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RADIOIMMUNOASSAY OF STEROIDS THE ROLE OF THE "BRIDGE" LINKING THE STEROID HAPTEN TO THE PROTEIN CARRIER

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SUMMARY

Two anti-oestradiol sera have been studied with respect to their ability to bind free oestrogens, oestrogen derivatives and oestrogen conjugates. Anti-oestradiol-6-O(carboxymethyl)-oxime serum bound free oestradiol with high specificity but did not cross react with any oestrogen glucuronides. Anti-oestradiol-3-glucuronide serum bound oestradiol-3-glucuronide, oestrone-3-glucuronide and also free oestradiol and oestrone to some extent. This antiserum did not cross react with ring-D glucuronides or with ring-A sulphates.

INTRODUCTION

The use of steroid–protein complexes as antigens to induce the formation of specific antisera forms the basis of many radioimmunoassay methods [1, 2]. In general, steroids cannot be joined directly to protein carriers and it is customary to convert these compounds into derivatives that contain a carboxylic acid group (—COOH), e.g. progesterone-11α-hemisuccinate (steroid-O·CO·CH_2·CH_2·COOH), oestradiol-6-O-(carboxymethyl)-oxime (steroid=N—O—CH_2·COOH) [3]. Such derivatives can then be joined covalently by the mixed acid anhydride [4] reaction to the ε-amino group of lysine residues in the polypeptide chain of the carrier protein.

Antigens formed in this way have the general form "(Steroid–bridge)$_n$-BSA" and it is clear from published data on the cross reactions of the induced antisera, that the hapten can be considered to be either the "steroid" or the "steroid–bridge". Two classes of naturally-occurring steroids, the bile acids and the steroid gluduronides, already contain a carboxylic acid group; they require no derivative formation and can be joined directly to carrier proteins [5]. Antisera raised against steroid glucuronides have the capacity to bind both free steroids (hapten) or steroid glucuronides (hapten–bridge) with considerable specificity although it is uncertain whether two set of antibodies are involved.

The present paper examines the ability of oestradiol antisera to bind free steroids and steroid conjugates.

MATERIALS

Steroid conjugates

Ring-A oestrogen glucuronides including 6-en-oestrone-3-glucuronide were prepared by the method of Conrow and Bernstein[6]. Ring-D oestrogen glucuronides were available within the department having been previously synthesized [7]. Oestrogen sulphates (oestrogen-3- and oestradiol-3-sulphates) were prepared freshly by the method of Joseph, Dusza and Bernstein[8].

Radioligands

Two radioligands were used. [2,4,6,7-^{3}H]-oestradiol (100 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. and was used as a ligand when labelled-oestradiol was being displaced. The second radioligand [6,7,-^{3}H]-oestradiol-3-glucuronide (36 Ci/mmol) was prepared by reduction of [6,7-^{3}H]-oestrone-3-glucuronide with sodium borohydride in methanol [8]. 6-n-Oestrone-3-glucuronide was reduced to [6,7-^{3}H]oestrone-3-glucuronide using tritium in the presence of 5% palladium on charcoal. [6,7-^{3}H]-oestradiol-3-glucuronide was used as a radioligand when the conjugate was being displaced.

Antisera

The work was carried out using two anti-oestradiol sera prepared by using antigens which differed only in the nature of the bridge linking oestradiol to the carrier protein (bovine serum albumin, BSA).

6-O(Carboxymethyl)-oxime link

This hapten was prepared by conventional synthesis of 6-oxo-oestradiol which was subsequently converted into oestradiol-6-O(carboxymethyl)-oxime. The derivative was linked covalently to BSA using the mixed anhydride method [9] and some purification of the antigenic complex was achieved by gel-filtration on a preparative colum of G-25 Sephadex [10]. The incorporation of the hapten into the carrier protein was 30 mol/mol and the product was stored as a lyophilized white powder at 0°C.

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3-Glucuronyl link

The second hapten, oestradiol-3-glucuronide was linked directly to BSA using the mixed anhydride method [5]; incorporation of the hapten into the BSA was 8·6 mol/mol. This product was also lyophilised and stored at 0°C.

