ISOLATION OF ANTIBODIES TO STEROID HORMONES BY AFFINITY CHROMATOGRAPHY ON ANTIGEN-LINKED SEPHAROSE : AN EFFICIENT ELECTROPHORETIC ELUTION PROCEDURE

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Received September 13,1977

SUMMARY

An electrophoretic elution procedure of antibodies retained on affinity columns is described. It afforded a 60% recovery of the binding activity of a high affinity (Ka $\sim 10^{10}$ M^{-1}) antiserum to 5α-dihydrotestosterone retained on antigen-linked Sepharose 4B affinity columns. These purified unbound antibodies, (Ka $\sim 10^{10}$ M^{-1}) when applied again on identical antigen-linked affinity columns, were all retained and totally recovered after a new electrophoretic elution. Comparable results were obtained by elution with 1M NH_4OH.

The residual 40% binding activity remaining on the antigen-linked Sepharose gel after electrophoretic elution was totally recovered by elution with an excess of 5α-dihydrotestosterone. It corresponded to antibodies of higher affinity (Ka $\sim 10^{11}$ M^{-1}). On the other hand the residual 40% fraction of antibodies resistant to NH_4OH elution was denaturated.

INTRODUCTION

The use of antigens immobilized on a solid matrix to achieve the isolation and purification of antibodies (1) was one of the pioneer experiments which led to the tremendous development of affinity chromatography techniques.

Antibodies can be retained on affinity columns prepared by covalent linkage of the whole antigen, or at least of its haptenic parts, to Sepharose gel. The major difficulty at this stage is to wash out all the uncoupled antigen from the columns in order to prevent any partial loss of apparent antibody activity.

The retained antibodies can always be released from the affinity columns by addition of an excess of free antigen which competes with the immobilized ligand. In the case of antibodies to steroid hormones, elution can be completed by an excess of steroid, and the steroid-bound antibodies so eluted must be thoroughly dialyzed to regenerate the purified unbound antibodies. This results in a considerable loss of activity.

This communication describes an elution procedure, based on the electrophoretic mobility of high-affinity (Ka $\sim 10^{10}$ M^{-1}) antibodies retained on antigen-linked affinity columns, which yields unbound antibodies. Results are

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ISSN 0006-291X

compared to the ones obtained by use of a recently reported method employing 1M NH_4OH (2).

MATERIALS AND METHODS

Antigens : Both 7-(O-carboxymethyl)oxime (7-CMO-DHT) (3) and 7β-hemisuccinamide (7β-Hem-DHT) (unpublished results of C1.Y. Cuilleron) derivatives of 5α-dihydrotestosterone were coupled to bovine serum albumin (BSA) (Armour Pharmaceutical Company) by the mixed anhydride procedure (4) leading to 18-20 moles of steroid fixed per mole of BSA (5). These antigens were extensively purified before use by 48 hours-dialysis against 0.1M $NaHCO_3$, pH 9.0 followed by two successive chromatographies on Sepharose 4B columns and a final 24 hours-dialysis against water neutralized to pH 7 by $NaHCO_3$. The radioimmunoassay of concentrated last dialysis water allowed a less than 10^{-2} mole estimation of the concentrations of free steroid per mole of BSA. A partial hydrolysis has been observed for the 7-CMO-DHT antigen at acidic pH.

Antisera : The 7-CMO-DHT-BSA antigen (0.5 mg) dissolved in complete Freund's adjuvant was injected subcutaneously once a week, for two months, to female New Zealand rabbits, to raise specific antibodies.

Unless specified, the binding activity measurements were performed in 0.1M phosphate buffer pH 7.4, 0.1% gelatine, 0.9% NaCl, 0.1% sodium azide (6). The binding affinities were determined by the Scatchard method (7).

Affinity columns : The two preceding purified antigens (100 mg) were coupled in a standard manner (8) to 50 ml of CNBr-activated Sepharose 4B gel suspension in 0.1M $NaHCO_3$ pH 9.0. The yield after coupling was estimated to be 75-80% by optical density measurement at 280 nm of uncoupled proteins. The Sepharose gel was washed extensively with 1 l 1M NaCl, with 0.5 l water brought to pH 7.0 by addition of $NaHCO_3$ and several times with a 3 l total volume of phosphate buffer.

The total elimination of any trace of released steroid or uncoupled antigen was verified by radioimmunoassay of concentrated last rinsage buffer and by the 100 ± 5% recovery of binding activities (sum of separately determined retained and unretained binding activities of anti-DHT antisera passed through the columns).

