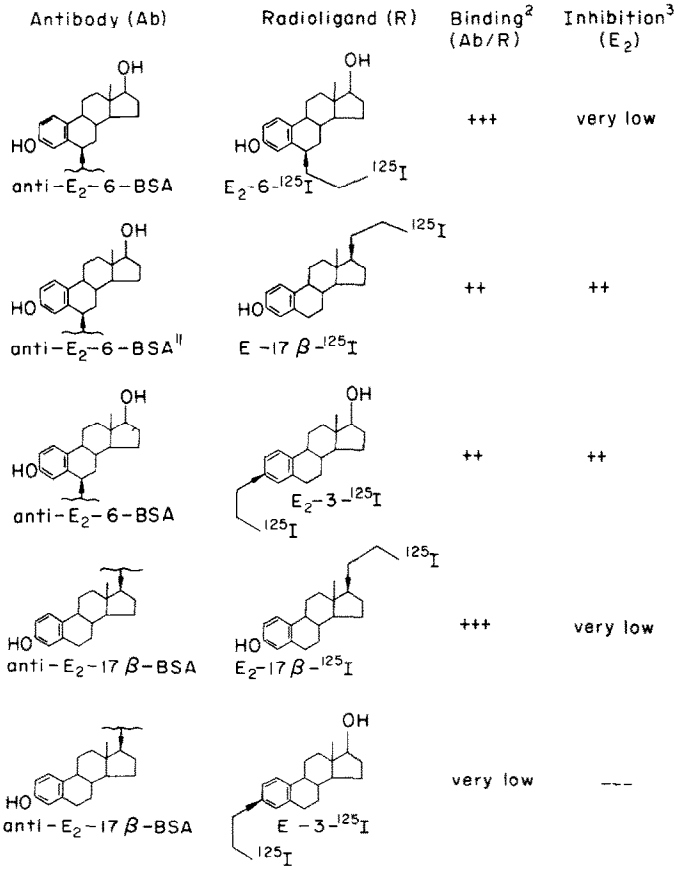

Fig. 2. Binding of 'typical' anti-E_2-6-BSA and anti-E_2-17β-BSA[1] sera to E_2-6-^{125}I, E_2-3-^{125}I and E_2-17β-^{125}I radio-ligands and inhibition by oestradiol.

[1]Anti-E_2-17β-BSA = anti-oestradiol-17β-hemisuccinyl-BSA; E_2-3-^{125}I = oestradiol-3-hemisuccinyl-[^{125}I]-iodohistamine; E_2-17β-^{125}I = oestradiol-17β-hemisuccinyl-[^{125}I]-iodohistamine; other abbreviations have same significance as in Fig. 1.

[2]Titre relative to ^{3}H-labelled radioligand.

[3]Use of inhibition of radioligand binding with 500 pg non-radioactive steroid relative to system using ^{3}H-labelled radioligand with same antiserum.

Table 2. Binding characteristics of "typical" antisera to various steroids to ^{125}I-labelled radioligands and their inhibition by the corresponding steroid hapten

Antiserum (Ab)	Radioligand (R)	Binding[1] (Ab/R)	Inhibition[2] (Steroid)
anti-T-3-BSA[3]	T-3-^{125}I	+ + +	+ + +
anti-T-3-BSA	T-11α-^{125}I	+	+ + +
anti-T-3-BSA	T-17β-^{125}I	N.D.[4]	N.D.
anti-T-11α-BSA	T-3-^{125}I	+ +	+ +
anti-T-11α-BSA	T-11α-^{125}I	+ + +	+
anti-T-11α-BSA	T-17β-^{125}I	N.D.	N.D.
anti-P-3-BSA	P-3-^{125}I	+ + +	+ + +
anti-P-3-BSA	P-11α-^{125}I	very low	—
anti-P-11α-BSA	P-3-^{125}I	+ +	+ +
anti-P-11α-BSA	P-11α-^{125}I	+ + +	+
anti-NE-11α-BSA	NE-3-^{125}I	+ +	+ +
anti-NE-11α-BSA	NE-11α-^{125}I	+ + +	very low
anti-NEA-3-BSA	NEA-3-^{125}I	+ + +	very low
anti-NG-3-BSA	NG-3-^{125}I	+ +	+ + +
anti-NG-11α-BSA	NG-3-^{125}I	+ +	+ +

