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"UNBOUND" LIGAND ADSORPTION ON DEXTRAN-COATED CHARCOAL: PRACTICAL CONSIDERATIONS

R. DE HERTOGH, I. VAN DER HEYDEN and E. EKKA
Laboratoire d'Endocrinologie et Nutrition, Cliniques Universitaires St. Pierre 69, Brusselsestraat, 3000 Louvain, Belgium

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SUMMARY

Some practical aspects of estrogen binding to Dextran-coated charcoal were studied. The binding capacity of the charcoal was very large and not limitative in most instances. The affinity depended on its degree of dispersion in the medium. At 40 mg% of charcoal less than 2% of free [^{3}H]-estradiol (^{3}H-E_2) remained in the supernatant. At high concentrations (more than 200 mg%), charcoal was able to precipitate most of the BSA bound ^{3}H-E_2, but none of the ^{3}H-E_2 bound to rat uterus cytosol prepared in Tris–EDTA buffer. BSA added to rat uterus cytosol decreased the amount of ^{3}H-E_2 not precipitated with charcoal.

Charcoal was able to remove ^{3}H-E_2 or ^{3}H-E_3 from some specific antibodies, but not from others. Time and temperature of incubation in the presence of charcoal were critical in this respect. Precipitated charcoal was still able to adsorb the estrogen moiety, slowly released from the antibody in function of time. Practical considerations are discussed concerning the use of dextran-coated charcoal in radioimmunoassay procedures.

INTRODUCTION

In studies dealing with binding of steroid hormones to antibodies or to specific tissue "receptor" proteins, Dextran-coated charcoal is widely used as an adsorbant of the unbound fraction. Several points concerning the adsorption capacity of the charcoal suspension, the competition between unspecific binding proteins and the charcoal, and the stability of the binding to specific proteins in the presence of charcoal deserve careful considerations. The latter point has been discussed in a report dealing with binding of steroid hormones to plasma specific proteins [1]. This point is also critical in radioimmunoassay techniques where the conditions of stability of the antigen–antibody binding in the presence of the charcoal should be well established before the precipitation procedure can be standardized.

In the present work, we have studied the relative affinity of Dextran-coated charcoal, bovine serum albumin (BSA) and rat uterus cytosol towards [2,4,6,7^{3}H]-estradiol, at several concentrations of the binders (or the adsorbant), and the ligand. The stability of the binding of [2,4,6,7^{3}H]-estradiol, and [2,4,6,7^{3}H]-estriol to specific antibodies has also been investigated under different experimental conditions.

METHOD

[2,4,6,7^{3}H]-estradiol-17β (^{3}H-E_2) and [2,4,6,7^{3}H]-estriol (^{3}H-E_3) (100 Ci/mmol NEN) were checked for radiochemical purity (97%) on celite or sephadex LH_{20} column chromatography. Charcoal (Norit A) was washed three times with distilled water and centrifuged each time at 700 *g* for 10 min in order to remove the fine particles. After drying in the oven at 50°C for 24 h, 100 parts of Norit A and 10 parts of Dextran T-70 (Pharmacia) were suspended in the buffer solution at appropriate dilutions (expressed in mg% of charcoal).

Homogeneity of the suspension was assured by continuous agitation with a magnetic stirrer. Buffer solutions, A: Tris (10 mM) – EDTA (1 mM) – Sucrose (250 mM) pH 7·4; B: 0·1 M Phosphate buffer, containing 1 g/l of gelatin, 9 g/l NaCl and 1 g/l of sodium azide. Unless stated otherwise, all incubations were performed in an icebath at 0–4°C.

RESULTS

1. *Influence of charcoal concentration on* ^{3}H-E_2 *adsorption efficiency*

Various amounts of ^{3}H-E_2 were incubated in the presence of charcoal* suspension at various concentrations. Figure 1 shows the results in % ^{3}H-E_2 not precipitated, against the total concentration of ^{3}H-E_2 per unit weight (mg) of charcoal. It can be seen that the adsorption efficiency of the charcoal was highly dependent on its dispersion in the suspension.

* In this experiment, the proportion of Dextran was 1% instead of 10% of the charcoal concentration. Comparative experiments utilizing these concentrations of Dextran showed no difference.

