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STUDIES ON AFFINITY CHROMATOGRAPHY OF ALDOSTERONE-BINDING MACROMOLECULES

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SUMMARY

Deoxycorticosterone-hemisuccinate competes with [^{3}H]-aldosterone for loci on mineralocorticoid-binding macromolecules found in the cytosol fraction of kidneys from adrenalectomized rats. Studies on deoxycorticosterone-hemisuccinate (ligand) covalently linked to aminoethyl-agarose (gel) show that exposure of cytosol to the ligand-gel results in loss of mineralocortocoid-binding activity from the cytosol. Substitution of estriol for deoxycorticosterone in the ligand-gel complex does not result in loss of mineralocorticoid-binding activity from the cytosol. Thus, deoxycorticosterone-hemisuccinate-agarose causes a steroid-specific loss of mineralocorticoid binding activity and satisfies at least two of the criteria we established to define the utility of the ligand-gel in affinity chromatography.

INTRODUCTION

CERTAIN biologically important macromolecules exhibit unique affinities for specific ligands. This property has been exploited recently to isolate and purify macromolecules by the relatively simple technique of "affinity chromatography" [1]. This technique involves the adsorption of the macromolecule to be purified on a column containing an insoluble polymer or gel to which a specific ligand has been covalently attached. Macromolecules not exhibiting appreciable affinity for the ligand are not adsorbed and pass unretarded through the column; whereas macromolecules which recognize the ligand are retarded to an extent related to the affinity constant for the ligand [1].

The following criteria must be met in order for a gel containing a covalently bound ligand to be useful in affinity chromatography: (1) It is essential that exposure of a preparation containing a specific solublized macromolecule to the ligand-gel results in loss of activity attributed to that particular molecule; (2) the loss of activity from a preparation must be produced only by a specific ligand; and (3) it is necessary that loss of activity from a preparation be due to an association between the ligand-gel and the macromolecule resulting in removal of that particular macromolecule from all other molecules. Beyond that, for the ligand-gel to be useful in purification, it is necessary that the association process be selective such that only one specific molecule becomes associated with the ligand-gel and that the complex, once formed, can be readily dissociated so that the specific macromolecule can be isolated from the gel.

This report describes our studies with an insoluble resin consisting of a mineralocorticoid, deoxycorticosterone, covalently attached to agarose. The purpose of these studies was to evaluate deoxycorticosterone-agarose with regard to the first and second criteria to determine its usefulness for affinity chromatography, i.e. to evaluate: (1) the ability of deoxycorticosterone-agarose to produce a loss of aldosterone-binding activity from a renal homogenate and (2) the steroid-specificity of the effect.

EXPERIMENTAL

Deoxycorticosterone-agarose. Aminoethyl-agarose was prepared from agarose (Sepharose 4B, Pharmacia, Uppsala, Sweden) as described by Cuatrecasas [1]. [^{3}H]-deoxycorticosterone 21-hemisuccinate was prepared from succinic anhydride and deoxycorticosterone (a trace of tritiated deoxycorticosterone, 30 Ci/mmol, New England Nuclear, Boston, Mass., was added to unlabelled deoxycorticosterone obtained from Sigma Chemical Co., St. Louis, Mo.) according to the procedure described by Erlanger, *et al.*[2]. Thin-layer chromatography, using two solvent systems (1 part Ethyl acetate: 20 parts Methanol; 20 parts Methanol: 1 part Ethyl acetate) indicated that more than 90% of the product migrated identically with commercial deoxycorticosterone 21-hemisuccinate (Steraloids Inc., Pawlings, N.C.). 300 mg (0·7 mM) of [^{3}H]-deoxycorticosterone 21-hemisuccinate (5,600 CPM/μmol) was added in 40 ml. of dimethylformamide to 40 ml. of the packed aminoethyl-agarose. The pH of this suspension was brought to 4·7 with 1 NHCl. 500 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was dissolved in 3 ml of water and added to the reaction mixture over a 5-min period. The reaction was allowed to proceed at room temperature for 20 h. The product was then packed in a column and washed extensively, first with 50% aqueous dimethylformamide and later with 0·25 M sucrose. Extensive washing was found to be necessary to eliminate detectable radioactivity in the wash and finally to eliminate from the wash a substance(s) that inhibited aldosterone-binding (Table 1). The washed product contained approximately 1 μmol of [^{3}H]-deoxycorticosterone per ml of packed deoxycorticosterone-agarose.