[1] Titre relative to ^{3}H-labelled radioligand.
[2] Ease of inhibition of radioligand binding with 500 pg non-radioactive steroid relative to system using ^{3}H-labelled radioligand with same antiserum.
[3] T, P etc. have same significance as in Table 1.
[4] Not done.

no assay system is practicable. *Heterologous* systems are more difficult to predict. For example, the anti-E_2-6-BSA serum will bind both E_2-3-^{125}I and E_2-17β-^{125}I ligands and this binding can be inhibited by oestradiol. However, anti-E_2-17β-BSA serum will not even bind E_2-3-^{125}I.

4-en-3-Oxosteroids

We have previously described some aspects of the use of ^{125}I-labelled derivatives in the radioimmunoassay of progesterone[6] and norethisterone[7]. We include here the data relevant to the total picture and also material related to systems involving testosterone and *D*-norgestrel. This part of the study was made possible by the generous provision of 4–3-oxo-steroid-11α-hemisuccinates and by the availability of a simple synthesis for 4-en-3-oxo-steroid-3-(O-carboxymethyl)oximes[9]. From both types of hapten, BSA conjugates and ^{125}I-labelled radioligands could be synthesized and the corresponding antisera raised. Thus different steroids could be compared directly in similar types of assay system.

Table 3. Percentage cross reactions of various steroids in potential assay systems using anti-testosterone-3-BSA and anti-testosterone-11α-BSA sera in combination with ^{3}H-testosterone and testosterone-3-^{125}I radioligands

Steroid	anti-T-3-BSA		anti-T-11α-BSA	
	^{3}H-T	T-3-^{125}I	^{3}H-T	T-3-^{125}I
Testosterone	100	100	100	100
5α-Dihydrotestosterone	65	75	15	27
4-androstene-3,17-dione	1·6	<4	2	2·4
Epitestosterone	6·3	0·8	<0·9	0·4
DHA	<0·06	0·07	<0·1	<0·01
5-androstene-3β,17β-diol	0·9	1·9	0·16	0·27
5α-androstane-3α,17β-diol	3·0	5·3	<0·1	2·2
Adrenosterone	—	<0·02	<0·1	0·01
11β-Hydroxy-4-androstene-3,17-dione	0·06	<0·2	<1·0	<0·1
17β-Hydroxy-4-androstene-3,11-dione	1·9	<1·9	17	16
11β,17β-Dihydroxy-4-androsten-3-one	2·5	<1·4	25	13
Cortisol	0·06	<0·02	<0·01	<0·01

Anti-T-3-BSA = anti-testosterone-3-(O-carboxymethyl)imino-BSA; anti-T-11α-BSA = anti-testosterone-11α-hemisuccinyl-BSA; ^{3}H-T = [1,2,6,7-^{3}H$_4$]testosterone; T-3-^{125}I = testosterone-3-(O-carboxymethyl)imino-[^{125}I]iodohistamine.

Table 4. Comparison of the cross reactions of 5α- and 5β-dihydro steroids in anti-steroid-3-BSA/steroid-3-^{125}I and anti-steroid-11α-BSA/steroid-3-^{125}I systems; and with either type of antiserum and a ^{3}H-labelled ligand

Antiserum	Radioligand	% Cross reaction 5α-	5β-
anti-T-3-BSA[1]	T-3-^{125}I	75	15
	^{3}H-T	65	13
anti-P-3-BSA	P-3-^{125}I	55	17
	^{3}H-P	60	14
anti-NG-3-BSA	NG-3-^{125}I	23	10
	^{3}H-NG	N.A.[2]	N.A.
anti-T-11α-BSA	T-3-^{125}I	27	4
	^{3}H-T	15	1·4
anti-P-11α-BSA	P-3-^{125}I	28	12
	^{3}H-P	14	6
anti-NE-11α-BSA	NE-3-^{125}I	63	23
	^{3}H-NE	18	3
anti-NG-11α-BSA	NG-3-^{125}I	3	20
	^{3}H-NG	N.A.	N.A.

[1] Abbreviations have same significance as in Tables 1 and 2.
[2] ^{3}H-*D*-Norgestrel was not available for comparison.