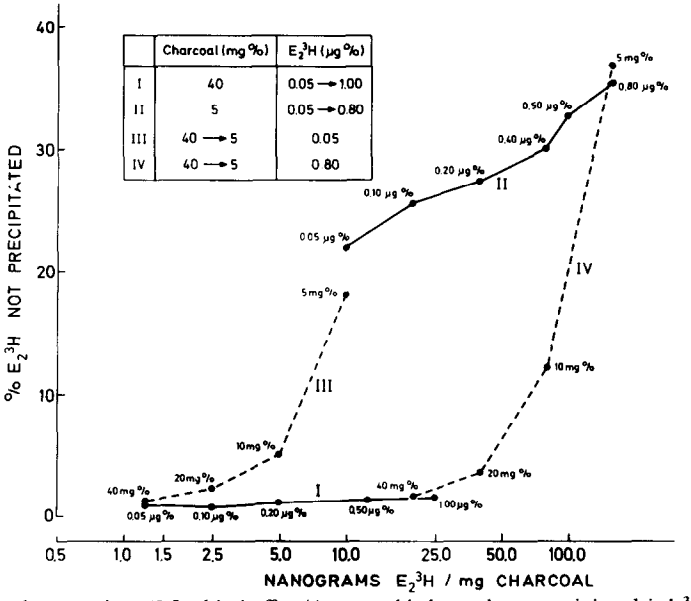

Fig. 1. Charcoal suspensions (0·5 ml in buffer A) were added to tubes containing dried ^{3}H-E_2. Charcoal and ^{3}H-E_2 final concentrations are indicated in the enclosure. Incubation time was 10 min. Charcoal was then precipitated by centrifugation at 700 *g* for 10 min. The percentage of ^{3}H-E_2 not precipitated was calculated from an aliquot of the supernatants, and is plotted against the concentration of ^{3}H-E_2 per unit weight of charcoal in the incubation mixture.

Indeed, for a charcoal concentration of 40 mg%, less than 2% of ^{3}H-E_2 was not precipitated at a concentration of 20 ng ^{3}H-E_2/mg charcoal. At the same relative concentration of ^{3}H-E_2, 26% of the steroid was recovered in the supernatant if the charcoal suspension was only 5 mg%.

2. Competition between BSA and Dextran-coated charcoal

BSA was able to retain important amounts of ^{3}H-E_2 in the supernatant, when low final concentrations of charcoal were used. (Fig. 2, curves 5,6 and 7).

3. Competition between rat uterus cytosol and Dextran-coated charcoal

Cytosol was prepared from adult rat uteri [2] in buffer A, and incubated with ^{3}H-E_2.

The results show that in this uterine preparation, 72% of the cytosol bound ^{3}H-E_2 was not precipitated, even at high final concentration of charcoal (Fig. 2, curve 1). On diluting the cytosol 12 times, 20% of the ^{3}H-E_2 resisted charcoal precipitation, which corresponded to the predicted fraction bound to one set of unsaturated binding sites. (Fig. 2, curve 2). Heated cytosol, as expected, had lost most of its binding capacity (Fig. 2, curve 3). BSA added to the diluted cytosol increased the percentage of ^{3}H-E_2 not precipitated at low charcoal concentration and decreased this percentage at high charcoal concentration (Fig. 2, curve 4).

4. Competition between cytosol and BSA

Cytosol from adult rat uteri was prepared as described above and incubated with ^{3}H-E_2.

The results (Fig. 3) show that the percentage of ^{3}H-E_2 not precipitated by high concentration of charcoal decreased with increasing BSA concentration in the medium.

