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STEROID GLUCURONOSIDE-BSA COMPLEXES AS ANTIGENS: THE RADIOIMMUNOASSAY OF STEROID CONJUGATES

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SUMMARY

A preliminary study has been made of the possibility of raising antisera to steroid glucuronosides. Using steroid glucuronosides as haptens, macromolecular complexes have been prepared by joining the carboxylic acid group of steroid conjugates covalently to the ϵ-amino groups of dibasic amino acids in bovine serum albumin. Three such complexes have been prepared from testosterone-17β-glucuronoside, oestradiol-17β-glucuronoside and dehydroepiandrosterone-3β-glucuronoside and these antigenic preparations have been used to raise antisera in rabbits.

The calibration graphs obtained by radioimmunoassay of these antisera against the corresponding steroid glucuronoside haptens indicated a sensitive response in the picogramme range. No cross-reaction of the antisera to steroid glucuronosides other than the hapten used was observed in the range 500 pg/300 μl equilibrium reaction volume. The anti-testosterone-17β-glucuronoside serum did cross-react with free testosterone and 5α-dihydrotestosterone, and the anti-oestradiol-17β-glucuronoside serum gave cross-reactions with free oestradiol, oestrone and oestriol. Evidence is presented that two different types of receptor sites may have been formed, one binding to the steroid glucuronoside and the other to free steroids.

A HIGH proportion of steroid assays requested as a routine clinical service are carried out on urine and are designed to measure conjugates of steroid hormones and their metabolites, e.g. 17-oxosteroids, 17-hydroxycorticosteroids, pregnanediol, pregnanetriol, oestrogens, etc.; the majority of these conjugates are glucuronosides. In all of these assays the initial step is the removal of the non-steroid part of the conjugate by hydrolysis, solvolysis or oxidation, and in this step the identity of the conjugate is lost (Fig. 1). Subsequently, the liberated steroid is measured by some colorimetric, gas–liquid chromatographic method or by competitive binding technique. There appears to be no good reason why these conjugates should not be determined directly by radioimmunoassay without preliminary hydrolysis. Such methods should be infinitely more sensitive than existing routine methods and might open the way to the detailed study of individual conjugates in urine and plasma. It is noteworthy that although free oestrogens can be determined by competitive binding to rabbit uterine cytosol, oestrogen glucuronosides and sulphates do not bind to this naturally-occurring uterine receptor and they cannot be determined by conventional competitive binding assay without preliminary hydrolysis[1]. Antibodies raised against oestrogen conjugates should not behave in this way.

An advantage of using steroid glucuronosides as haptens is that they already contain a carboxylic acid group and do not require modification in any way for covalent linkage to the antigenic macromolecule (c.f. Lieberman *et al.*[2]). Using steroid glucuronosides as haptens, macromolecular complexes have been prepared

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	Glucuronosides	Sulphates
17-Oxosteroids	~ 80%	~ 20%
17-Hydroxycorticosteroids	> 95%	traces
Oestrogens	> 95%	traces
Pregnanediol	100%	–
Pregnanetriol	100%	–

Acid hydrolysis
Enzyme hydrolysis
or Solvolysis

Free steroid for assay

Non-steroid abandoned

Fig. 1. Conventional destruction of steroid conjugates before routine determination.

by joining the carboxylic acid group of glucuronosides to the ϵ-amino groups of dibasic amino acid residues in bovine serum albumin (Fig. 2). Three such complexes have been prepared from oestradiol-17β-glucuronoside, testosterone-17β-glucuronoside and dehydroepiandrosterone-3β-glucuronoside (Fig. 3) using the mixed acid anhydride reaction[3, 4] in dimethylformamide solution. In each case the protein-steroid-glucuronoside macromolecular complex was separated from low molecular weight reagents and from unreacted steroid glucuronoside by gel filtration on a Sephadex G25 column (30 cm × 2 cm i.d.). The column was developed with water and the elution of protein and steroid glucuronoside was monitored in the case of testosterone-17β-glucuronoside by recording the absorbance at 280 and 240 nm and calculating the concentration of each component on the assumption that the molecular extinctions of albumin and testosterone glucuronoside are not affected by covalent linkage (Fig. 4). In an analogous manner the incorporation of oestradiol-17β-glucuronoside into complex formation

