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GENERAL DISCUSSION

Exley:

The assessment of immunological activity of [^{125}I]-labelled antibodies by radioimmunoassay using [^{3}H]-labelled steroids demands measurement of ^{3}H often in the presence of larger amounts of ^{125}I. Unfortunately to date no method appears to be available for this assessment.

The measurement of ^{3}H is normally made by setting the amplification and discriminator controls of a β-scintillation counter to obtain maximum square efficiency/background readings. These settings were found unsuitable when ^{3}H was accompanied by ^{125}I on our β-counter instrument.

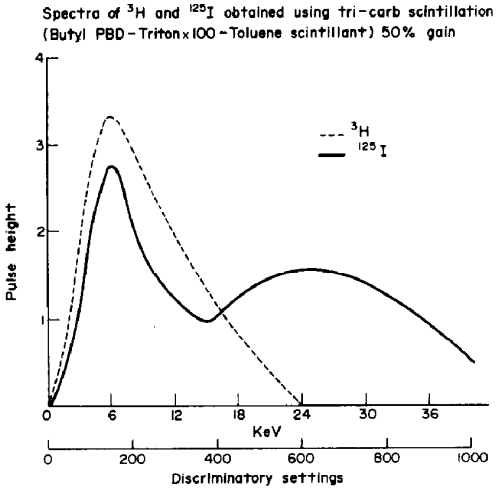


Fig. 1. (Exley).

Figure 1 shows the pulse height spectra of ^{3}H and the Compton Edge pulse height spectra of ^{125}I. As can be seen the spectra of ^{3}H coincides with the lower energy peak of the ^{125}I Compton Edge spectra and it was found that quenching increased the total ^{125}I + ^{3}H cpm reading within the normal ^{3}H discriminator setting due to a quench induced shift of the higher energy ^{125}I Compton Edge spectra into this setting. The use of a wider discriminator setting of 20–1000 (08–40 KeV) and adjustment of amplification for maximum ^{3}H counting overcame this difficulty. A further difficulty was encountered with the preparation of the appropriate quench curve for ^{125}I. Whilst the quench curve for ^{3}H was found to be suitable for different levels of quenching a 5% apparent increase was found for the quench curve of ^{125}I. Initially it was considered that this was due to an effect of γ radiation on the Automatic External Standard assessment, but later it has been considered to be possibly due to the fact that ^{125}I energy is dissipated by multiple collisions with the gel-buffer-scintillator mixture required for radioimmunoassay in contradistinction to ^{3}H whose energy is mainly dissipated by single collision events. Due to this difficulty construction of accurate ^{125}I quench curves could not be made since without buffer and gel we obtained an erroneous curve and with buffer and gel the quench did not enable us to obtain the efficiency for quenched samples above the mean of our experimental conditions. Quench curves corrected for this 5% discrepancy were therefore constructed using the scintillant with buffer-gel.

Using these above parameters our department has developed a method for counting ^{3}H in the presence of ^{125}I.

Table 1 shows a few results obtained using this method. Assessment of ^{3}H in the presence of ^{125}I was made as

Table 1. Data showing results used in assessing ^{3}H in the presence ^{125}I

(1) dpm ^{125}I	Corrected % eff ^{125}I	cpm ^{125}I + ^{3}H	(2) Apparent dpm	Δ 2–1	cpm ^{3}H	% eff ^{3}H	dpm ^{3}H	% recovery
1000	50	750	1500	500	250	25	1000	100
17082	50·4	11270	22361	5279	2661	29·6	8989	95·8
17349	49·4	11147	22565	5216	2577	32·4	7953	84·7
30705	51·7	18769	36304	5599	2895	31·5	9189	97·9
29367	52·7	18234	34600	5233	2758	27·2	10137	108
30610	52·5	18840	35886	5276	2770	30·8	8993	95·8
60336	54·8	35333	64476	4140	2269	26·7	8498	90·5
60995	54·3	35653	65659	4664	2533	28·1	9013	96·0
20697	51·9	13165	25366	4669	2423	24·0	10096	108
41938	53·2	24668	46368	4430	2357	25·9	9100	96·9
41772	51·6	23626	45787	4014	2072	23·0	9007	96·0
83630	53·2	46951	88922	5292	2794	28·0	9980	106

follows: The true ^{125}I of a mixture of ^{125}I + ^{3}H was first counted using a γ counter and appropriately corrected to true ^{125}I dpm. The total ^{125}I + ^{3}H cpm was then counted using a β-scintillator counter using the previously mentioned adjusted discriminator and amplification settings. This total cpm was then assessed as total apparent ^{125}I dpm using the efficiency for ^{125}I by the corrected ^{125}I quench curve. The true ^{125}I dpm previously obtained on the γ counter was then subtracted from this apparent ^{125}I dpm. The remaining dpm was then corrected back by the corrected ^{125}I efficiency to cpm and this then represents ^{3}H which was finally corrected for ^{3}H efficiency to ^{3}H dpm.

