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ELECTROPHORETIC DESORPTION: PREPARATIVE ELUTION OF STEROID SPECIFIC ANTIBODIES FROM IMMUNOADSORBENTS

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SUMMARY

A technique of electrophoretic desorption has been used to separate antibodies to oestriol-16α-glucuronide from a complex with oestriol-16α-glucuronide. The steroid was immobilized by covalent attachment to Sepharose-4B. The yield of the desorbed antibody was high, furthermore the desorbed antibody showed no loss of affinity for the steroid. Similarly, oestriol-16α-glucuronide has been recovered from an immobilized antibody preparation. The procedure involves the combination of affinity chromatography and electrophoresis, and has considerable potential applications in the purification of antisera generally.

INTRODUCTION

There are good reasons for wanting to fractionate steroid antisera. Although the production of antisera has improved [1] the affinity and specificity of some samples are still often less than desirable for particular steroid estimations. There is evidence [2] that antisera raised to a particular steroid consist of more than one antibody species. Antibody purification could therefore improve specificity and thereby improve the performance of radioimmunoassay.

Affinity chromatography involves the immobilization of an appropriate ligand to an insoluble phase. Material will bind to the ligand according to the affinity of the material for that ligand. Thus high affinity compounds can be retained on the support, and the impurities of low or zero affinity can be removed either by washing with suitable solvent or by displacement using low concentrations of free ligand. This technique has proved to be useful in the purification of many biological compounds, for example, oestradiol receptor proteins from calf uterus [3], corticosteroid binding globulin from serum [4], and testosterone binding globulin from serum [5].

Many attempts have been made to purify antibodies to steroids by affinity chromatography, but problems have been encountered in the elution of the antibodies bound to the affinity columns. Massaglia *et al.*[6] used buffer at pH 3 and a similar buffer containing 10% dioxane to elute testosterone-binding activity, and Exley and Avakian[7] used oestradiol-6-(O-carboxymethyl)oxime in an attempt to remove oestradiol antibodies. In both cases the recovery of high affinity antibody was poor. In addition, the use of free ligand introduces problems of assay and use for any eluted antibody.

It is suggested that the comparative lack of success in the fractionation of steroid antisera by affinity chromatography is due to a combination of the strength of the binding of a steroid to antibody and the effects produced by the small, rigid nature of the steroid ligand.

In order to use the potential of affinity chromatography for steroid antisera purification, we have looked at alternative methods of sample elution, conscious of both the need to avoid conditions harmful to the protein and the strength of the binding forces to be overcome. We have studied the effects of an electric field with the possibility of removing charged material from the affinity support into a gel, such as polyacrylamide.

This preliminary work has been carried out with oestriol-16α-glucuronide and with antisera raised to this steroid as a hapten.

EXPERIMENTAL

[6,9(n)$^{-3}$H]-Oestriol-16α(β-D-glucuronide), specific activity 30 Ci/mmol, was obtained from the Radiochemical Centre, Amersham. Scintillation fluid (NE 260) was obtained from B.D.H. Limited, Poole, and samples were counted in 4 ml of this fluid using a Nuclear Chicago Isocap 300 Scintillation Counter. The counting efficiency for tritium was 48%.

The non-radioactive oestriol-16α-glucuronide (a gift from Professor A. Klopper) was coupled via the carboxyl group to bovine serum albumin (BSA) as previously described [8].

The antiserum to oestriol-16α-glucuronide used in these experiments was that described by Park *et al.*[9] and Kerr *et al.*[10].

The isoelectric point (*pI*) of oestriol-16α-glucuronide antibody activity was determined by thin layer gel electrofocusing in a polyacrylamide gel, using an LKB 2117 Multiphor.

Both oestriol-16α-glucuronide-BSA and its antisera were immobilised to Sepharose 4B (Pharmacia, Croydon) after activation with cyanogen bromide as described for Ultrogel® by Doley *et al.*[11]. Antisera (0.2 ml) were incubated with the cyanogen bromide-activated Sepharose 4B (2 g) for 24 h at 4°C. The antibody–matrix complex was then washed thoroughly.