The maximum concentration of free steroid was estimated to be 10^{-10} mole of steroid per ml of antigen-linked Sepharose gel.

The antigen-Sepharose gel (1 ml) was introduced in small glass columns (diameter 0.6 cm) and was equilibrated with 20 ml of 0.1M phosphate buffer, pH 7.4, 0.9% NaCl.

2 ml of antiserum diluted into 15 ml of phosphate buffer was allowed to pass through the column with a slow (1 ml/hr) flow rate to ensure maximum adsorption of the antibodies. The column was then washed with 15 ml of phosphate buffer in order to recover all unretained proteins and antibodies. A final rinsage was performed with 50 ml of phosphate buffer.

First elution method : by competition with an excess of DHT. The two 7-CMO-DHT and 7β-Hem-DHT-BSA-Sepharose gels (1 ml) impregnated with antibodies were incubated for 15 minutes at 30°C in 2 ml phosphate buffer containing 5 nmoles of DHT and 1 μCi of tracer [1,2-^{3}H]DHT, then filtered and washed with 2 ml of phosphate buffer. The filtrates were passed through Sephadex G25 columns to

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Abbreviations used : DHT = 5α-dihydrotestosterone ; BSA = bovine serum albumin ; 7-CMO-DHT = 7-(O-carboxymethyl)oximino-5α-dihydrotestosterone ; 7β-Hem-DHT = 7β-hemisuccinamido-5α-dihydrotestosterone.

remove the excess of unbound DHT. The macromolecular-associated radioactivity measurements showed a total elution of retained antibodies on both columns.

Second elution method : by addition of 1M NH_4OH (2). The two 7-CMO-DHT- and 7β-Hem-DHT-BSA-Sepharose gels impregnated with antibodies were equilibrated with 0.05M NH_4HCO_3 buffer pH 7. Then, 50 ml of 1M NH_4OH was passed through the gel at 4°C and allowed the recovery after 5 hours of a 60% maximum of antibody binding activity (90% of which was eluted with the first 15 ml). The use of 0.5M NH_4OH increased the volume of solution necessary for the elution without any advantage.

The NH_4OH-eluted unbound antibodies were lyophilized in order to remove NH_4OH and dissolved in phosphate buffer.

Third elution method : by direct electrophoresis of the affinity gel. The 7β-Hem-DHT-BSA-Sepharose gel impregnated with antibodies was first equilibrated with 0.033M CH_3COONa buffer pH 5.6 and introduced in a glass tube (diameter, 0.6 cm ; length, 12 cm) at the bottom of which 0.8 ml of 4% acrylamide was polymerized at 4°C with 5 mg sodium persulfate. The electrophoresis was performed at pH 4.5 (the isoelectric pH of antibodies lies between 5 and 8).

The tube was filled with 2 ml of 0.1M β-alanine - 0.1M CH_3COOH buffer pH 4.5 and its extremities were plunged into two glass cells containing 15 ml of the same buffer. A platinum electrode was placed in each cell. The whole system was cooled in an ice-bath in order to maintain the temperature of the gel and of the bottom cell between 5 and 10°C.

The top electrode (anode) and bottom electrode (cathode) were connected to a DC power supply (LKB 2103).

Optimal conditions were determined : best results were obtained by applying an intensity of 30 mA (V ∿ 200 V) during 4 hours, leading to the elution of 50-60% of the antibody activity retained on the affinity column. When electrophoresis was prolonged for 4 hours more, less than 5% additional activity was eluted.

The electrophoretically eluted unbound antibodies were dialyzed against phosphate buffer and concentrated by vacuum dialysis.

RESULTS AND DISCUSSION

The crude anti 7-CMO-DHT antiserum used throughout this study had a titer of 1:20,000 and an apparent association constant with DHT around $10^{10} M^{-1}$. This antiserum (2.0 ml) was passed through the 7-CMO-DHT-BSA-Sepharose column (1 ml) which retained 100% of the binding activity.

Since a partial release of cross-reacting 7-keto-DHT was observed with this 7-CMO-DHT column at slightly acidic pH, a more stable 7β-Hem-DHT-BSA-Sepharose gel was also used. This retained only 60% of the binding activity in the same conditions as above.

Elution of antibodies retained on both 7-CMO-DHT- and 7β-Hem-DHT-BSA-Sepharose columns was first achieved with an excess of DHT and allowed a total recovery of the binding activity. The DHT-bound antibodies obtained were completely dissociated after 72 hours of dialysis at 37°C. This dissociation was accompanied with a 70% loss of binding activity.