Thus homologous and heterologous combinations of antisera and radioligands involving the 3- and 11α-positions were examined and Table 1 shows the results obtained with a variety of steroids. As described earlier, inhibition was examined by addition of 500 pg of the appropriate steroid per tube in a duplicate titration curve as exemplified in Fig. 1. From an examination of the data it can be seen that corresponding combinations of antiserum and radioligand appear to behave in a similar fashion. An anti-steroid-3-BSA/steroid-3-^{125}I homologous system is potentially useful in an assay since there is substantial binding of ligand which is reversible by inhibition with the steroid hapten. On the other hand, an anti-steroid-11α-BSA serum/steroid-11α-^{125}I is valueless since although high binding of the ligand occurs it cannot be reversed by addition of steroid. There are two curious anomalies–whereas the anti-steroid-11α-BSA/steroid-3-^{125}I system has obvious potential, the anti-steroid-3-BSA/steroid-11α-^{125}I system has none. Secondly the anti-norethisterone acetate-3-BSA/norethisterone acetate-3-^{125}I behaves differently from other anti-steroid-3-BSA/steroid-3-^{125}I systems in that the binding cannot easily be inhibited by addition of norethisterone acetate.

Cross-reactions of various steroids in the potential assay systems for progesterone and norethisterone have been reported in detail elsewhere [6, 7] and Table 3 shows corresponding data for testosterone which may be regarded as fairly typical for 4-en-3-oxosteroids when comparing one potential assay system with another. It can be seen that the use of the T-3-^{125}I radioligand causes an increase in the cross-reaction of steroids which resemble testosterone around the 3-position, e.g. 5α-dihydrotestosterone or 5α-andostan-3α,17β-diol. Hence an important factor in the degree of cross-reaction is the site of attachment of the ^{125}I-bearing group. Moreover, the ^{3}H-T almost always gave the lower cross-reaction. The major exception to this was the greatly reduced value for 11β, 17β-dihydroxy-4-androsten-3-one (11β-hydroxytestosterone) with the anti-T-11α-BSA/T-3-^{125}I system.

Comparison of the cross-reactions of 5α- and 5β-dihydro derivatives (Table 4) was performed to see how well the assay systems distinguished between the epimeric steroids. The evidence strongly suggests that with all three types of radioligand the antisera distinguish between them very readily and discriminate clearly against 5β-dihydro compounds. The only exception to this rule was with the anti-*D*-norgestrel-11α-BSA/*D*-norgestrel-3-^{125}I system. In all cases the ^{3}H-steroid gave lower cross-reactions than either ^{125}I-labelled radioligand.

Glucuronosides

The three anti-steroid-glucuronoside-BSA sera prepared using haptens testosterone-17β-glucuronoside, androsterone-3α-glucuronoside and aetiocholanolone-3α-glucuronoside were examined in conjunction with their homologous ^{125}I-radioligands. Where possible the results were compared with those obtained using the corresponding ^{3}H-labelled materials. The cross-reactions of the corresponding free steroids were always high and particularly so when a ^{125}I-labelled ligand was used (Table 5). No clear pattern emerged from an examination of the three types of antisera and

Table 5. Titration and cross-reaction data for anti-steroid-glucuronoside-BSA sera with ^{3}H- and ^{125}I-labelled radioligands

Antiserum	Radioligand (pg/tube)	Titre	Cross-reaction* steroid	%
anti-T-17β-gluc-BSA†	^{3}H-T (30)	8×10^{3}	—	—
	^{3}H-T-17β-gluc (60)	8×10^{3}	testosterone	45
	T-17β-gluc-^{125}I (2)	$2{\cdot}6 \times 10^{4}$	testosterone	170
anti-aetio-3α-gluc-BSA	^{3}H-aetio (60)	5×10^{4}	—	—
	^{3}H-aetio-3α-gluc (240)	—	aetio	48–95
			andro-3α-gluc	2–12
	aetio-3α-gluc-^{125}I (2)	5×10^{4}	aetio	27–105
			andro-3α-gluc	5–9
anti-andro-3α-gluc-BSA	^{3}H-andro (60)	1×10^{4}	—	—
	andro-3α-gluc-^{125}I (2)	8×10^{4}	andro	321
			aetio-3α-gluc	64

* Respective steroid glucuronoside taken as 100%.