Soluble aldosterone-binding macromolecules. Kidneys from adrenalectomized male rats (Charles River Breeding Laboratories, Wilmington, Mass.) were homogenized in either 0·25 M sucrose or 0·3 M KCl with teflon-glass homogenizers. The cytosol fraction was obtained by sequential centrifugation at 15,000 × g for 15 min and 104,000 × g for 60 min. Throughout the study, aldosterone-binding in the various cytosol fractions was determined by the addition of tritiated aldos-

Table 1. Removal from deoxycorticosterone-agarose of an inhibitor of binding of [^{3}H]-aldosterone

	After wash with*	Percent inhibition†
A.	4 L Dimethylformamide + 1 L 0·25 M Sucrose	84%
B.	8 L 0·25 M Sucrose	71%
C.	5 L Dimethylformamide + 4 L 0·25 M Sucrose	35%

*40 ml. of deoxycorticosterone-agarose in a column (dia. 2·5 cm) was sequentially washed with the volumes of agents indicated under A, B and C.

†To test for inhibitor(s) in the wash, one-half of a kidney from an adrenalectomized rat was homogenized in the 0·25 M sucrose wash while the other half of the kidney (the control) was homogenized in 0·25 M sucrose. [^{3}H]-aldosterone (6×10^{-9} M) was added to the cytosol from both homogenates and bound aldosterone was determined after a 3 h incubation period. The values refer to the percent inhibition of binding of [^{3}H]-aldosterone when compared to the control.

terone ([1,2-^{3}H] d-aldosterone, 50 Ci/mmol, New England Nuclear, Boston, Mass.) to the cytosol such that the final aldosterone concentration was $6{\cdot}0 \times 10^{-9}$ M. Following a 3 h incubation, the amount of aldosterone bound per 0·5 ml of cytosol fraction was determined using a gel-filtration method [3]. Homogenization, centrifugation, incubation and filtration were all performed at 0–5°C.

Analyses. [^{3}H]-aldosterone was quantitated with a Nuclear-Chicago Unilux II-A liquid scintillation spectrometer. External standardization and channels ratio were used to correct for quench. A dioxane base [4] or a toluene base counting solution containing Triton X-100 was used [3].

Where applicable, data were analyzed statistically with students' t-test, grouped comparisons. The 0·05 level of probability was the criterion of significance.

Procedures. In order to obtain an approximation of the relative affinity of the covalently bound ligand for mineralocorticoid binding sites, we assessed the ability of deoxycorticosterone, deoxycorticosterone-hemisuccinate and estriol hemisuccinate to compete with aldosterone for renal mineralocorticoid-binding sites. [^{3}H]-aldosterone and one of the other steroids were added simultaneously to the cytosol fraction from a sucrose homogenate. The samples were incubated for three hours. A control received [^{3}H]-aldosterone only. Bound [^{3}H]-aldosterone was measured by gel-filtration.

The effect of exposure to deoxycorticosterone-agarose on aldosterone-binding activity was determined by passing the cytosol fraction from either a sucrose or KCl homogenate through columns (i. d. of 0·6 cm × length of 15 cm) containing deoxycorticosterone-agarose. From 8 to 15 ml of cytosol fraction was passed through the column at a flow rate of approximately 0·4 ml/min. Cytosol eluted from the column after the first 6 ml (which was discarded routinely) was collected to determine aldosterone-binding. Aldosterone-binding activity was measured in a 1 ml aliquot of the cytosol fraction that had passed through the column and in the appropriate control, i.e. a 1 ml aliquot of the same cytosol fraction that had not been exposed to the deoxycorticosterone-agarose column.

In some experiments the cytosol fraction was passed through columns containing agarose or aminoethyl-agarose and deoxycorticosterone-agarose. Aldosterone-binding in the cytosol eluted from the various columns was determined and compared to aldosterone-binding in the untreated cytosol fraction (control).

All deoxycorticosterone-agarose columns used in this study were washed with either 0·3 M KCl or 0·25 M sucrose (depending on whether a KCl or sucrose homogenate was used) just prior to application of the cytosol. To determine the degree of contamination of the wash with substance(s) that inhibited aldosterone-binding (Table 1), 1 ml of the respective washes was added to 1 ml of cytosol after which [^{3}H]-aldosterone was added so that the final aldosterone concentration was $6{\cdot}0 \times 10^{-9}$ M. This was compared with the addition of 1 ml of 0·3 M KCl or 0·25 M sucrose, not exposed to the various columns, to 1 ml of cytosol plus 6×10^{-9} M [^{3}H]-aldosterone. Aldosterone-binding was determined after the usual 3 h incubation period.