This method is now being used in our department for radioimmunoassay involving ^{125}I -labelled antibodies with tritiated steroids.

De Hertogh:

One step in the translocation of the estradiol-cytosol receptor complex into the uterine nucleus is described by several authors, as an "activation" process which would enable the complex to associate with the chromatin acceptor sites. This "activation" has been described as being temperature dependent. We tried to see whether temperature "activation" of the complex produced some modification in the binding affinity of the receptor moiety.

Fig. 1. (De Hertogh).

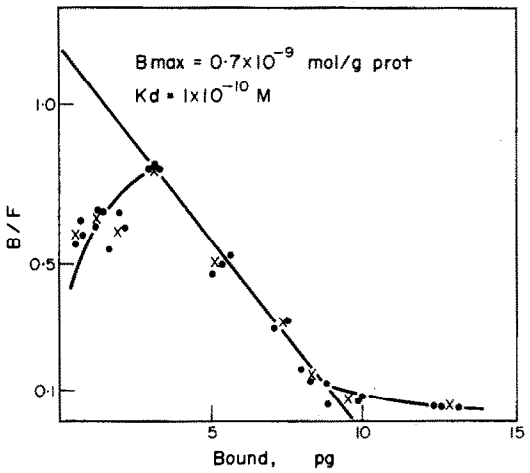


Fig. 1. Binding of [^{3}H]-estradiol to a Tris-EDTA-Sucrose cytosol preparation from immature rat uteri. Scatchard type of representation. Protein concentration: 0·22 mg/ml.

Figure 1 shows a Scatchard plot derived from the binding equilibrium obtained after 30 min incubation at 22°C, followed by 30 min incubation at 4°C, of [^{3}H]-estradiol with a cytosol preparation of immature rat uteri (28 days old). The cytosol was prepared in Tris (10 mM)-EDTA (1 mM)-sucrose (0·25 M) buffer, pH7·4. The bound and unbound fractions were separated by the charcoal method (10 min incubation). Total protein concentration was 0·22 mg/ml.

The figure shows essentially one binding component and a cooperative effect at low ligand concentration. The stability of the complex in the presence of charcoal at 4°C (from 10 s to 29 h) is indicated in Fig. 2 I, curve C.

Fig. 2. (De Hertogh).

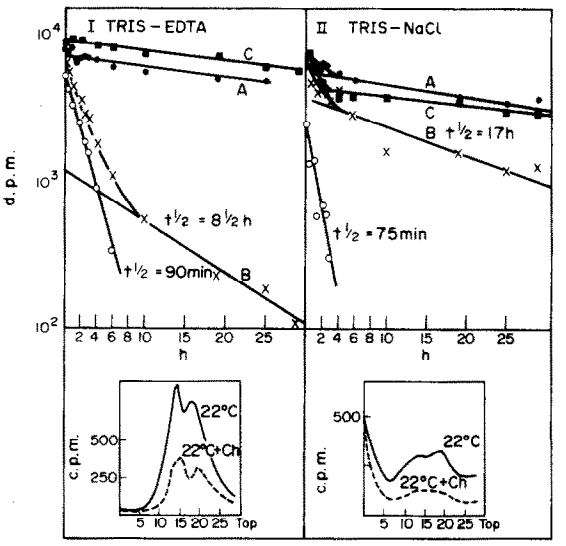


Fig. 2. *Upper part*: Effect of time on binding stability of [^{3}H]-estradiol to immature rat uterus cytosol.
A: 4°C in the presence of dextran-coated charcoal.
B: 22°C.
C: 22°C in the presence of 300-fold excess of [^{14}C]-estradiol.
In ordinate: DPM bound [^{3}H]-estradiol.
Lower part: High salt (0·4 M KCL) 8·5 to 28·5% sucrose density gradients after 2 h incubation in the absence (22°C) or in the presence (22°C + Ch) of 100-fold excess of [^{14}C]-estradiol. Protein concentration: 0·39 mg/ml.
See text for experimental details.
I: Cytosol prepared in Tris—0·01 M—EDTA 1 mM—Sucrose 0·25 M, buffer pH 7·4.
II: Cytosol prepared in Tris—0·01 M—NaCL 0·16 M—Sucrose 0·25 M, buffer pH 7·4.