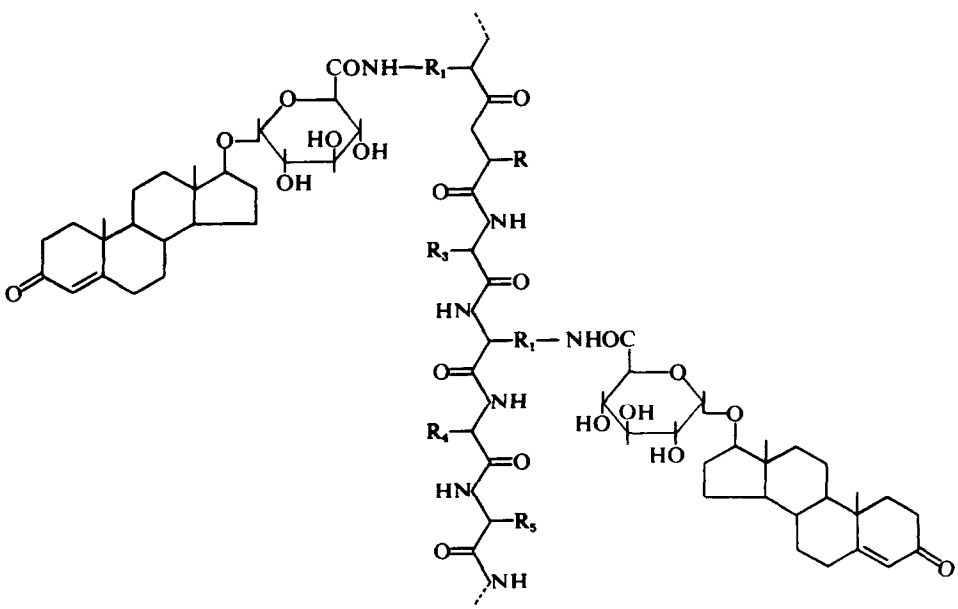
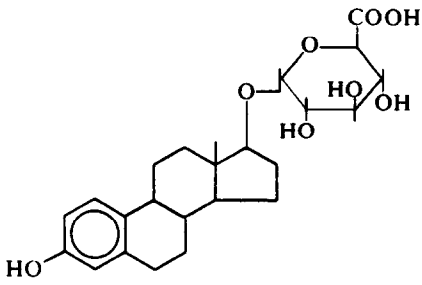
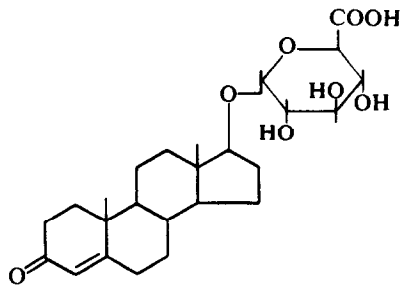


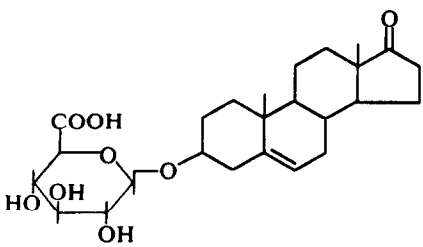
Fig. 2. Fragment of a steroid-glucuronoside-BSA complex.



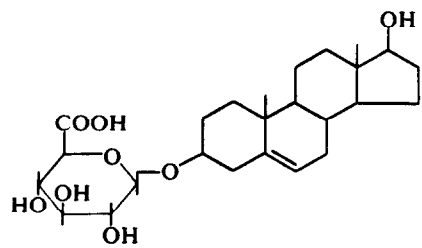
Oestradiol-17β-glucuronoside



Testosterone-17β-glucuronoside



Dehydroepiandrosterone-3β-glucuronoside



5-Androstene-3β,17β-diol-3β-glucuronoside

Fig. 3. Structures of steroid glucuronosides used for complex formation.

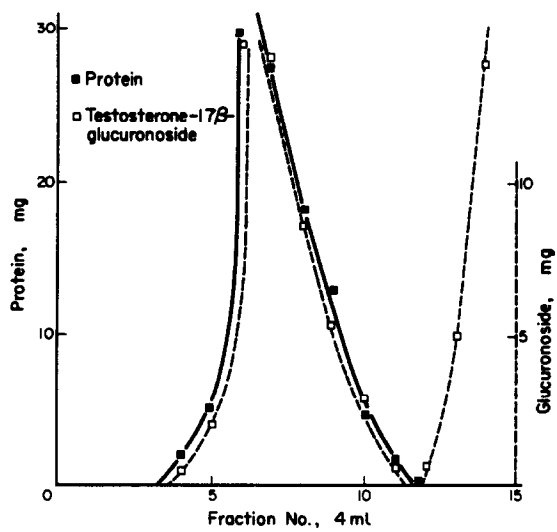


Fig. 4. Gel filtration of reaction products on Sephadex G-25. Elution of testosterone-17β-glucuronoside-BSA complex and unreacted testosterone-17β-glucuronoside.

was determined by Kober reagent[5] and that of dehydroepiandrosterone-3β-glucuronoside by Zimmermann reaction[6].