Immunization

Random-bred male New Zealand white rabbits (3 kg) were used. The soluble antigenic complex (2 mg) was emulsified in Freund's complete adjuvant and was administered intramuscularly and subcutaneously at multiple sites on the flanks of the animals. A booster dose was given after 2 weeks and subsequently similar doses were given at intervals of 4 weeks; test samples of antiserum were drawn at intervals after 3 months. These samples were tested for antibody titre using the appropriate radioligand in the absence and in the presence of cold steroid. Figure 1 shows a serum dilution curve using anti-oestradiol-3-glucuronide serum with 0·12 pmol of [6,7-^{3}H]-oestradiol-3-glucuronide (radioligand) in the absence of (●——●) and in the presence of (■——■) 1·06 pmol of oestradiol-3-glucuronide (cold steroid). The antiserum was usedful for radioimmunoassay tests at a final dilution of 1/72,000 and at this dilution bound 60% of the radioligand. The antioestradiol-6-O(carboxymethyl)-oxime serum was used at a final dilution of 1/12,000, binding 60% of the radioligand (0·073 pmol [2,4,6,7-^{3}H]-oestradiol).

Cross reactions of steroids, steroid derivatives and steroid glucuronides against the two anti-oestradiol sera were carried out in phosphate buffer (0·1 mol/l; pH 7·0) containing 0·9% NaCl and 0·1% gelatine. Equilibrium was established by incubation for 0·5 h at 30°C and separation of free and bound forms was achieved by dextran–charcoal adsorption of the free form. Labelled oestradiol was determined by β-scintillation counting (Packard Model. 3375 and 3385) in a toluene-based phosphor but because of the insolubility of oestradiol-3-glucuronide in this medium a modified solvent mixture was employed when [6,7-^{3}H]-oestradiol-3-glucuronide was the radioligand. The composition of the phosphor was (3·5 g PPO; 100 g naphthalene; 230 ml ethanol; 385 ml xylene and 385 ml dioxan) and the counting efficiency for tritium was 35%.

RESULTS AND DISCUSSION

The method of calculating the degree of cross-reaction between antisera and the compounds tested is based on that described by Weinstein *et al.*[11] and is illustrated in the comparative graphs in Figs. 2 and 3.

The percentage cross reaction is defined as $100x/y$ where x is the mass of cold homologous steroid and y the mass of the heterologous cold compound required to produce 50% inhibition of the binding of the labelled ligand by the antiserum. The results of cross reaction tests summarized in Table 1 illustrate the following points. Antioestradiol-6-O(carboxymethyl)-oxime serum reacts with the steroid hapten (oestradiol), and with the steroid–bridge [oestradiol-6-O(carboxymethyl)-oxime] and somewhat unexpectedly cross reacts strongly with 6-oxo-oestradiol. The corresponding derivatives of oestriol show virtually no cross reaction yet they differ only in respect of an additional 16α-hydroxyl group on the D-ring.

It is noteworthy that this discriminant feature is remote from the point of linkage. None of the oestrogen glucuronides conjugated in the ring-A or ring-D showed any capacity to displace oestradiol from this antiserum.

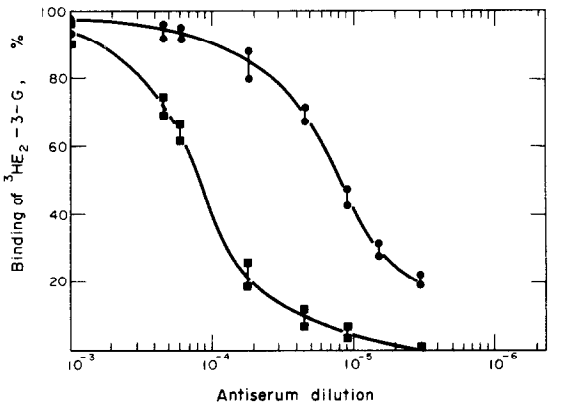

Fig. 1. Serum dilution curve for anti-oestradiol-3-glucuronide serum. The upper curve (●——●) represents the binding of the radioactive ligand [^{3}H]oestradiol-3-glucuronide (0·12 pmol). The lower curve (■——■) represents the binding of the radioactive ligand (0·12 pmol) in the presence of (1·06 pmol) of oestradiol-3-glucuronide.