The steroid–BSA conjugate (1 mg) was added to the cyanogen bromide-activated Sepharose 4B (2 g) and similarly incubated and washed to form the immobilized steroid matrix.

The immobilized antibody matrix (0.5 g) was complexed with tritiated oestriol-16α-glucuronide (500,000 c.p.m.) by incubating the two materials for 15 h at 4°C prior to use, together with 0.01 M-Tris [2-amino-2-(hydroxymethyl)propane-1,3-diol]/0.08 M-glycine buffer (pH 8.6, 0.5 ml). The matrix was then washed with 8 × 5 ml of the buffer. Each wash was retained and its radioactivity determined.

The immobilized steroid (0.4 g) was complexed with antibody (0.2 ml) by incubating the two materials in the same way with 0.01 M-Tris/0.04 M-glycine buffer (pH 9.0, 0.5 ml). The matrix was then washed with 8 × 5 ml of this buffer. Each wash was retained and the antibody activity present was determined by the method of Kerr *et al.*[10]. Tritiated oestriol-16α-glucuronide (5000 c.p.m.) was added to duplicate 0.2 ml samples of each wash.

Gel electrophoresis was performed on 7% polyacrylamide gels as described by Davies and Ornstein [12]. The gels were 0.5 cm in diameter, with the length varying as described. The electrophoresis was carried out in an appropriate tank (Shandon, London NW10) at room temperature and with a constant current density of 2 mA per gel. The washed, immobilized complexes were placed on top of the gels after pre-electrophoresis. Tris/glycine buffers were used in the upper and lower buffer reservoirs, 0.09 M at pH 8.6 for the desorption of steroid, and 0.05 M at pH 9.0 for the desorption of the antibody. The apparatus was arranged so that the negatively charged ions would move from the matrix through the gel towards the anode.

On occasions, antibodies were desorbed through the gel into a small volume of buffer separated from the rest of the reservoir by a dialysis membrane fitted over the bottom of the glass cylinder containing the gel.

After electrophoresis, the affinity matrix on top of the gel was removed and retained. The gels were removed, sliced and each piece soaked in water (1.0 ml) overnight. To measure the tritiated steroid content of the gel, scintillation fluid was added and the radioactivity determined. To assay antibody activity, tritiated oestriol-16α-glucuronide (5000 c.p.m.) was added to duplicates (0.2 ml) from each sample. The assay described by Kerr *et al.*[10] was used.

RESULTS

(a) *Thin layer gel electrofocusing*

The major part of the oestriol-16α-glucuronide antibody activity was found to be at pH 6.8.

(b) *Removal of unbound material from the affinity support*

The removal of unbound material from the affinity support was carried out as described previously, prior to each desorption experiment. Figure 1 shows the tritium content of the washes of immobilized antibody after incubation with 500,000 c.p.m. of tritiated oestriol-16α-glucuronide. Figure 2 shows the amount of antibody-bound tritiated steroid present (after

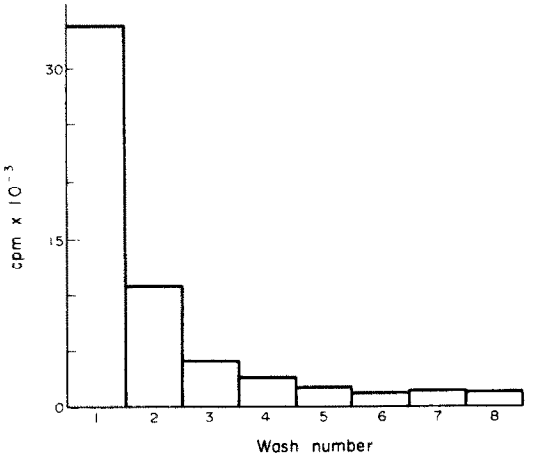

Fig. 1. The total tritium content (c.p.m.) of each of the 8 × 5 ml washes of immobilized antibody matrix after incubation with 500,000 c.p.m. of tritiated oestriol-16α-glucuronide. Approximately 90% of the steroid is bound to the antibody.