Assuming a molecular weight of 70,000 for bovine serum albumin and the presence of 61 ε-amino acid groups per molecule, the incorporation of oestradiol-17β-glucuronoside into the protein was 60 per cent of the theoretical maximum; the corresponding figures for testosterone-17β-glucuronoside and dehydro-

epiandrosterone-3β-glucuronoside were 29 and 32·6 per cent respectively. The ratio of protein to steroid in fractions eluted from the column was fairly constant (Table 1) although the value may represent a mixed population of molecules possibly including some unreacted bovine serum albumin. This material in aqueous solution, as eluted from the column, was used as the antigenic dose for injection into 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 sera were collected from the rabbits 10 days after the last injection; a booster dose (2 mg) was given and the animals were finally bled after three weeks.

Table 1. The composition of eluates from the Sephadex G 25 column after the formation of testosterone-17β-glucuronoside-BSA complex

Fraction no.	Protein (calc.) mg.	Testosterone-17β-glucuronoside (calc.) mg.	Protein/steroid ratio
F 4	3·2	0·25	12·8
F 5	5·2	0·50	10·3
F 6	29·7	2·90	10·3
F 7	27·3	2·80	9·8
F 8	20·7	2·20	9·4
F 9	6·3	0·7	9·0
F 10	0·0	0·1	—
F 11	0·0	2·0	—
F 12	0·0	10·7	—

Incubation of the antisera in serial dilution against the corresponding radioactive steroid glucuronosides at 30°C for 30 min (or 4°C for 16 h) in the absence and presence of the non-radioactive glucuronoside indicated that the percentage binding of the radioactive ligand was dependent on protein dilution and that the addition of the non-radioactive glucuronoside progressively lowered the percentage of the total radioactivity bound (Fig. 5). The equilibrium data presented as a Scatchard plot[7] were linear corresponding to the following dissociation constants (K_d): oestradiol-17β-glucuronoside $6{\cdot}3 \times 10^{-10}$ M, testosterone-17β-glucuronoside 10×10^{-10} M and dehydroepiandrosterone-3β-glucuronoside 8×10^{-10} M at 30°C. With the anti-oestradiol-17β-glucuronoside serum, a useful final dilution of 1/1500 gave 60 per cent binding with [^{3}H]oestradiol glucuronoside (50 pg), and 375 pg gave a 50 per cent decrease in the percentage binding (Fig. 6). A useful dilution of the anti-testosterone-17β-glucuronoside serum at 1/12,000 gave 45 per cent binding with [^{3}H]testosterone glucuronoside, and 510 pg gave a 50 per cent decrease. At a dilution of 1/3000, anti-dehydroepiandrosterone-3β-glucuronoside serum gave 45 per cent binding with [^{3}H]dehydroepiandrosterone glucuronoside (100 pg), and 1,000 pg of the non-radioactive glucuronoside gave a 50 per cent decrease.

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 300 μl reaction mixture. The binding of [^{3}H]oestradiol-17β-glucuronoside to anti-oestradiol-17β-glucuronoside serum was unaffected by testosterone-17β-, dehydroepiandrosterone-3β-, oestriol-17β-,

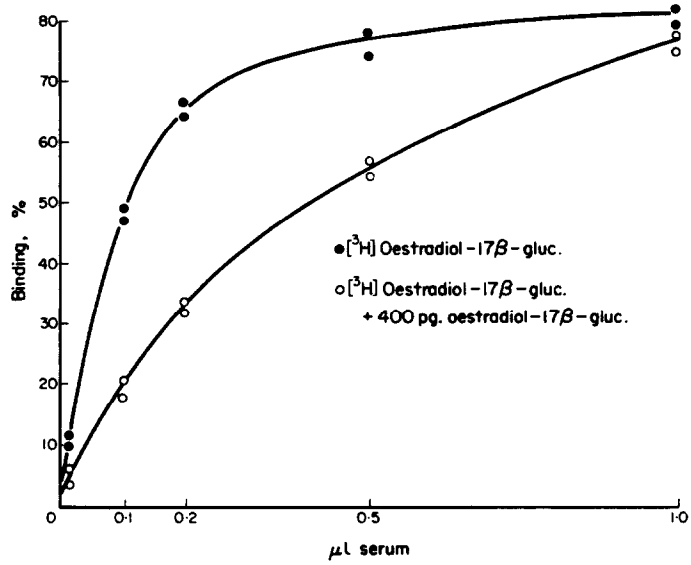


Fig. 5. Protein dilution curve for anti-oestradiol-17β-glucuronoside serum. The upper curve represents the binding of the radioactive ligand [³H]oestradiol-17β-glucuronoside in the absence of non-radioactive oestradiol-17β-glucuronoside. The lower curve represents the binding of the radioactive ligand in the presence of 400 pg of non-radioactive oestradiol-17β-glucuronoside. The vertical distance between the upper and lower curves gives the change in percentage binding of the radioactive ligand produced by 400 pg of non-radioactive oestradiol-17β-glucuronoside.

