DEMONSTRATION OF STEROID SPECIFIC HORMONE RECEPTORS BY CHROMATOGRAPHY

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1. Introduction

Drug–ligand interaction is a fundamental preamble of pharmacological hierarchy and steroid–receptor complex formation in a variety of tissues (liver, kidney, thymus, muscle, lung) is currently believed to constitute an initial step in the mechanism of adrenocortical hormone action [1]. Although studies with displaceable binding indicate the presence of steroid-specific hormone receptors, earlier efforts [2,3] at physicochemical characterization of these intracellular binders has largely been frustrating and ambiguous, due largely to technical difficulties. As early as 1970, when this field was still accepted with scepticism, we had established techniques for chromatographic identification of steroid-binding proteins and accomplished 300-fold purification of rat liver corticosterone receptors [4–7], and recently extended them to revealing renal mineralocorticoid specific 'MR' receptors [8,9]. Physicochemical comparison reveals here that aldosterone specific binders present in the kidney, but not the liver, cytosol elute at lower ionic strengths than corticosterone specific receptors identical in both organs, followed lastly by serum transcortin, although all three sorts of macromolecules exhibit comparable monomeric mol. wts. Thus, corticosteroid specificity may indeed reside in the recognition of the steroid by chemically distinct groups of closely related proteins. Furthermore, procedures established here may profitably be exploited in future elucidation of the various types of steroid-specific ligands.

2. Materials and methods

Male Wistar rats (150–200 g) were bilaterally adrenalectomised at least 48 h before use, exsanguinated under light ether anesthesia, and perfused with the appropriate initial buffer by aortic cannulation. The excised organs were homogenized in the same buffer and the 105 000 *g* supernates were equilibrated, for 60 min at 4°C, with the desired concentration of the steroid. The free radioactivity was thereafter removed by additional incubation in presence of 100 mg/ml cell sap of activated charcoal (Sigma C-5260) for 10 min and centrifugation at 3000 *g*. The supernate was then passed through glass wool to remove the remaining traces of carbon and then charged on to the appropriate resin.

The techniques for chromatography on Sephadex G-200 and DEAE-Cellulose-52 have been described in our previous reports [4–7,9]. The chromatography on Sephadex-DEAE-A-50 was performed after modification of other procedures [3]. Further details are given in the text and the legends.

Aliquots of 1 ml were mixed with 10 ml Unisolve (Koch-Light Laboratories) and counted in a Packard Tricarb Scintillation Spectrometer with corrections for quenching, background and spilling as before [10]. The optical density was measured by continuous absorbance at 280 nm.

[1,2,^{3}H]aldosterone (lot 1172; 55 Ci/mmol), [1,2,^{3}H]corticosterone (batch 57072; 26 Ci/mmol) were both purchased from Saclay, France, and diluted with ethanol which was evaporated in the incubation vessel prior to incubation. All other chemicals were high purity reagent grade.