Elution with 50 ml 1M NH_4OH, for 5 hours at 4°C, of antibodies retained on both 7-CMO-DHT and 7β-Hem-DHT-BSA-Sepharose columns, yielded in the two

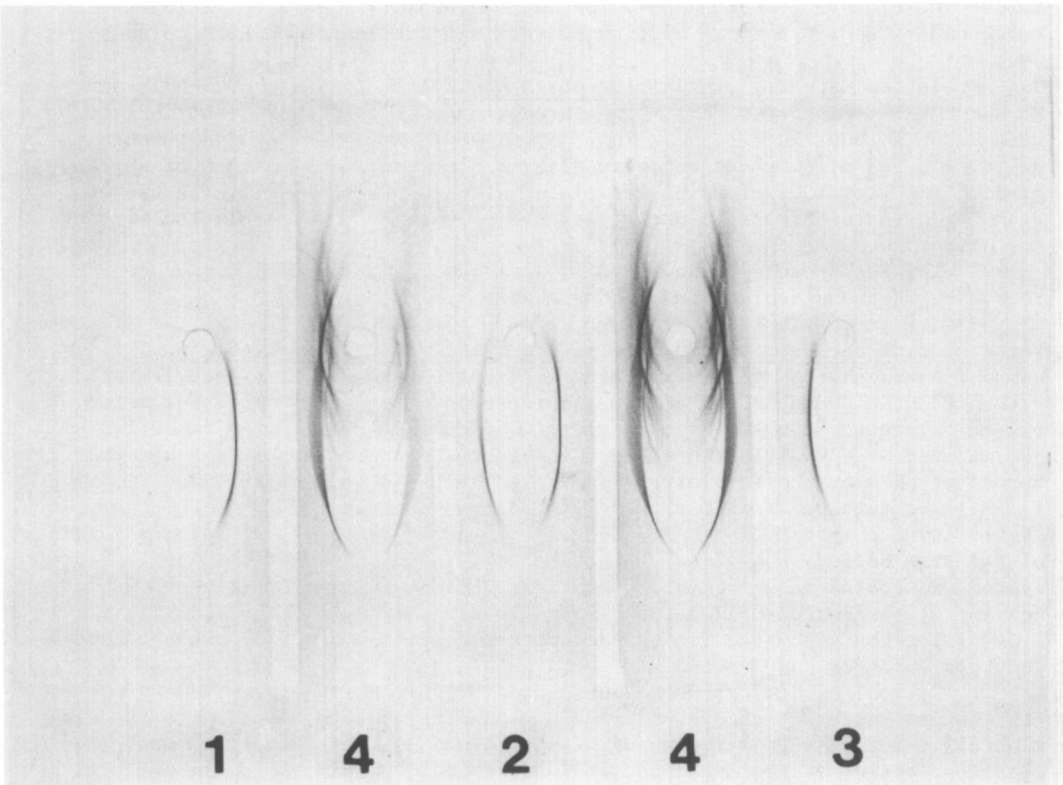

Fig. 1. Immunoelectrophoresis on agar plates of anti-7-CMO-DHT antibodies purified on 7β-Hem-DHT-BSA-Sepharose affinity columns and eluted by : 1) DHT ; 2) NH_4OH ; 3) electrophoresis ; 4) total serum. The central wells contained donkey anti-rabbit serum.

cases a maximum 60% recovery of binding activity. But if these NH_4OH-eluted antibodies were applied again on identical affinity columns, all the binding activity was retained and was then totally recovered by NH_4OH elution.

All attempts to elute with an excess of DHT the 40% binding activity of antibodies remaining on both affinity columns after NH_4OH elution, failed. No binding activity could be detected for these elution-resistant antibodies which were presumably denaturated by 1M NH_4OH as well as by 0.5M NH_4OH which was also tried as elution solvent.

Elution by electrophoresis was performed with the best yields at 5-10°C in a pH 4.5 buffer and allowed, after 4 hours, a maximum 60% recovery of the binding activity of antibodies retained on a 7β-Hem-DHT-BSA-Sepharose gel column. A test experiment showed no elution induced by the acidic pH itself. The electrophoretically eluted antibodies were totally retained when applied