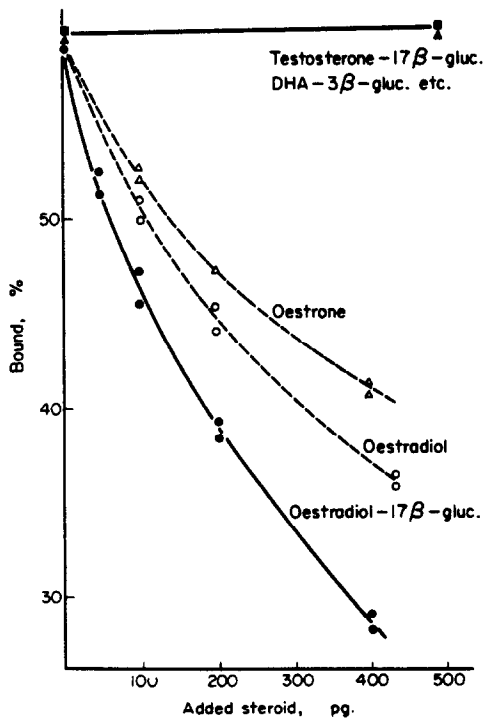
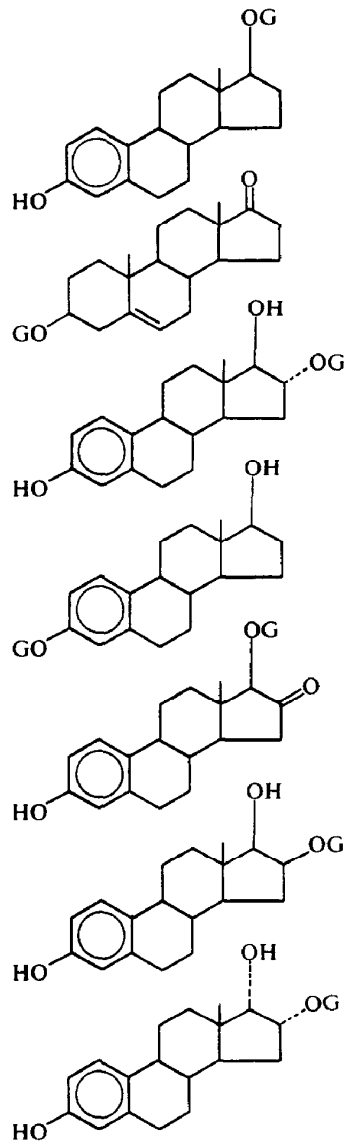
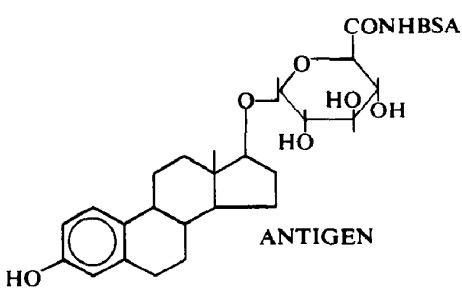


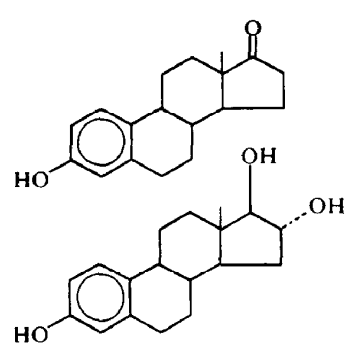
Fig. 6. Calibration graph of oestradiol-17β-glucuronoside. Reaction of anti-oestradiol-17β-glucuronoside serum to cognate structures.