The dissociation kinetics of the binding at 22°C were studied after incubation of [^{3}H]-estradiol with the cytosol at 4°C for 1 h, followed by a 30 min incubation at 22°C. After cooling at 4°C, the excess unbound [^{3}H]-estradiol was precipitated by charcoal. Incubation of the cytosol was then continued at 22°C in the presence or the absence of a 300 fold excess in weight of [^{14}C]-estradiol. At the end of the incubation times, the charcoal method was again used to determine the amount of bound [^{3}H]-estradiol. This amount was corrected for the unspecific binding, calculated from the ^{14}C counts in the bound fraction.

Curve A (Fig. 2 I) shows the stability of the binding at 22°C for as long as 25 h. Curve B shows the loss of bound [^{3}H]-estradiol in the presence of ^{14}C-competitor. The dissociation of [^{3}H]-estradiol from the receptor appeared to be biphasic. The slowly dissociated component amounts for less than 20% of the total sites.

On high salt (0·4 M KCL) sucrose density gradient, the bound [^{3}H]-estradiol migrated as a double peak in the 4 to 5 S region, before and after a 2 h chase with [^{14}C]-estradiol at 22°C. In the latter case, the two peaks were proportionally reduced in height, showing that both had similar dissociation kinetics.

The sedimentation pattern of the cytosol preparation incubated with [^{3}H]-estradiol at 4°C only (not shown here) was usually also biphasic, with a lesser relative importance of the heavier component.

When the same experiment was run with cytosol prepared in Tris (0·01 M)–NaCl (0·16 M)–sucrose (0·25 M) buffer, pH 7·4, different observations were made (Fig. 2 II).

The estradiol-receptor complex could be partially disrupted by charcoal incubation at 4°C up to 4 h (curve C).

The exchange kinetics with [^{14}C]-estradiol was biphasic (curve B). The first component had the same half-time of dissociation as in the previous experiment, but the second component was more important and had a much longer half-time of dissociation. On high salt sucrose density gradient, aggregates were obtained in which estradiol exchange was limited.

In conclusion of these parallel observations, it seems that the binding of estradiol to immature rat uterus cytosol involves multiple forms. Temperature "activation" does not seem to modify the binding kinetics although other experimental condition (presence of 0·16 M NaCL or KCL in the homogenesing medium) may alter not only the sedimentation pattern but also the dissociation kinetics of the binding components.

Fotherby:

Some advantages of iodine labelled ligands have been mentioned by Dr. Cameron. One major advantage of iodine labelling is that it enables radioimmunoassays to be developed for compounds, for example many synthetic steroids, for which no tritiated preparation with a suitably high specific activity for use as a labelled ligand exists. We, (Warren and Fotherby, 1974 *J. Endocr.* In press) have recently described the use of iodinated tyrosine methyl esters of norgestrel and norethisterone in the development of radioimmunoassays for these synthetic progestogens. Antigens were prepared as described by Erlanger (1967) by coupling the 3(O-carboxymethyl) oxime of the steroid to bovine serum albumin using a mixed anhydride technique. Tyrosine methyl esters of the steroid oximes were prepared and iodinated. Typical standard curves obtained for norgestrel and norethisterone are shown in Fig. 1. The sensitivity of the assays was about 25 pg/ml and 15 pg/ml for norgestrel and norethisterone respectively.

During preparation of the steroid tyrosine methyl esters, two isomers, the cis and trans, are produced. After iodination the iodinated forms of the two isomers can be separated from each other and also from chloramine T and free iodine as we have previously described. The amount of ^{125}I incorporated into the steroid tyrosine methyl ester increases up to a reaction time of 2½ min. Both for norethisterone and norgestrel the amount of iodine incorporated into the less polar isomer was always greater than that incorporated into the more polar one suggesting that the less polar isomer was more readily iodinated or that a greater proportion of the less polar isomer was produced during the synthesis. The amounts of ^{125}I incorporated produced labelled ligands with specific activities of at least 100 Ci/mmol. Although these values are considerably lower than those which can be obtained by iodination of steroid-protein conjugates they are higher than the specific activities obtained by incorporation of four atoms of tritium into a steroid molecule. We have no indication for the incorporation of significant amounts of iodine into the ethynyl side-chain of the synthetic progestogens.