† T-17β-gluc = testosterone-17β-β-D-glucuronoside, etc.; T-17β-gluc-^{125}I = testosterone-17β-β-D-glucuronoside-6-[^{125}I]-iodohistamine, etc.; aetio = aetiocholanolone; andro = androsterone; other abbreviations have the same significance as in Tables 1 and 2.

their affinities for different types of radioligand. Indeed there were considerable variations among sera raised in different animals against the same antigen *vide* the range of cross-reaction of aetiocholanolone in the anti-aetio-3α-gluc-BSA/aetio-3α-gluc-^{125}I system.

DISCUSSION

The purpose of the present study was to exploit a readily available source of ^{125}I-labelled radioligands for steroid immunoassay and investigate their use in comparison with the corresponding ^{3}H-labelled materials. A number of general conclusions may be drawn. The systems using radio-iodine labelled ligands lend themselves particularly to situations where large numbers of samples require to be analysed and the advantages of scale considerably reduce total cost and counting time. Furthermore, if a material, steroid or otherwise, is to be analysed and no ^{3}H-labelled ligand is commercially available, a radio-iodine labelled tracer may be the only practicable answer. From the data in Tables 2 and 4, however, it is obvious that the anti-steroid-3-BSA/steroid-3-^{125}I homologous system and the anti-steroid-11α-BSA/steroid-3-^{125}I heterologous system cannot compete with the corresponding ones using a ^{3}H-labelled ligand in terms of specificity. In the majority of instances, homologous systems involving the "non-functional" positions on the steroid molecule, e.g. 6-oxo-oestradiol and progesterone-11α-hemi-succinate probably do not succeed because the structure of the original antigenic determinant and the steroid radioligand are so similar that the affinity of the antiserum for the ligand greatly exceeds that of the free steroid. Why this should not apply to the anti-steroid-3-BSA/steroid-3-^{125}I system is currently inexplicable. As mentioned earlier, antisera with unique properties occur, e.g. the 'Exley' anti-oestradiol-6-BSA[3, 15] or the 'Tenovus' anti-progesterone-11α-BSA sera[6] with which homologous systems can be established. When found and used with the homologous ^{125}I-labelled ligand these antisera compete very favourably with the corresponding ^{3}H-system in terms of antiserum titre and specificity. It may be that perseverance in the search for such sera is the way to secure the best advantages in the use of ^{125}I-labelled steroid radioligands.

It is also apparent that with the physical phenomena of ^{125}I-labelled radioligand/antibody binding and cross-reactions lie clues to the nature, size and shape of the binding sites, and to the corresponding characteristics of steroid antigenic determinants. In this regard it is interesting to speculate on the reasons for the occurrence of binding of oestradiol-3-^{125}I and oestradiol-17β-^{125}I to the anti-oestradiol-6-BSA serum yet the lack of binding of oestradiol-3-^{125}I to anti-oestradiol-17β-BSA (Fig. 2). It is perhaps useful to consider the steroid on the antigenic protein as akin to the serations on a key and the bridging group as its handle. The position of the 'handle' appears to be crucial. In the first two instances described above, the iodinated groups are at the extreme edges of the key and may not influence the fit so much as in the latter instance when the iodinated group is virtually opposite the end of the shaft. It is also possible that similar criteria can be used to explain why binding occurs with an anti-steroid-11α-BSA/steroid-3-^{125}I system but not with an anti-steroid-3-BSA/steroid-11α-^{125}I system (Table 2). The greater cross-reactions of the ^{125}I-systems in Table 2 compared with the ^{3}H ones may be due to the fact that only the antibody population which is relatively

blind with respect to the 3-position is featuring in the assay.

In these terms, however, it is difficult to explain the very high cross-reaction of the free steroids[16] in the anti-steroid-glucuronoside-BSA / steroid-glucuronoside-^{125}I systems although the rules of antibody/antigen binding may be different when hydrophobic (steroid) and hydrophilic (glucuronoside) determinants are in close proximity to one another.