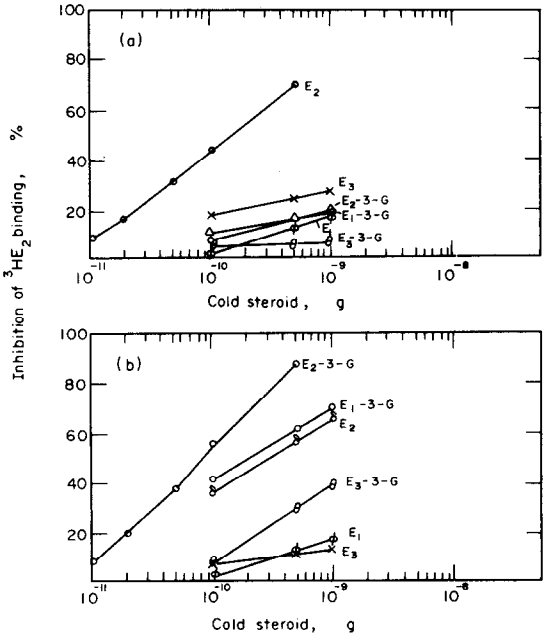

Fig. 2a. Antiserum to oestradiol-6-O-(carboxymethyl) oxime (diluted 1:12,000); Effect of free oestrogens and oestrogen-glucuronides on the binding of tritiated oestradiol (20 pg). (b) Antiserum to oestradiol-3-glucuronide (diluted 1:72,000); Effect of free oestrogens and oestrogen-glucuronides on the binding of tritiated oestradiol-3-glucuronide (60 pg). Abbreviations: E_1 = oestrone; E_2 = oestradiol; E_3 = oestriol; E_1-3-G = oestrone-3-glucuronide; E_2-3-G = oestradiol-3-glucuronide; E_3-3-G = oestriol-3-glucuronide.

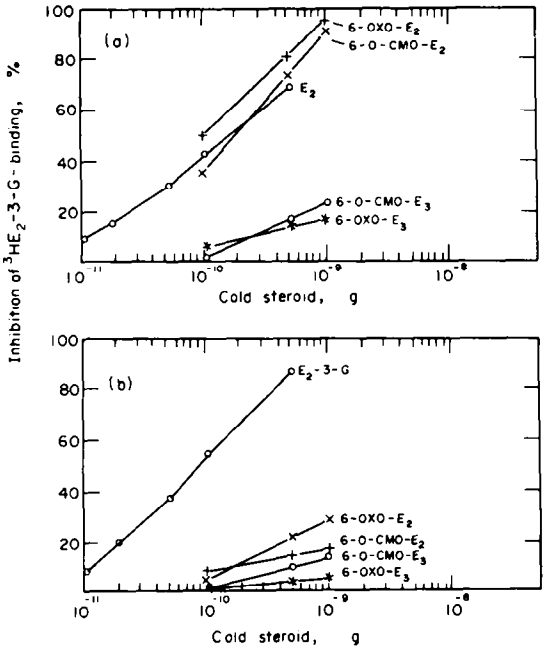

Fig. 3a. Antiserum to oestradiol-6-O-(carboxymethyl) oxime (diluted 1:12,000). Effect of oestrogen derivatives on the binding of tritiated oestradiol (20 pg). (b) Antiserum to oestradiol-3-glucuronide diluted (1:72,000). Effect to oestrogen derivatives on the binding of tritiated oestradiol-3-glucuronide (60 pg). Abbreviations: E_2 = oestradiol; E_2-3-G = oestradiol-3-glucuronide; 6-oxo-E_2 = 6-oxo-oestradiol; 6-O-CMO E_2 = oestradiol 6-O-(carboxymethyl) oxime; 6-oxo-E_3 = 6-oxo-oestriol; 6-O-CMO-E_3 = oestriol-6-O-(carboxymethyl) oxime.