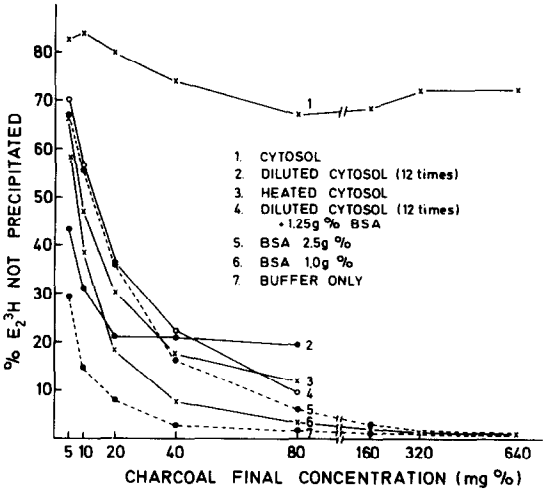

Fig. 2. 0·1 ml aliquots of an adult rat uterus cytosol prepared in buffer A (corresponding to about 1/10 uterus) were incubated for 1 h with 12·5 pg of ^{3}H-E_2, either as such (curve 1), or after a 1/12 dilution (curve 2), or after heating for 10 min at 60°C (curve 3), or after 1/12 dilution and addition of BSA to attain a final concentration of 1·25 g% (curve 4). 0·1 ml aliquots of buffer A containing 2·5 g% (curve 5), 1 g% (curve 6) or no BSA (curve 7), were similarly incubated with 250 pg of E_2^3H. 0·4 ml aliquots of charcoal suspensions (6·25 to 800 mg% in buffer A) were added at the end of the incubation time. The tubes were shaken for 30 s and centrifuged at 700 g for 10 min. The percentage of ^{3}H-E_2 not precipitated is plotted against the final concentration of charcoal in the mixture.

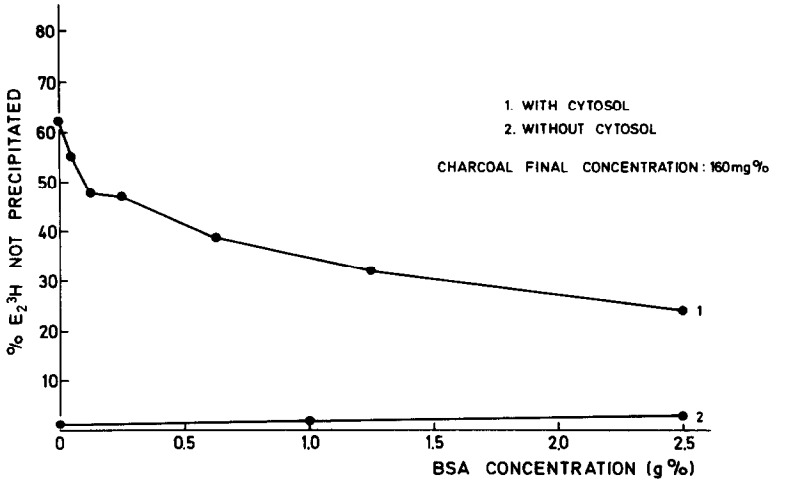

Fig. 3. 0·1 ml aliquots of an adult rat uterus cytosol prepared in buffer A were incubated for 1 h with 12·5 pg ^3H-E_2 in the presence of increasing concentrations of BSA (0·05 to 2·5 g%), (curve 1). BSA without cytosol was similarly incubated (curve 2). 0·4 ml aliquots of a 200 mg% charcoal suspension in buffer A were added. The tubes were shaken for 30 s and centrifuged at 700 *g* for 10 min. The percentage of ^3H-E_2 not precipitated is plotted against the concentration of BSA in the mixture.

5. *Stability of ^3H-E_2 binding to cytosol in the presence of charcoal*

Cytosol from immature rat uteri, prepared in buffer A [2] was incubated with ^3H-E_2 for 1 h. Aliquots of the ^3H-E_2-cytosol preparation were then incubated in the presence of charcoal suspension (160 mg%, final concentration). Incubation time varied from 15 s to 24 h, with continuous shaking to avoid sedimentation of the charcoal particles.

At the end of the incubation times, the aliquots were centrifuged at 700 *g* for 10 min. The results plotted on a semi-logarythmic scale showed that the amount of ^3H-E_2 not precipitated by the charcoal decreased linearly with time. After 24 h, the decrease was, however, not more than 25% of the $E_2{}^3H$ not precipitated after 15 s.

However, when cytosol was prepared in Tris (10 mM) – NaCl (0·15 M) – Sucrose (250 mM) pH 7·4, or in Tricine (10 mM) – NaCl (0·15 M) – Sucrose (250 mM) pH 7·4 buffers, 30% or more of the bound ^3H-E_2 were removed from their binding sites by the charcoal during the first 3 or 4 h of incubation. Afterwards the decrease was slow and linear, as with buffer A, up to 24 h.*

6. *Stability of ^3H-estrogen-binding to antibodies in the presence of charcoal*

Figure 4 shows the amount of antibody-bound [^3H]-estrogen in function of the incubation time and temperature in the presence of charcoal, relative to the 3 min value considered 100%. Some antibodies appeared to have very stable binding (more than 90%