In one series of experiments a sucrose cytosol was incubated for three hours with [^{3}H]-aldosterone prior to exposure to columns of either deoxycorticosterone-agarose or aminoethyl-agarose. Bound-aldosterone in this "pre-labelled" cytosol eluted from these columns was measured with gel-filtration. Bound aldosterone was also measured in control cytosol, i.e. cytosol that did not come in contact with deoxycorticosterone-agarose or aminoethyl-agarose columns.

RESULTS

Competition studies indicated that both deoxycorticosterone and deoxycorticosterone-hemisuccinate compete with aldosterone for mineralocorticoid binding sites (Fig. 1). The fact that the ability of deoxycorticosterone-hemisuccinate to compete with aldosterone for binding sites was only slightly less than the ability of deoxycorticosterone indicated that a major portion of the affinity for mineralocorticoid-binding sites was retained in spite of the added bulk at the 21-position of the steroid. Therefore, it seemed reasonable that attachment of deoxycorticosterone to agarose via a hemisuccinate might produce an insoluble resin with an affinity for mineralocorticoid binding sites. In contrast, estriol-hemisuccinate did not compete with aldosterone for binding sites (Fig. 1).

Figure 2 shows the effect on aldosterone binding produced by passing cytosol

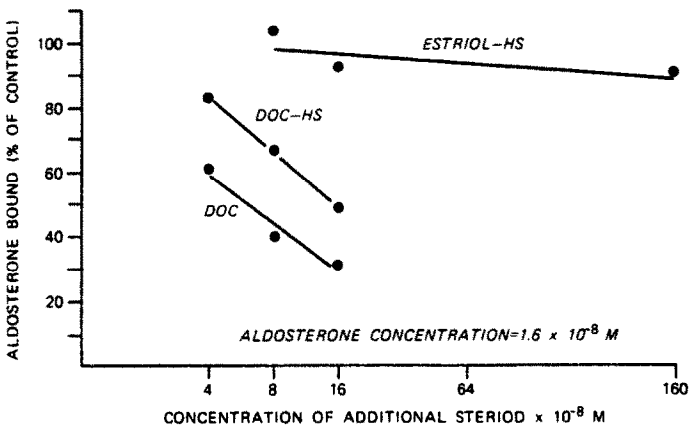

Fig. 1. Inhibition of [^{3}H]-aldosterone-binding in cytosol fraction of kidney from adrenalectomized rats. Values are percent of binding in the absence of non-radioactive deoxycorticosterone (DOC), deoxycorticosterone-hemisuccinate (DOC-HS), or estriol-hemisuccinate (estriol-HS). See text for details.

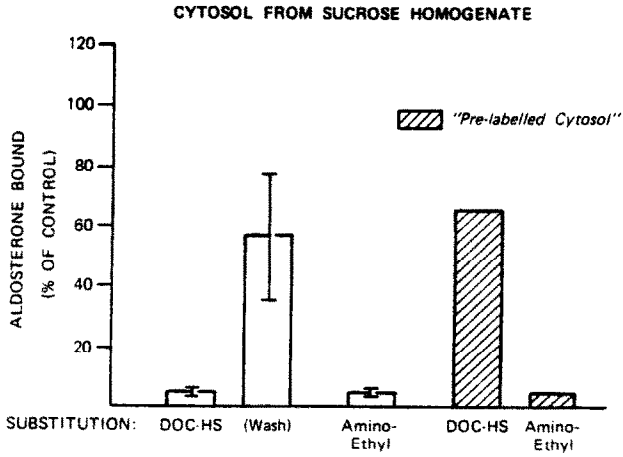

Fig. 2. Effects of various substitutions on agarose on the binding of [^{3}H]-aldosterone by the cytosol fraction of a sucrose homogenate of kidney from adrenalectomized rats. Values are percent of binding in the absence of exposure to substituted agarose. DOC-HS: deoxycorticosterone-hemisuccinate. See text for definition of aminoethyl substitution and "pre-labelled cytosol". Bars represent mean and SEM.