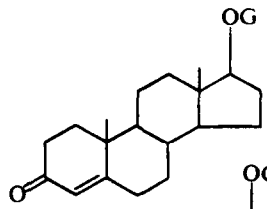
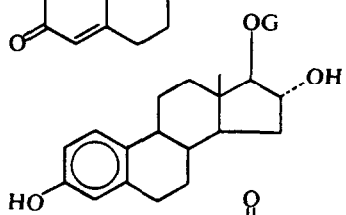
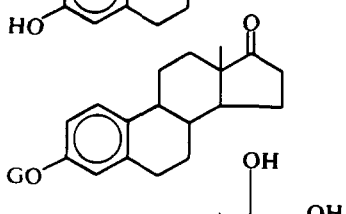
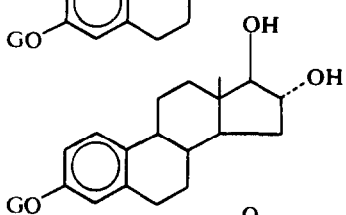
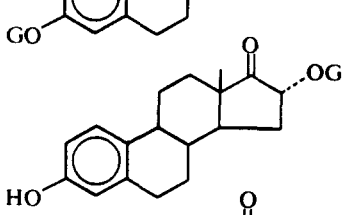
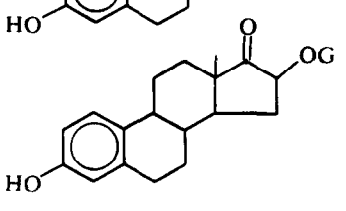
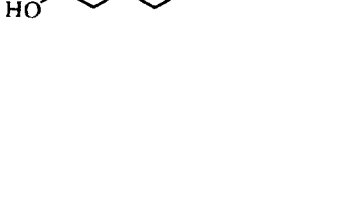
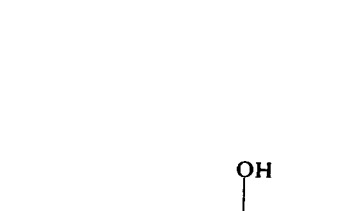
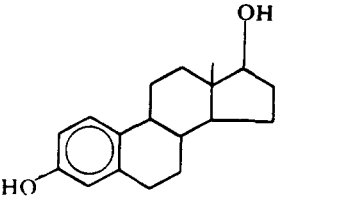
Compound ref. no.	Name
G 8	Oestradiol-17β-glucuronoside
G 1	Testosterone-17β-glucuronoside
G 2	Dehydroepiandrosterone-3β-glucuronoside
G 9	Oestriol-17β-glucuronoside
G10	Oestriol-16α-glucuronoside
G11	Oestrone-3-glucuronoside
G12	Oestradiol-3-glucuronoside
G13	Oestriol-3-glucuronoside
G14	16-Oxo-oestradiol-17β-glucuronoside
G15	16α-Hydroxyoestrone-16α-glucuronoside
G16	16-Epioestriol-16β-glucuronoside
G17	16β-Hydroxyoestrone-16β-glucuronoside
G18	17-Epioestriol-16α-glucuronoside



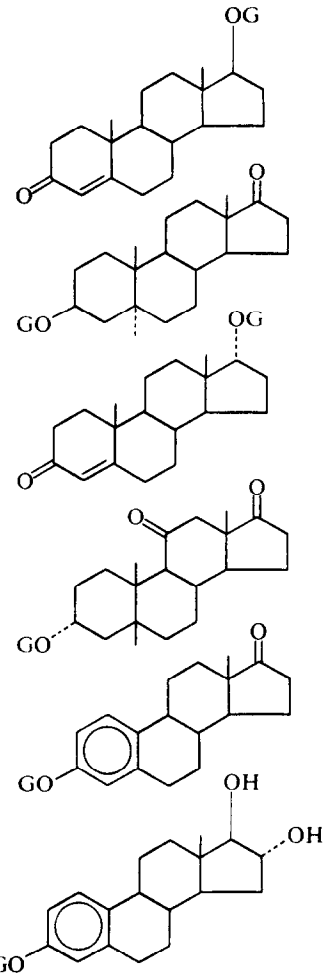


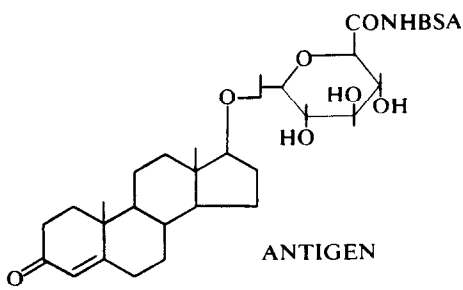
S11	Oestrone
S27	Oestradiol
S28	Oestriol



	From 50% fall in % Bdg. pg	Relative cross reaction
	375	1·0
	No cross reaction	0·0
	No cross reaction	0·0
	No cross reaction	0·0
	No cross reaction	0·0
	No cross reaction	0·0
	No cross reaction	0·0
	No cross reaction	0·0
	No cross reaction	0·0
	No cross reaction	0·0
	19%/400pg	—
	24%/400pg	—
	12%/400pg	—

Compound Ref. no.	Name
G 1	Testosterone-17β-glucuronoside
G 2	Dehydroepiandrosterone-3β-glucuronoside
G 3	Epiandrosterone-3β-glucuronoside
G 4	Androsterone-3α-glucuronoside
G 5	Epitestosterone-17α-glucuronoside
G 6	Aetiocholanolone-3α-glucuronoside
G 7	11-Oxoaetiocholanolone-3α-glucuronoside
G 8	Oestradiol-17β-glucuronoside
G11	Oestrone-3-glucuronoside
G12	Oestradiol-3-glucuronoside
G13	Oestriol-3-glucuronoside