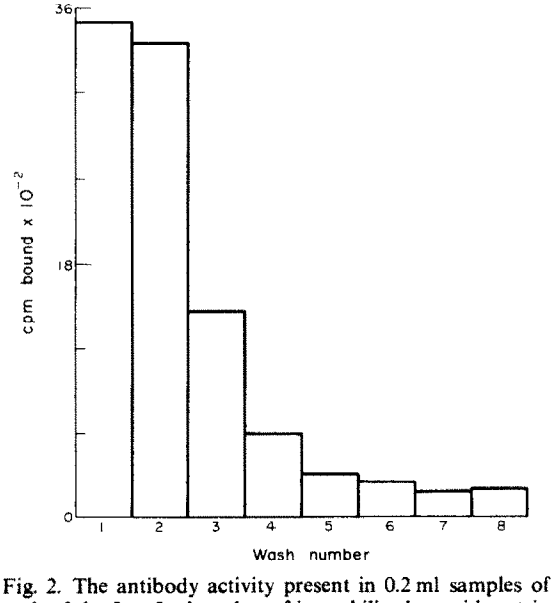

Fig. 2. The antibody activity present in 0.2 ml samples of each of the 8 × 5 ml washes of immobilized steroid matrix after incubation with antiserum (0.05 ml). The results shown are averages of duplicate measurements and show the tritium content (c.p.m.) of the bound fraction after assay.

adding 5000 c.p.m. initially) in 0.2 ml samples of the washes of immobilized steroid after incubation as described.

The figures show that the washing procedure has in both cases reached the stage where all initially unbound material had been removed. Any material that could now be removed from the matrix can be assumed to be that which was initially bound to it.

(c) *Desorption of tritiated steroid from immobilized antisera*

After washing off the unbound steroid, the complex of steroid with antibody immobilized to Sepharose 4B was electrophoretically desorbed on top of polyacrylamide gels as previously described. Figure 3 shows the distribution of tritium in gel slices after carrying out the desorption for 1 h and 2 h upon separate samples. The initial amount of steroid bound to the support is indicated in each case.

On another occasion, the desorption was carried out for 6 h on a sample containing 250,000 c.p.m. bound to the matrix. After this time a total of 44% of the initially-bound steroid was found either in the gel or in the anode buffer reservoir.

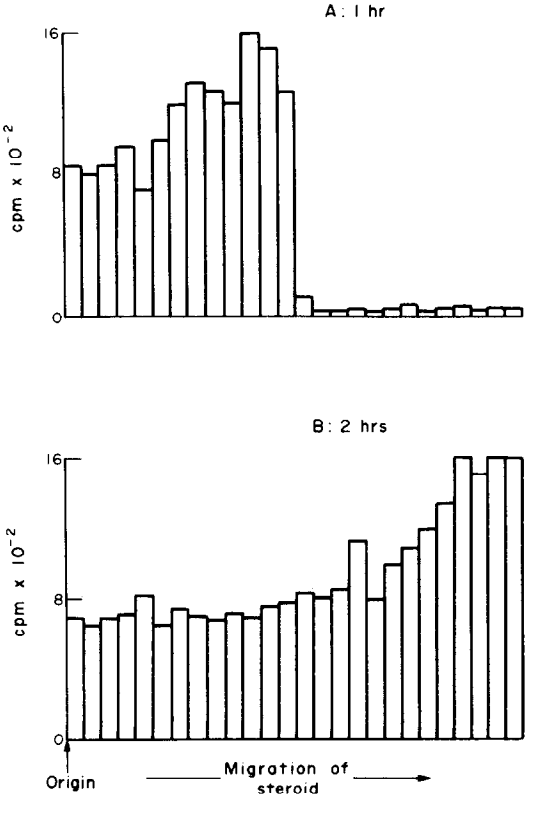

Fig. 3. The tritium content (c.p.m.) of 0.2 cm slices of gel after electrophoretic desorption of a matrix containing tritiated oestriol-16α-glucuronide bound to the immobilized antibody (A) after 1 h, (B) after 2 h. Each desorbed sample initially contained 200,000 c.p.m.