from sucrose homogenates through columns containing either deoxycorticosterone-agarose or aminoethyl-agarose. Aldosterone-binding was reduced to 5% of the control following exposure to deoxycorticosterone-agarose. When sucrose was passed through this column ("wash") and added to unexposed cytosol, aldosterone binding decreased to 57% of the control. This decrease, although substantial, was significantly less than the decrease in aldosterone binding produced by direct exposure of cytosol to the deoxycorticosterone-agarose. Surprisingly, aminoethyl-agarose also decreases aldosterone-binding to 5% of the control in spite of the fact that the aminoethyl-agarose did not contain a ligand with an affinity for mineralocorticoid binding sites. This result suggested a non-specific effect of the insoluble resins. A definitive answer was obtained by first labelling the aldosterone-binding sites with [^{3}H]-aldosterone and then passing the "pre-labelled cytosol" through the columns. As shown in Fig. 2, only 5% of the pre-labelled aldosterone-binding macromolecules passed through aminoethyl-agarose. Since the mineralocorticoid-binding sites were already occupied with [^{3}H]-aldosterone prior to exposure to aminoethyl-agarose, we assumed attachment to the resin was due to non-specific effects unrelated to specific mineralocorticoid binding sites. In contrast, when "pre-labelled cytosol" was exposed to a deoxycorticosterone-agarose column, 66% of the bound aldosterone passed through the column. We found that the aldosterone-binding macromolecules from a sucrose cytosol, attached in this non-specific manner to aminoethyl-agarose, could be eluted from the column with 0·3 M KCl. Therefore, this particular non-specific effect was eliminated in subsequent studies by including KCl in the cytosol and eluent. We have previously reported on the effects of 0·3 M KCl on various characteristics of cytosol aldosterone-binding activity [5].

The results obtained when cytosol from KCl homogenates was applied to the various columns are shown in Fig. 3. Aldosterone binding was reduced to 9% of the control following exposure to deoxycorticosterone-agarose. When KCl passed through the deoxycorticosterone-agarose column ("wash") was added to unexposed cytosol, aldosterone-binding was reduced to 51% of the control. This reduction to 51% of the control was similar to the results with cytosol from sucrose homogenates and, although substantial, was significantly less than

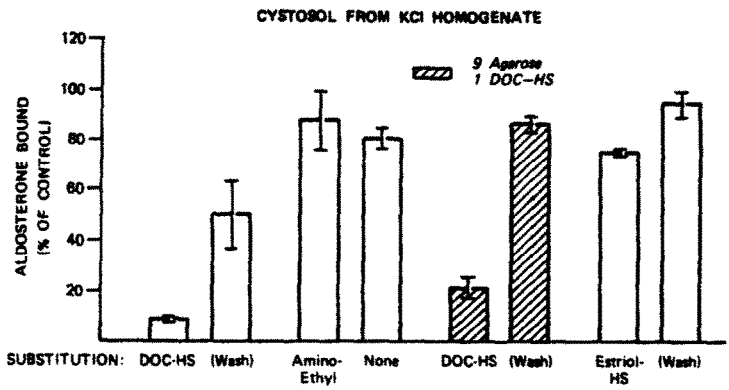

Fig. 3. Effects of various substitutions on agarose on the binding of [^{3}H]-aldosterone by the cytosol fraction of a KCl homogenate of kidney from adrenalectomized rats. None: unsubstituted agarose; estriol-HS: estriol-hemisuccinate. Other conventions used in the construction of this figure are given in the legend to Fig 2 or the text.

the decrease in aldosterone binding produced by direct exposure of KCl cytosol of the deoxycorticosterone-agarose. Unlike the results with cytosol from sucrose homogenates, when KCl was present in the homogenate and eluent, aminoethyl-agarose had little effect on aldosterone-binding in that 88% remained after exposure to aminoethyl-agarose. Exposure of KCl cytosol to an agarose column also had little effect on aldosterone-binding, as 81% of binding remained.

We attempted to minimize the inhibitory effect of deoxycorticosterone-agarose that is present in the solutions washing the column by preparing a column containing 1 part deoxycorticosterone-agarose and 9 parts agarose. The wash from this column (Fig. 3) decreased aldosterone-binding to only 87% of the control. As expected, direct exposure of cytosol to this column produced a significantly greater loss of aldosterone-binding activity to 22% of the control.