S12	Testosterone
S16	Dihydrotestosterone

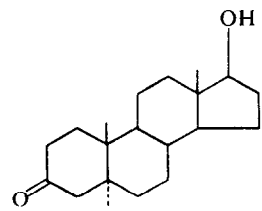
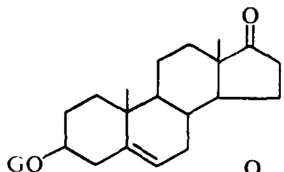
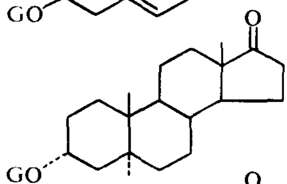
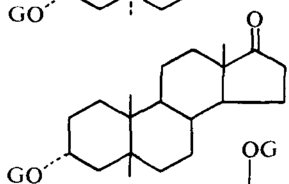
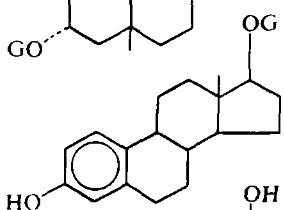


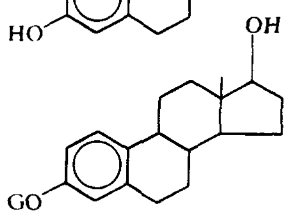
Fig. 8. Cross-reaction of cognate glucuronosides



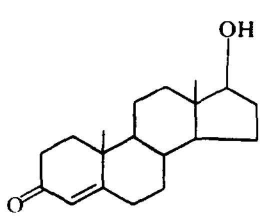








For 50% fall in % Bdg. pg	Relative cross reaction
510	1·00
No cross reaction	0·0
No cross reaction	0·0
No cross reaction	0·0
No cross reaction	0·0
No cross reaction	0·0
No cross reaction	0·0
No cross reaction	0·0
No cross reaction	0·0
No cross reaction	0·0



920	0·55
5%/100pg	—

to anti-testosterone-17β-glucuronoside serum.

oestriol-16α-, oestrone-3-, oestradiol-3-, oestriol-3-, 16-oxo-oestradiol-17β-, 16α-hydroxyoestrone-16α-, 16-epi-oestriol-16β-, 16β-hydroxyoestriol-16β- and 17-epi-oestriol-16α-glucuronosides (Fig. 7) (for synthetic routes see [8, 9]). Free oestrone, oestradiol and oestriol caused some depression of the percentage binding of the radioactive ligand. In an analogous manner antisera raised against testosterone-17β-glucuronoside did not cross-react with cognate steroid glucuronosides including the following compounds: dehydroepiandrosterone-3β-, epiandrosterone-3β-, androsterone-3α-, aetiocholanolone-3α-, 11-oxoaetiocholanolone-3α-, epitestosterone-17α-, oestradiol-17β-, oestrone-3-, oestradiol-3-, and oestriol-3-glucuronosides (Fig. 8). The antiserum did cross-react with some free steroids, notably with testosterone and 5α-dihydrotestosterone.

Of the many forms of comparison of the degree of cross-reaction between antisera and cognate steroid structures, we have elected to present the data by plotting on a log/log scale the function $[Rf/b - R_0 f/b]$ against the amount (pg) of non-radioactive steroid added. Where,

Rf/b is the ratio of free/bound for the radioactive ligand in the absence of non-radioactive steroid and,

$R_0 f/b$ is the ratio of free/bound for the radioactive ligand in the presence of non-radioactive steroid.

In this form the graphs were linear[10], but it was observed that the slopes of graphs representing the cross-reaction to free steroids which were parallel as a group, differed from that of the steroid glucuronoside ligand. As this was true of both the oestrogen and testosterone forms it is possible that at least two types of antibody were present, one reacting with the steroid glucuronoside and the other with free steroids. Further evidence in support of this hypothesis was provided by

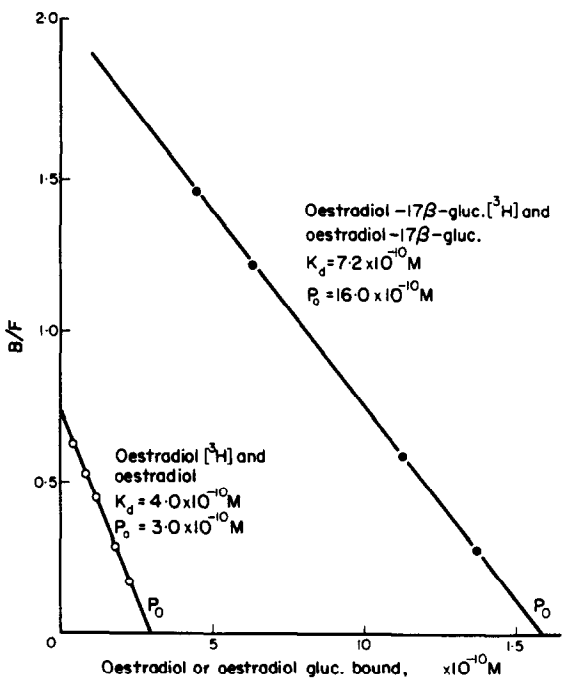


Fig. 9. Scatchard plot of anti-oestradiol-17β-glucuronoside serum. [^{3}H]Oestradiol-17β-glucuronoside/oestradiol-17β-glucuronoside, ● [^{3}H] oestradiol/oestradiol, ○.