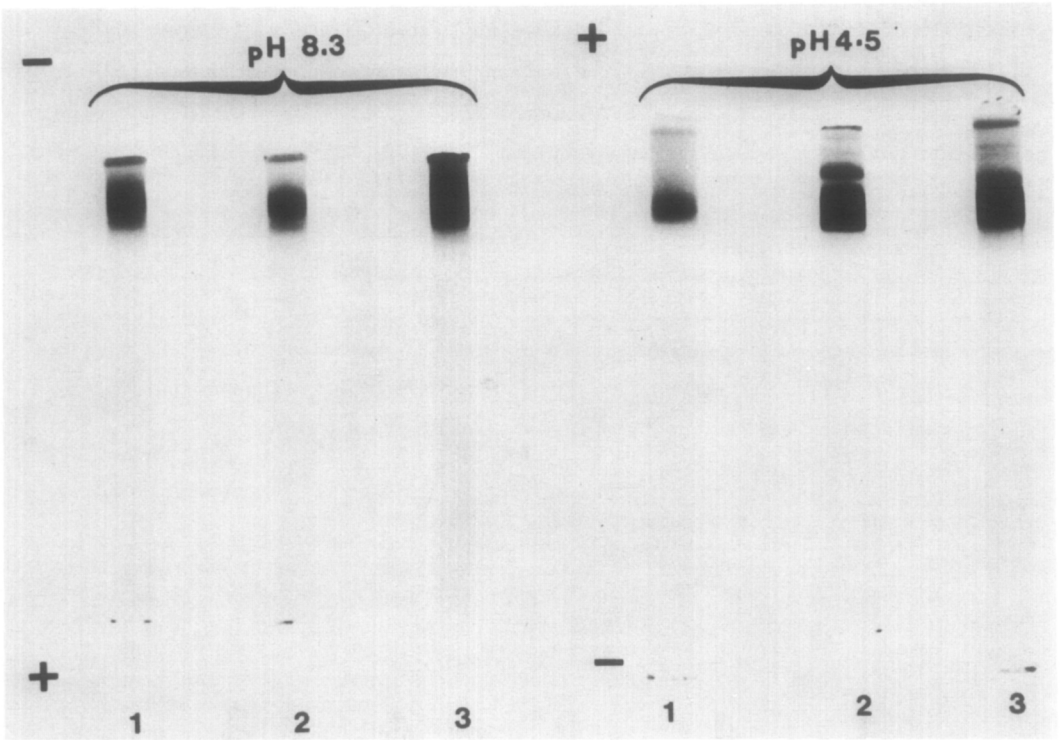

Fig. 2. Disc electrophoresis of anti-7-CMO-DHT antibodies purified on 7β-Hem-DHT-BSA-Sepharose affinity columns and eluted by : 1) DHT ; 2) NH_4OH ; 3) electrophoresis.
7% acrylamide, 3 mA/tube. Coomassie blue stain.

back onto an identical affinity column, and 100% of binding activity was recovered after a new electrophoretic elution of these purified antibodies.

After electrophoretic elution, the affinity gel was incubated with an excess of DHT which allowed the total recovery of the 40% remaining binding activity of the electrophoretic elution-resistant antibodies in DHT-bound form.

These DHT-bound antibodies were dissociated as above, with a 70% loss of binding activity, by prolonged dialysis and showed an apparent association constant around 10^{11} M^{-1}, about ten times higher than the one determined for both NH_4OH and electrophoretically eluted unbound antibodies.

A more direct comparison between NH_4OH and electrophoretically eluted antibodies was made by passing again NH_4OH-eluted antibodies through a 7β-Hem-DHT affinity column which retained all the binding activity. Elution by NH_4OH as well as by electrophoresis allowed a total recovery of the purified antibodies. The same experiment was done with electrophoretically eluted antibodies and the same results were obtained.

The binding tests precedently described confirm the absence of any detectable denaturation of unbound antibodies eluted in 60% yield from the affinity column, either by NH_4OH or electrophoretic procedures. In addition, the more classical tests, agar plates immunoelectrophoresis (Fig. 1) and polyacrylamide gel electrophoresis at pH 4.5 and 8.3 (Fig. 2) were performed and indicated an efficient separation of antibodies from total serum.

One of the major advantages of the electrophoretic method over the NH_4OH method is that the elution-resistant antibodies of higher affinity remaining on the column are not denaturated. Improvements of the electrophoretic elution procedure are under investigation in order to achieve the elution of these higher affinity antibodies. More detailed studies of specificities of purified antibodies are in progress in our laboratory.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Pr. Jean Bertrand for his stimulative interest in this work, to Dr. Maguelone Forest for helpful advice on steroid antibodies binding activity measurements and to Pr. René Got for giving us access to his laboratory for immunoelectrophoretic analyses. The help of Dr. Guy Tell, Dr. José Saez and Dr. Carlos Sonnenschein in editing the manuscript and the expert secretarial assistance of Miss Joëlle Bois are greatly acknowledged.

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