Acknowledgements—The authors wish to thank the Tenovus Organisation for generous financial support throughout these investigations. One of us (E.H.D.C.) also received a grant for technical assistance from Schering AG, Berlin, who also kindly provided the synthetic steroids and their derivatives. Dr. G. F. Woods, Organon Laboratories Ltd., Newhouse, Lanarkshire, Scotland, generously donated 11α-hemisuccinates of testosterone and progesterone. Prof. G. D. Niswender, Dept. of Physiology and Biophysics, Colorado State University, Fort Collins, Co., U.S.A. provided the anti-progesterone-3-BSA serum, Dr. D. Exley, Dept. of Biochemistry, Queen Elizabeth College, London, one of the anti-oestradiol-6-BSA sera, and Dr. Vernon C. Stevens, Dept. of Obstetrics and Gynecology, The Ohio State University Hospital, 410, West 10th St., Columbus, Ohio, U.S.A. the anti-oestradiol-17β-BSA serum. Prof. A. K. Kellie, Courtauld Institute of Biochemistry, Middlesex Hospital, London, kindly advised on the preparation of steroid glucuronosides and provided [6,7-^{3}H] aetiocholanolone glucuronoside. The authors also wish to thank Prof. K. Griffiths, Tenovus Institute for Cancer Research for his advice and criticism throughout.

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DISCUSSION

Adlercreutz:

Have you used selenium isotopes?

Cameron:

I think the answer is, not yet, but Dr. Evans from the Radiochemical Centre at Amersham is here and he may want to talk about selenium isotopes. I have no information. I have never used them but we hope to be able to use them when they become available.

Kuss:

I would like to discuss the affinity of anti-estrogen-C6-conjugate antisera for estrogen-C6-conjugates. This has been tested systematically also in the progress of our studies on steroids as immunochemical probes. In summary, the C6-histamine derivative and even the C6-lysine derivative, which is most strongly related to the steroidal immunogenic determinants of the macromolecular antigen, exhibits practically the same K_{Ass}-values as 6-oxo-estrogen did (Kuss, E. and Dirr, W., unpublished data). Therefore, we concluded that the steroid-protein bridge of the antigen is not really recognized by the immunocompetent system as a part of the steroidal immunogenic determinant. The problem has been discussed previously in detail (Kuss, E., Goebel, R. and Enderle, H., *Hoppe-Seyler's Z. physiol. Chem.*: **354**, (1973) 347). In our opinion, this hypothesis is also supported by Kellie's data from experiments with glucuronic acid as steroid-protein bridge in the antigens (Kellie, A. E. *et al.*, *J. steroid Biochemistry*: **3**, (1972) 275): the unconjugated steroid exhibited nearly the same affinity to the antisera as the corresponding steroid glucuronide, although it lacks all of the numerous bridge component hydroxyl groups. On the other hand, we all know that only minimal affinity is exhibited by steroids lacking nothing but just one hydroxyl group of the steroidal nucleus as compared to the corresponding hapten.

Now, also Nars and Hunter (*J. Endocrinology*: **57**, (1973) xlvii) reported the particular high affinity of antisera for the ^{125}I-labeled histamine derivative. Regarding the fact that the size of the big iodine atom equals about the size of a benzene ring, one expects that the hapten must be recognized by the antiserum as less complementary than the unlabeled histamine or lysine derivatives mentioned above, if the bridge component is recognized at all as a part of the hapten. Since in our experiments the affinity of these derivatives did not exceed the affinity of 6-oxo-estrogens significantly, I am not finally convinced, that the reported strong binding of the iodine labeled estrogen haptens to antiserum is really caused by the antigen-antibody interaction of the bridge component.

Cameron:

The problem is that we're possibly talking about two different situations. On the one hand you're talking about inhibition of binding of estradiol 6-(O-carboxymethyl)oxime linked to[^{25}I]-iodo-histamine, and on the other hand about a situation where you're allowing free estradiol to compete

with [^{3}H]-estradiol. Now to my way of thinking there is a strong possibility that we may be using different populations of antibodies depending on what our detection system is. For instance if you are using [^{3}H]-estradiol, I could well believe that you're using a certain population of antibodies which, if you'll excuse the expression, "sees" the estradiol well. If we are talking about systems using the iodine tracer, then by definition surely we are talking about a different spectrum of antibodies. Would you agree with that?

Kuss:

Perhaps, but I believe, at least, that the antigen binding site is filled completely by the steroid and if you fractionate your population and you isolate the steroid binding fraction you perhaps don't get affinity to the bridge.