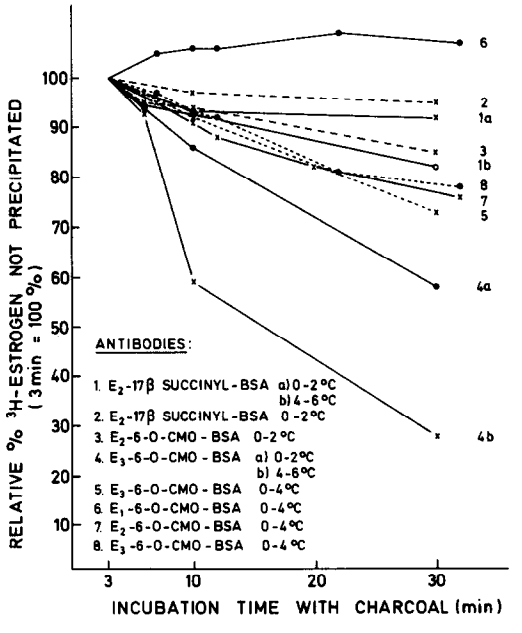

Fig. 4. Antibodies to estrone (E_1), estradiol-17β (E_2) or estriol (E_3), diluted in buffer B, were incubated with 25 pg of their respective tritiated ligand for 16 h, as previously described [3] (0·2 ml final incubation volume). 1 ml aliquots of 250 mg% Dextran-coated charcoal, prepared in buffer B, were added to the samples. The tubes were shaken for 15 s and left for variable periods of time without any further shaking, before centrifugation at 700 *g* for 10 min. The radioactivity recovered in the supernatants after 3 min of incubation in the presence of charcoal was set as 100% for each antibody. (The actual binding varied between 50 and 85% of the total amount of incubated estrogens). The percentage of [^3H]-estrogen not precipitated, relative to the 3 min value, is plotted against the incubation time in the presence of charcoal. The temperature of incubation with the charcoal is indicated for each experiment.

* Ekka and De Hertogh (in preparation).

after 30 min); others were reasonably stable (75–90%); and others were poorly stable (less than 60%), the more so on raising the incubation temperature from 2 to 6°C (less than 30% after 30 min).

Figure 5 showing standard curves obtained with antibody no. 7, for various times of incubation in the presence of charcoal, illustrates the practical influence of this charcoal effect on the radioimmunoassay technique.

A progressive lowering of the curve occurred when incubation time was increased. As an example: one sample giving 600 c.p.m. would read either 16 or 32 pg on the curves incubated with charcoal for 32 or 3 min respectively. Similarly, a sample giving 200 c.p.m. would read either 170 or 300 pg.

7. Stability of ^{3}H-E_2 binding to antibodies in the presence of precipitated charcoal

Figure 6 shows that precipitated charcoal continued to adsorb most of the ^{3}H-E_2 slowly released from the ^{3}H-E_2–antibody complex.

The deviation of the standard curve was already critical after 3 h.

DISCUSSION

A number of practical points may be noted as conclusions from the above experiments.

1. The "capacity" of the Dextran-coated charcoal appears to be very high, and except for low concentrations (less than 40 mg%), not more than 1% of the "free" estradiol would remain in the supernatant in the physiological range of tissue or plasma concentrations. Even at much higher estradiol concentration (as used for instance in competitive displacement studies), only a minor per cent of free estradiol would remain in the supernatant, provided higher charcoal concentrations are used.

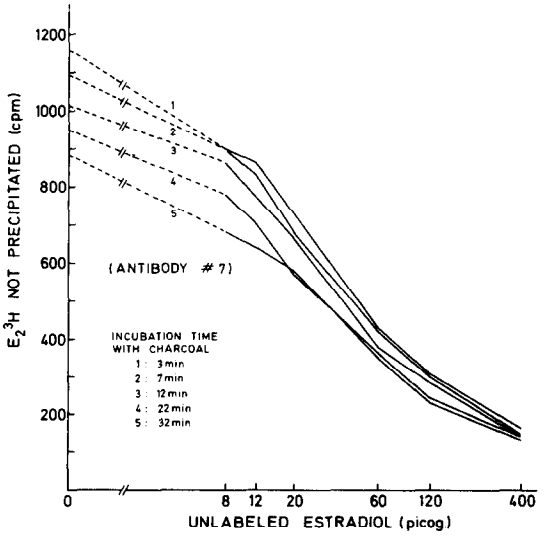

Fig. 5. Standard curves were performed as previously described [3], with antibody 7 (see Fig. 4). Incubation with charcoal suspension was done as indicated for Fig. 4 and lasted from 3 to 32 min, before centrifugation at 700 *g* for 10 min.