In view of the different amounts of iodine incorporated into the two isomers it was of interest to see whether their interaction with the antiserum was similar. When solutions of the two labelled isomers with equal counting rates were

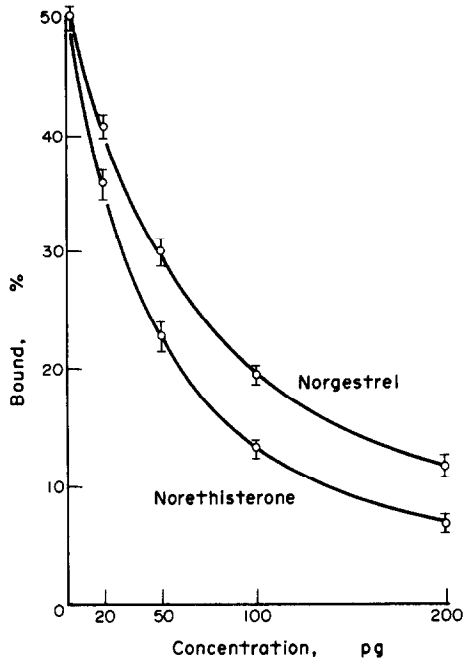


Fig. 1. Standard curves obtained for percentage binding of [^{125}I]-norgestrel TME and [^{125}I]-norethisterone TME with their respective antisera. (Values shown are means ± SEM for analysis of 5 replicate samples at each concentration). (Fotherby).

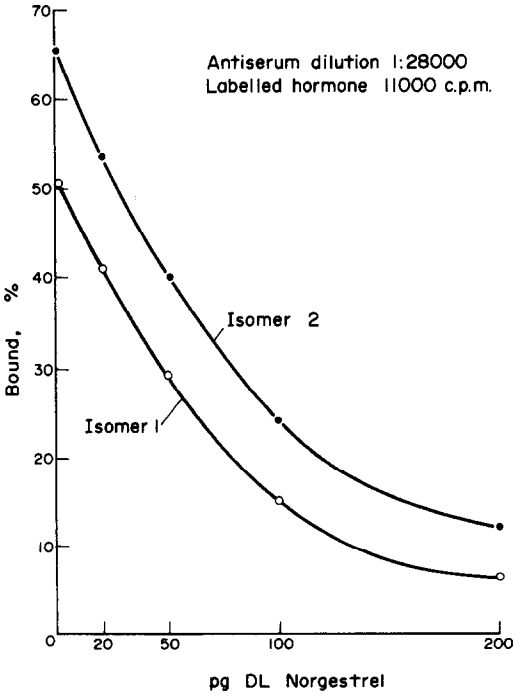


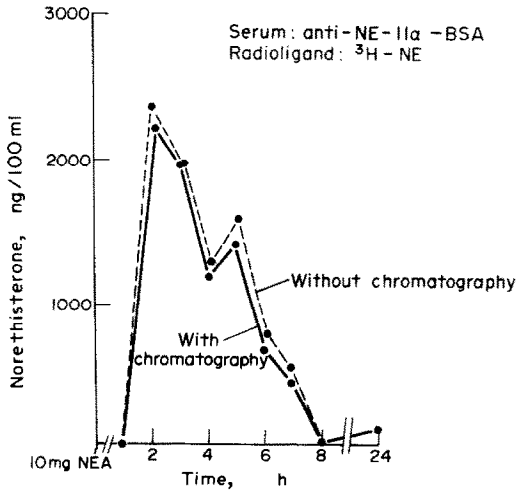
Fig. 2. Binding of the two isomers of [^{125}I]-norgestrel TME with the norgestrel antiserum. (Fotherby).

used with the antiserum against norgestrel, standard curves obtained over the 0 to 200 pg range were parallel (Fig. 2). With no unlabelled steroid added to the assay tubes, 65% of the less polar isomer was bound compared with 50% of the more polar isomer. This difference in binding could be accounted for by the difference in specific activity of the two isomers. That the avidity of the antisera for the two isomers was similar was shown by measuring the affinity constants from a Scatchard plot of the ratio of bound : free radioactivity against the total amount bound. For the two isomers of norgestrel, the affinity constants were similar ($0{\cdot}5 \times 10^9$ l/mol).