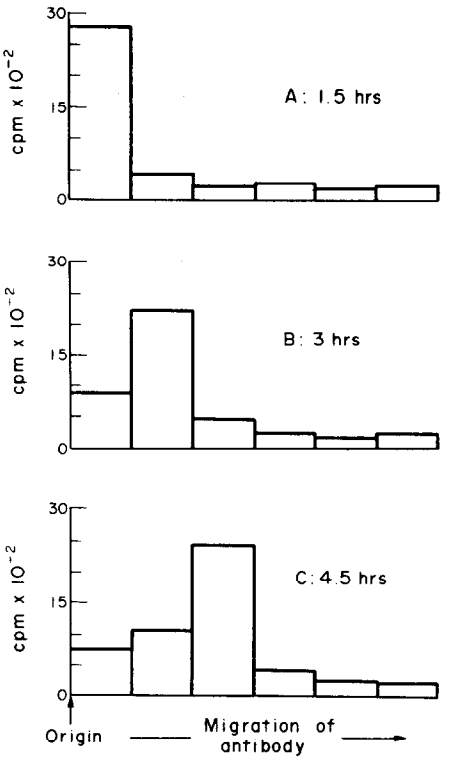

Fig. 4. The antibody activity present in 1 cm slices of gel after electrophoretic desorption of an antibody from immobilized steroid for (A) 1½ h, (B) 3 h, and (C) 4½ h. Each gel column received the same amount of washed matrix.

(d) *Desorption of antibody from immobilized steroid BSA*

After washing off the unbound antibody, the complex of antibody with steroid immobilized to Sepharose 4B was electrophoretically desorbed on top of a polyacrylamide gel as previously described. Figure 4 shows the distribution of antibody activity in gel slices after desorption times of 1.5, 3, and 4.5 h.

The procedure was then carried out with two gel columns, 4 cm in length, set up so that there were 1 ml volumes of buffer between the bottoms of the gels and dialysis membranes over the end of the glass tubes. The matrix was set up and washed as usual, divided into two equal portions, and each placed on top of a gel. Electrophretic desorption was carried out for 6 h on one sample. No current was passed through the other, control sample. After this time the matrix on top of each gel was removed and retained in 2.5 ml of buffer. The gels were removed, sliced into 1 cm pieces and retained. The 1 ml buffer volumes at the bottom of each gel were retained and made up to 2.5 ml. Assays for antibody activity were then carried out as usual.

No antibody was detectable in the sliced gels of either sample. The solution with the retained matrix from the top of the experimental sample showed no antibody activity; 0.2 ml from the control sample was able to bind 1400 c.p.m. (out of 5000 c.p.m.) of the

initially added steroid. The buffer volume from the control sample showed no antibody activity; 0.2 ml from the experimental sample was able to bind 1375 c.p.m. (out of 5000 c.p.m.) of the initially added steroid.

DISCUSSION

The results show that oestriol-16α-glucuronide can be removed from a complex with immobilized antibody by the described electrophoretic desorption method. Similarly, specific antibody can be removed from immobilized oestriol-16α-glucuronide. Previous attempts to purify steroid specific antibodies by conventional affinity chromatographic techniques have failed due to the lack of a suitable elution technique. The proposed electrophoretic desorption procedure may prove to be useful in overcoming these problems.

The patterns of elution of the steroid and antibody with respect to time would appear to be similar. An initial peak amount is followed by adsorbed "trail". Although the protein is removed from its complex much faster than the steroid, once in the gel it moves at a slower rate. There would seem to be no reason why the yield of steroid should not be increased if desired by increasing the time of desorption further. The antibody would appear to be removed in high yield, there being no detectable activity remaining on the immobilized ligand after six hours desorption; and with little loss of affinity, since the binding ability of the desorbed sample is the same as that of the initially added material. The latter are two of the problems that have been encountered in earlier attempts at steroid antibody purification by affinity chromatography; their solution by this electrophoretic desorption method therefore has considerable implications for the usefulness of the method. Similarly the desorption of steroid from immobilized antibody could be used for the specific extraction of charged labile steroids from biological fluids.

Brown *et al.*[13] have used the electrophoretic desorption method for the purification of ferritin. From this work it appears that the technique could have a wide application. The method does not involve chaotropic reagents or the addition of free ligand to remove the bound material; yet it still retains all of the advantages of affinity chromatography and is powerful enough to resolve previously difficult problems.

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