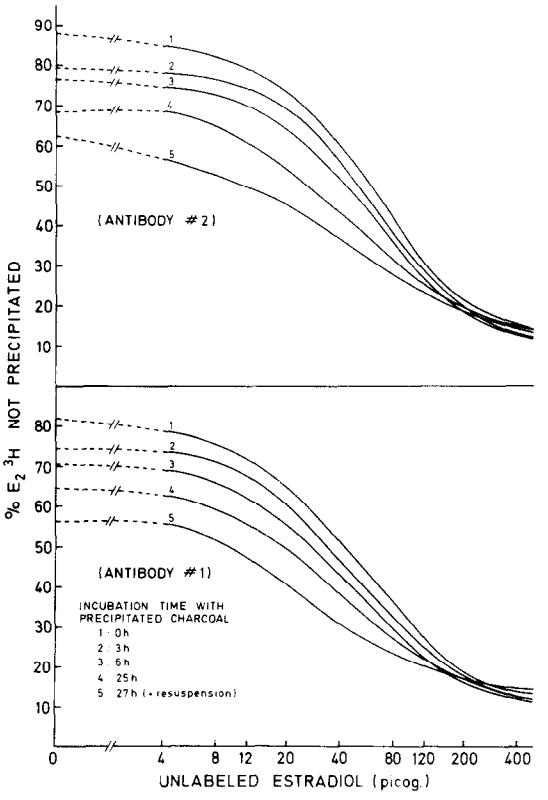

Fig. 6. Standard curves were obtained as previously described (3) with antibodies 1 and 2 (see Fig. 4). After precipitation of the charcoal at 700 *g* for 10 min, aliquots were taken from the supernatant either immediately (curve 1) or after 3, 6 and 25 h (curves 2, 3 and 4). Before each of these samplings the tubes were again centrifuged at 700 *g* for 10 min. After 27 h, the tubes were shaken for 15 s, before the last centrifugation and sampling (curve 5). The percentage of ^{3}H-E_2 not precipitated (on the ordinate) was calculated for each incubation time relative to the corresponding control tube, not treated with charcoal, in order to correct for eventual glass-wall adsorption.

2. The "affinity" of the charcoal preparation for estradiol is somehow intermediary between the affinities of BSA and of rat uterus cytosol preparation. Evidently, this crude estimate cannot be expressed on a molar basis. However, it appears that concentrations of charcoal above 200 mg% can efficiently remove more than 95% of estradiol bound to physiological concentrations of BSA, whereas cytosol bound hormone would resist charcoal competition up to 640 mg%. At lower charcoal concentrations, BSA would be able to retain part of the originally bound estradiol.

Tissue preparations always contain a much higher concentration of proteins other than the specific "binders" or "receptors", which may compete for steroid binding, as shown for BSA in the present experiments (Fig. 2). After *in vitro* incubation of such tissue preparation with labelled estradiol, high concentrations of

charcoal would probably remove most of the "non specifically" bound hormone and leave most of the "specifically" bound form in the supernatant. This procedure would then measure the number of occupied sites, but not necessarily the total number of sites (see Fig. 3).

In vitro competition with non specific proteins may involve an underestimation of specific sites present in small amounts in some tissues, where *in vivo* compartmentation may have been destroyed by tissue grinding.

3. The stability of the estradiol binding to tissue "receptors" may be altered by experimental procedures, and the incubation time in the presence of charcoal may become critical.

4. In radioimmunoassay techniques, the use of charcoal must be carefully checked, with respect to the stability of the hormone binding to the antibody. This stability was observed to be highly variable among several specific antibodies, in the presence of charcoal. The temperature of incubation was also critical.

Meaningless results are to be expected if time and temperature of incubation in the presence of charcoal are not carefully systematized in a routine procedure.

This necessity concerns several practical steps like: amount of samples to be treated in one series (time interval of charcoal addition between the first and the last tube); emplacement of the standard curve in the assay; rapidity of pipetting aliquots after charcoal precipitation; validity of intra-assay variations for samples processed at the same time *vs* interassay variations.

All these technical problems can be solved, and reproducible results can be obtained when properly adapted procedures are used.

Some commercially available antibodies (no. 6, 7 and 8, Fig. 4) were included in this study, and indeed users cannot avoid the necessity of adapting the techniques properly.

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