Dr. Cameron has already discussed the comparison of iodine labelled and tritium labelled ligands. We have found that both for the untreated and the rivanol-treated antiserum against norethisterone, 50% binding of the ligand was achieved at a higher dilution of antiserum using the iodine labelled than the tritium labelled steroid. With dilutions of antiserum similar to those required for 50% binding, the amount of unlabelled norethisterone required to reduce the relative binding of the ^{125}I ligand by 50% was much lower than the amount required to reduce the relative binding of [^{3}H]-norethisterone by this amount. In agreement with results of previous investigators we find that the degree of cross-reaction depends upon the labelled ligand which is used. Better specificity is obtained using the ^{3}H ligand than the ^{125}I ligand. Using the latter many metabolites of norethisterone will displace this ligand from the antiserum whereas the relative activity is greatly reduced when [^{3}H] norethisterone is used as ligand.

Cameron:

I simply wanted to show 2 slides very quickly to support Dr. Fotherby's data. The first one shows the effect of chromatography on plasma analysis for norethisterone. With this particular antiserum and this particular tritiated ligand, you get a highly specific system. There's very little difference with the introduction of a chromatography step. The 2nd slide compares the same sort of data obtained using a tritiated ligand and also using the iodine ligand. In each case the peak obtained using the ^{3}H-labelled traces is the slightly higher of the two, but it shows that the method using either ligand is specific for determination of norethisterone.



Concentrations of NE in plasma of volunteers given 10 mg NE acetate orally

Serum : anti-NE-IIa - BSA
(^{3}H-NE, dil. 1: 4000)
(NE-3-^{125}I, dil. 1: 10000)

Fig. 1. (Cameron).

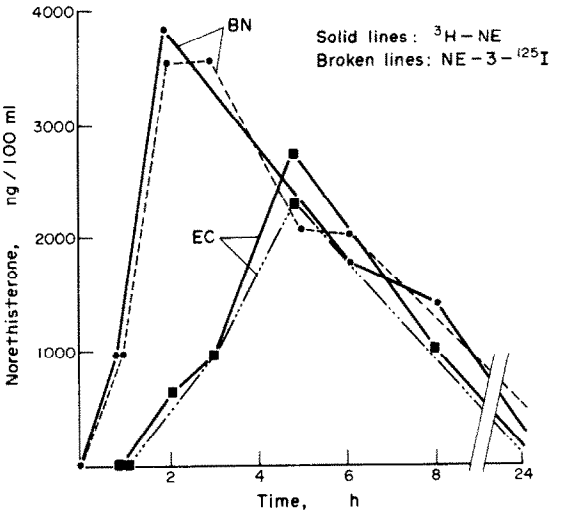


Fig. 2. (Cameron).

Crabbé:

I would like to address myself to Dr. Carstensen and ask him whether he had the opportunity to examine to what extent the secretion of testosterone by the testis would be influenced by surgery performed on dogs. Was there a sizeable decrease in the concentration of testosterone in the spermatic vein of the animals after surgery?

Carstensen:

No, we did not collect spermatic vein blood after the surgery in the dogs. I mean in order to collect spermatic vein blood you have to operate and we wanted to see how surgery in connection with anesthesia affected it. But I can tell you that we've collected samples in human males that were exposed to trauma without anesthesia and we get a pronounced decrease in the plasma testosterone concentrations in response to this. This is a very complicated matter. Actually, we have also been able to decrease the plasma concentration and the production rates in dogs by using 100% oxygen alone. Letting dogs breathe 100% oxygen for $2\frac{1}{2}$ h will decrease plasma testosterone and testosterone production rates to very low values. This can also be done in male rats but the rat does not respond to surgical trauma and does not respond to barbiturates.

Adlercreutz:

My question is also to Dr. Carstensen. We made two observations recently which may have some relation to your studies. First, we gave young people alcohol and measured testosterone during their alcohol intoxication phase and during the hangover and there was no change during intoxication but a highly significant decrease during the hangover period. This was related to the degree of hangover. Those who did not have very much hangover had normal testosterone levels (*Br. Med. J.*, **2** (1974) 443). We have also

investigated amateur marathon runners participating in the Athens marathon of 42 km. In 12 of 13 participants, testosterone decreased very significantly but there was one man who was 3rd in the whole race who had a slight increase but he had a high level from the start also. One man collapsed and his level went down to female plasma testosterone levels (*Br. Med. J.*, in press). Can you comment on this?