Anti-oestradiol-3-glucuronide serum cross reacts strongly (40%) with oestrone-3-glucuronide and to a small extent (4%) with oestrial-3-glucuronide. No cross reactions with ring-D glucuronides were observed and this is consistent with the idea that the ring-D and the 17β-hydroxyl substituent are important discriminants in the hapten. Cross reaction with free oestradiol is substantial at 33% but interference from free oestrone and oestriol is unlikely to have much practical effect on the radioimmunoassay of the conjugate. The C-6 oxo-derivatives (steroids and steroid bridge compounds) were without effect on the binding of oestradiol-3-glucuronide. Oestrone-3-sulphate and oestradiol-3-sulphate had no effect on the binding of either of the radioligands to the antioestradiol sera.

Table 1. Per cent cross-reactivity of antisera raised against E_2-6-O-CMO-BSA and E_2-3-Gluc-BSA

	E_2-6-O-CMO-BSA	E_2-3-Gluc-BSA
E_1	1	1
E_2	100	33
E_3	0·1	1
E_1-3-G	0·1	40
E_2-3-G	0·1	100
E_3-3-G	0·1	4
6-oxo-E_2	170	1
6O-CMO E_2	90	1
6-oxo-E_3	1	1
6-O-CMO E_3	1	0·1
16α-OH E_1-G	0·1	0·1
E_2-17β-G	0·1	0·1
E_3-16α-G	0·1	0·1
E_3-17β-G	0·1	0·1

Abbreviations: E_1 = oestrone; E_2 = oestradiol; E_3 = oestriol; E_1-3-G = oestrone-3-glucuronide; E_2-3-G = oestradiol-3-glucuronide; E_3-3-G = oestriol-3-glucuronide; 6-oxo-E_2 = 6-oxo-oestradiol; 6-O-CMO E_2 = oestradiol 6-O-(carboxymethyl) oxime; 6-oxo-E_3 = 6-oxo-oestriol; 6-O-CMO-E_3 = oestriol-6-O-(carboxymethyl) oxime; 16α-OH E_1-G = 16α-hydroxy-oestrone-16α-glucuronide; E_2-17β-G = oestradiol-17β-glucuronide; E_3-16α-G = oestriol-16α-glucuronide; E_3-17β-G = oestriol-17β-glucuronide.

It may be concluded that when anti-oestradiol-6-O(carboxymethyl)-oxime serum is used for the determination of free oestradiol neither ring-A nor ring-D oestrogen conjugates are likely to interfere with the assay. On the other hand, when anti-oestradiol-3-glucuronide serum is used for the assay of this conjugate, free steroids must be removed, e.g. by simple partition between benzene and water. With this antiserum there is likely to be considerable interference from oestrone-3-glucuronide but little from oestriol-3-glucuronide.

When antigens are designed to produce specific antibodies to free steroides some kind of bridge is a necessity to join hapten to the carrier and insofar as the bridge is usually an organic structure not normally occurring in nature it does not interfere with the assay of the free steroid as long as a tritium-labelled steroid is used as the radioligand. Indeed the presence of the "foreign" bridge may be an asset as it may prevent naturally occurring steroid conjugates from any form of cross reaction. This advantage may be lost if an iodine (^{125}I) labelled radioligand is employed in which the same bridge is used in the antigen and in the radioligand (cf. homologous antisera in [2]. The assay of steroid glucuronides using anti-steroid glucuronide serum approximates to the perfect model of competitive binding as the hapten (oestradiol-3-glucuronide) and radioligand ([6,7-^3H]-oestradiol-3-glucuronide) and the non-radioactive competing steroid (oestradiol-3-glucuronide) are almost identical.

Analogous antisera against oestrone-3-glucuronide and oestriol-3-glucuronide have already been prepared and a comparison of the properties of these preparations will be presented in a subsequent publication.

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