steroids. Further evidence in support of this hypothesis was provided by Scatchard plots[7] using the anti-oestradiol-17β-glucuronoside serum for competition between [^{3}H]oestradiol-17β-glucuronoside and non-radioactive oestradiol-17β-glucuronoside, and competition between [^{3}H]oestradiol and non-radioactive oestradiol (Fig. 9). The dissociation constants (K_d) for these reactions were not the same, and molar concentrations of the two types of binding sites (P_0) in the antiserum were also different. The analogous series of experiments with testosterone, free and conjugated forms, also indicated the existence of two types of receptor sites. In view of the similarity in the structure of the antigens used in the present work, steroid-glucuronoside-BSA complexes, with those used by the Lieberman group[3, 4], steroid-hemisuccinyl-BSA complexes to raise antibodies to free steroid forms, the result is not surprising.

The three steroid glucuronosides used in the present study are relatively unimportant for diagnostic purposes, although as unique metabolites of oestradiol, testosterone and dehydroepiandrosterone they could be used to determine secretion rates. The choice of steroid glucuronoside haptens was limited by the availability of [^{3}H]-labelled conjugates of sufficiently high specific radioactivity for assay by competitive binding and this limitation may remain until the necessary radioactive glucuronosides have been prepared. Some priority should be given to

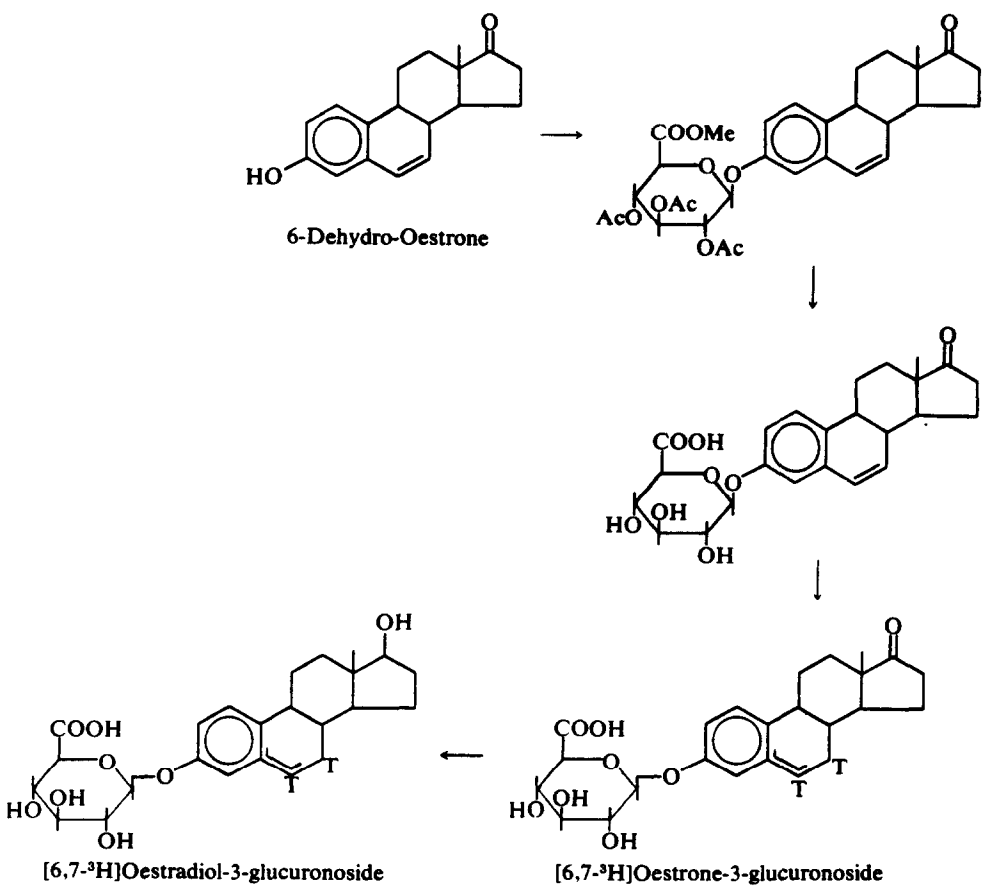


Fig. 10. Scheme for the synthesis of [^{3}H]-labelled steroid glucuronosides.

the preparation of [^{3}H]-labelled oestriol-16α-glucuronoside, pregnane-3α,20α-diol-3α-glucuronoside, pregnane-3α,17α,20α-triol-3α-glucuronoside, aetiocholanolone-3α-glucuronoside and possibly tetrahydrocortisol-3α-glucuronoside: in view of the high specificity of the immunological reactions it seems unlikely that cognate steroid glucuronosides will cross react. Because of the difficulty associated with the preparation of steroid glucuronosides using radioactive intermediates of high specific radioactivity, a more practical approach appears to be the synthesis of a series of glucuronosides incorporating at least one double bond, which on catalytic reduction with hydrogen or tritium would yield the required non-radioactive and radioactive steroid glucuronosides. For example, the synthesis of 6-dehydro-oestrone-3-glucuronoside would yield [6,7-^{3}H]oestrone-3-glucuronoside, and by borohydride reduction, [6,7-^{3}H]oestradiol-3-glucuronoside (Fig. 10). If the antibodies to the steroid glucuronosides could be labelled with ^{125}I, alternative radioimmunoassay procedures would be available and the [^{3}H]-labelled glucuronosides could be used as internal standards. The formation of antibodies against steroid sulphates also seems possible.