To determine that the loss of aldosterone-binding activity we had observed in the previous studies was a specific result of deoxycorticosterone-agarose and not merely a non-specific effect produced by any gel-ligand, we synthesized estriol-agarose. Estriol-hemisuccinate replaced deoxycorticosterone-hemisuccinate in the procedure for preparing estriol-agarose that was otherwise identical to the procedure used to prepare deoxycorticosterone-agarose. The results are shown in the right-hand portion of Fig. 3. Exposure to an estriol-agarose column had little effect on aldosterone-binding activity in the cytosol of a renal homogenate.

DISCUSSION

Our studies demonstrated the effectiveness of deoxycorticosterone-agarose in removing aldosterone-binding activity from the cytosol of a homogenate prepared from the kidneys of adrenalectomized animals. The results also showed that the loss of aldosterone-binding activity was a specific result of deoxycorticosterone-agarose rather than a non-specific result that could be produced by any gel-ligand, since estriol-agarose had little effect on aldosterone-binding activity. Therefore, the first and second criteria regarding the utility of a ligand-gel in affinity chromatography are clearly met by deoxycorticosterone-agarose.

Previous investigators working with other steroid-binding fractions have similarly demonstrated that specific steroids covalently attached to insoluble polymers effectively remove steroid-binding activity. An estrogen covalently attached to cellulose or polystyrene was shown to produce a loss of estradiol-binding activity in the cytosol from a uterine homogenate[6] and an androgen (3-β-aminoandrostan-17-β-ol) attached to agarose was found to remove testosterone-binding activity from plasma[7]. However, the third criterion for utility of a ligand-gel in affinity chromatography – that the loss of steroid-binding activity must be due to an association of the steroid-binding macromolecule with the ligand-gel – was not satisfied in these earlier studies.

Similarly, whether or not the third criterion is met by deoxycorticosterone-agarose is not established at the present time. To date, several attempts to recover aldosterone-binding macromolecules from dexoycorticosterone agarose columns have failed. For example, we attempted to recover aldosterone-binding macromolecules from ligand-gel columns which had been exposed to large volumes of cytosol by introducing 6×10^{-6} M [^{3}H]-aldosterone (deoxycorticosterone-hemisuccinate was present linked to agarose at $1{\cdot}2 \times 10^{-5}$ M). Since the affinity of the aldosterone-binding macromolecules for aldosterone is considerably greater than it is for deoxycorticosterone-hemisuccinate, we postulated that the [^{3}H]-aldosterone

might strip the mineralocorticoid-binding protein from the ligand gel. Similar use of a higher affinity ligand to recover a protein from a lower affinity column had been successfully employed in the case of acetylcholinesterase [8]. However, we were unable to recover aldosterone-binding activity by this method. This raises the question of whether or not the macromolecules with aldosterone-binding activity were retained by the column containing deoxycorticosterone-agarose. Indeed, preliminary studies suggest that the cytosol from renal homogenates may be capable of freeing deoxycorticosterone-hemisuccinate from agarose. If this proves to be the case, loss of aldosterone-binding activity after exposure of cytosol to the ligand-gel may simply be the result of competition of free deoxycorticosterone-hemisuccinate with [^{3}H]-aldosterone for the binding loci rather than the result of an association between the ligand-gel and the steroid-binding macromolecule. This alternative explanation for the loss of steroid-binding activity after affinity chromatography is the subject of current investigation.

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DISCUSSION

Funder: You find that the material eluted from your column inhibited the binding of [^{3}H]-aldosterone in your test system, and that this interference was greater when cytosol was passed through the column than when it was washed with buffer. We have found that kidney cytosol has the ability to cleave rapidly an ester-DOC iodocaetate – similar but not identical to your DOC hemisuccinate. Is it possible that the interfering material in the column eluate is DOC?, the hemisuccinate having been split off and remaining attached to the column? Do you know the proportion of DOC and DOC hemisuccinate in the column eluate?

Fanestil: (Note added in proof: We have analyzed the radioactivity which was bound to protein after a cytosol preparation was incubated for 3 hours with 6×10^{-9} M [^{3}H]-DOC-hemisuccinate. Approximately half of the bound radioactivity is ^{3}H-DOC and half is [^{3}H]-DOC-hemisuccinate.)

Snart: Professor Meuller, using affinity chromatography to separate the estradiol

receptor, has reported that although he is unable to elute the active receptor from his resin he was able to extract some lipid components from the receptor bound to the resin. Have you considered whether his system would be subject to the same problems?

Fanestil: I have no information regarding your suggestion.