Carstensen:

I can't comment on the alcohol intoxication, but actually we have tested a lot of factors and have found that many factors will decrease plasma testosterone. Sometimes, but not always, one of these is corticosteroid injection and I believe that some people have also tried ACTH and some get a decrease and some not, so there must be a common explanation for this and we think this is the peripheral circulation in the testes. We are just going to investigate that in rats. There is an anatomical possibility of a shunting mechanism in the testes so the peripheral circulation may be very sensitive to many different agents.

Korenman: I can't avoid pursuing this issue. Is this reduction of testosterone in association with elevation of LH or reduction of LH?

Adlercreutz:

There is a secondary rise in LH.

Carstensen:

In the human being we have tested FSH and LH and there is no explanation. There is no reduction of gonadotropins that can explain the decrease in testosterone, but like Prof. Adlercreutz said there is maybe a secondary reaction, but in the post-operative decrease there is an early increase of LH which doesn't last very long and there is no secondary increase either.

Sjövall:

I had one question to Dr. Siekmann about the estrone peak. Did it represent 200 ng and what was the concentration in plasma? Was that peak one compound? The shape of the peak in the *m/e* 342 chromatogram and the shape of the peak of internal standard, *m/e* 344, did not seem to be the same.

Siekmann:

First I would like to tell you that this fragmentogram was a re-drawing for the purpose of explanation. The other fragmentograms were original and they were photographed from the paper. With respect to the amount of oestradiol-17 I would like to mention that this was measured in the plasma of pregnant females. We have used 2·5 ml of plasma and we have found about 115 to about 250 n in 2·5 ml of plasma.

Bertrand:

I would like to ask one question to Dr. Lindner. How could you explain the high cross reactivity between the 5α-derivatives and the unsaturated derivatives on antibodies raised against testosterone-6-derivative or 7 derivative?

Lindner:

The overall skeletal shape of the two compounds (testosterone and 5α-DHT) is remarkably similar, so it's really asking quite a lot of the antibody to discern the difference. We tried to couple not too close to the A/B ring junction. When we couple testosterone through position 7, this cross-reaction is about 42%, and when we shift the attachment to the 1 position it is reduced to 8%. This is all I can say at this stage. Dr. Kellie suggested that it may also matter whether we couple the steroid to protein through axial or equatorial bonds and present the α or β surface of the steroid to the lymphocyte.

Bertrand:

Dr. Maguelone Forest in our laboratory has observed a high cross reactivity between the 5-reduced compound and the unsaturated compound when studying antibodies obtained from C.7 derivative-testosterone-BSA complexes.

My question was that maybe the testosterone-BSA complex has been transformed or more precisely a 5α-reduction of the coupled testosterone could have taken place. This would then result in a production of antibodies to the 5α-dihydrotestosterone–BSA complex.

Lindner:

I have no evidence that these conjugates could be still amenable to enzymatic transformation after injection to the animal, but there are situations where this may be a problem, for instance with some prostaglandins. To examine this question further it would be important to fractionate the antibody population by use of immunoadsorbants and see whether you are dealing with an antibody binding site that has sufficient wobble to accommodate both testosterone and 5α-dihydrotestosterone or whether you have a mixed population of specific antibodies, one adapted exclusively to testosterone and the other to the metabolite.

Exley:

Dr. Lindner. You are still using a single bond conjugation of your bridge to the steroid. Don't you think that you might endow more rigidity to your molecule (and hence greater specificity of the elicited antisera) if this conjugation was made by a double bond, or do you feel this is not important?

Lindner:

We have really used both. We used the oxime attachment where we have a double bond, and in the case of the thioethers they would be single bonds. We haven't enough information where we compare the two for the same compound and attachment site to answer this question.

Exley:

We know that steroids conjugated to the bridge by double bonds have been reasonably successful to date.

Lindner:

Yes, well the others, too, for instance the thioether bond at the 1-position gives excellent antisera against testosterone. The double bond has one drawback, namely of causing cross-reaction with the corresponding ketosteroid. But then 11-hemisuccinate conjugates of testosterone, progesterone and oestradiol, i.e. antigens with single bond attachments, also give rise to antisera cross-reacting with the corresponding 11-oxygenated steroids, though perhaps less strongly.