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DISCUSSION

Munck: I wasn't clear whether you have looked at cross-reactivity of other conjugates, like the sulphates, with the antigens for the glucuronides?

Kellie: Yes, in the case of the anti-oestradiol-17β-glucuronoside serum we have tried oestrone-3-sulphate, oestradiol-3-sulphate and oestradiol-17β-sulphate, and we do not get any cross-reaction.

Siiteri: Drs. Gurpide and Kelly in the United States have recently prepared an antibody to oestriol conjugated at the 17 position, I believe, and they found something very remarkable; not only do the free oestrogens (oestriol, oestrone, oestradiol) cross-react with this antibody, but also the glucuronides and the sulphates cross-react very nicely, so that one can use this antibody to free oestriol for the measurement of the conjugates. This contrasts with your situation, in which you show that there was no cross-reaction between oestrone and oestriol

glucuronides with the antibody to oestradiol glucuronide, whereas the free compounds did in fact cross-react. I thought this was rather remarkable. Perhaps you might comment further on this, the fact that the glucuronides of oestrone and oestriol do not cross-react in your system.

Kellie: Yes, this is perhaps a little surprising. We are aware that when you raise antibodies to free oestrogens you get an antiserum that cross-reacts with glucuronosides. We do not necessarily regard this as being desirable; we think this may be a disadvantage, if the object is to estimate glucoronosides. This is a new approach and I think that one will have to examine very carefully the specificity of this type of antiserum reaction, but as far as we have been able to determine, the antisera seem to be specific to the steroid glucuronoside that we use, and not to cross-react.

Adlercreutz: I think, Dr. Kellie, that you are right to think in terms of conjugates, and not only in terms of the free moiety, at least with regard to the oestrogens in pregnancy. For example, the oestriol-3-glucuronide is excreted very late in the urine, because the oestriol moiety passes first into the bile and then is conjugated in the 3 position in the intestinal tract, so it may appear in the maternal urine from several hours up to more than a day after the oestriol has been produced in the foetal organism. For this reason it is primarily much better to estimate oestriol-16-glucuronide, which passes very rapidly into the urine, and I think that the future for the determination of oestriol in pregnancy is just to determine the oestriol-16α-glucuronide.

Kellie: Thank you very much, Professor Adlercreutz. We have simultaneously been measuring oestrogen conjugates in late pregnancy plasma by competitive binding, and it's a very tedious process, because one has to add isotopically labelled steroid conjugates as internal standards and one has to hydrolyze. As far as we can determine, in late pregnancy plasma the main conjugated form is oestriol-16α-glucuronoside, and this rises very steeply towards the end of pregnancy. It is very much easier to measure oestriol glucuronoside in plasma than it is in urine, and yet it is difficult to satisfy a department of obstetrics and gynaecology. I think that if we can determine oestriol glucuronoside in plasma by radioimmunoassay it will simplify matters considerably.

Grant: While I would agree with you, Professor Kellie, I think we must be careful. One can find large variations in plasma oestrogens in late pregnancy. This is related to uterine blood flow, which seems to do curious things. One can get different values if the patient has come into the clinic and lain down for an hour than if she has just arrived. Currently there are arguments, as you probably know, as to the relative merits of the old-fashioned urinary oestriol measurements and the kind of modern thing you are trying to do. We need much more physiological research before we can make use of the plasma steroids.

Kellie: Yes, Dr. Grant. I think you would agree that it's going to be easier to do this if we have the methods.

Munck: What do you actually have to do with the urines? Do you first purify the glucuronides?

Kellie: I should explain that we have not applied the work that we have carried out on radioimmunoassay of steroid glucuronosides to biological fluids. When I have spoken of the assay of oestrogen conjugates in urine and plasma in my department, up to the present time, they have been done by hydrolyzing the conju-

gate and measuring the oestrogen by competitive binding to the uterine receptor. Naturally, we intend to go over to the other method as soon as we can.

Munck: Do you think it will be possible to use the urines without any extraction?

Kellie: Yes. In the case of oestriol glucuronoside I think it's extremely likely because the concentration in late pregnancy is in the region of 40 mg/l, and you have to dilute this about at least a million times in order to get into the right range.