Results in fig.1A confirm our initial findings that

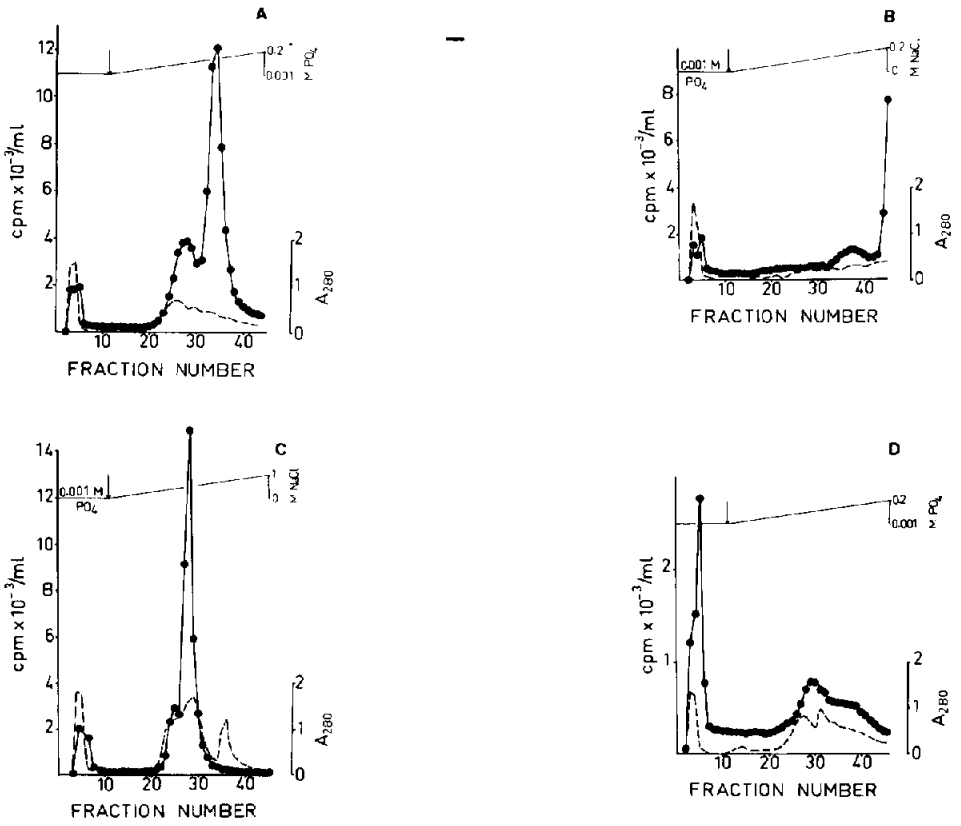

Fig.1. Optimum conditions for ion exchange separation of corticosterone binding proteins from rat liver. A 50% liver cytosol (4 ml) was equilibrated with 10^{-7} [^{3}H]corticosterone and loaded onto the DE-52 column (1 × 25 cm), prepared and packed as described in the text. After an initial prewash with 80 ml of the 0.001 M phosphate, pH 7.5, buffer (until fraction 11), elution was effected by a linear gradient between 60 ml of this buffer (mixing chamber) and 60 ml of either 0.2 M phosphate, pH 7.5 (A), or 0.2 M NaCl (C) in the reservoir; the NaCl was prepared in 0.001 M Na phosphate, pH 7.5, in all cases. (D) Liver cytosol (5 ml) was equilibrated with 10^{-7} M ^{3}H-aldosterone and eluted under the standard conditions, as for (A). For further details, and previous manipulations prior to establishing this procedure, see text and [4–6]. The total procedure required 3 h, at 4°C.

rat liver conticosterone receptors elute as a distinct peak in the 0.001 M prewash followed by another between 0.01–0.02 M phosphate on DE-52; the highly acidic transcortin binds tightly to the DE-cellulose and peaks around 0.06 M PO_4. With 0–0.2 M NaCl gradient, although peaks I and II (the latter greatly diminished) could be eluted, the corticosteroid binding globulin (CBG) still remained bound to the resin (fig.1B); complete elution was possible only at concentrations of about 0.5 M NaCl but the resolution between the various components was much less clear than with phosphate (fig.1A) such that peak II was reduced to a mere shoulder of the CBG peak (fig.1C); it is surprising that serum transcortin (CBG) could previously be eluted with 0.2 M NaCl [2]. Contrary to other suggestions [2], the peak in the 0.001 M prewash does not represent radioactivity dissociated from the components eluting in the gradient (since prolonged washing did not eliminate the latter and since the prewash peak was charcoal resistant). The elution profile further suggests that liver glucocorticoid binders in the 0.001 M phosphate prewash had greater affinity for aldosterone whereas the reverse was true of binders eluting around 0.02 M PO_4 and the transcortin (fig.1A, 1D).

It is evident from fig.2A that mineralocorticoid-specific receptors in the kidney are resolved as a more abundant peak in the prewash followed by a less abundant species eluting around 0.006 M phosphate (which is absent from the liver, fig.1D); the third peak represents binding of 10^{-8} M aldosterone to gluco-corticoid receptors and to transcortin. As with liver, this last peak was mostly lost with 0–0.2 M NaCl gradient (fig.2B); it is conclusive that an aldosterone binding component elutes at 0.06 M NaCl from kidney cytosol which could not be revealed by either aldosterone in the liver (fig.1D) or corticosterone in the kidney (fig.2D). This clear distinction between the steroid-specific receptors was lost when 0–1 M NaCl gradient was used, in keeping with the known tendency to disaggregate with high salt [6,11] and resulting in comparable elution profiles in the liver and the kidney. The fact that no radioactivity could be eluted in the 0.001 M prewash when kidney cytosol was equilibrated with 10^{-8} M corticosterone (fig.2D), in place of aldosterone, constitutes a formal proof that, when observed, the same does not represent an artifact of the washing procedure. Rather, gluco-(liver) and mineralo-(kidney) corticoid specific receptors elute in this region with great abundance (compare figs.1 and 2).

Next, separation was attempted on Sephadex A-50 columns, based both on mol. wt and charge density. It is clear from fig.3 that aldosterone was bound to two distinct components; the first, more abundant, eluted between 0.35–0.45 M KCl, and the second at 0.6 M KCl (which coeluted with [^{14}C]corticosterone bound to serum transcortin, in separate experiments). Thus, although the peak at 0.6 M KCl may represent transcortin, resolution between the mineralo- and gluco-corticoid receptors

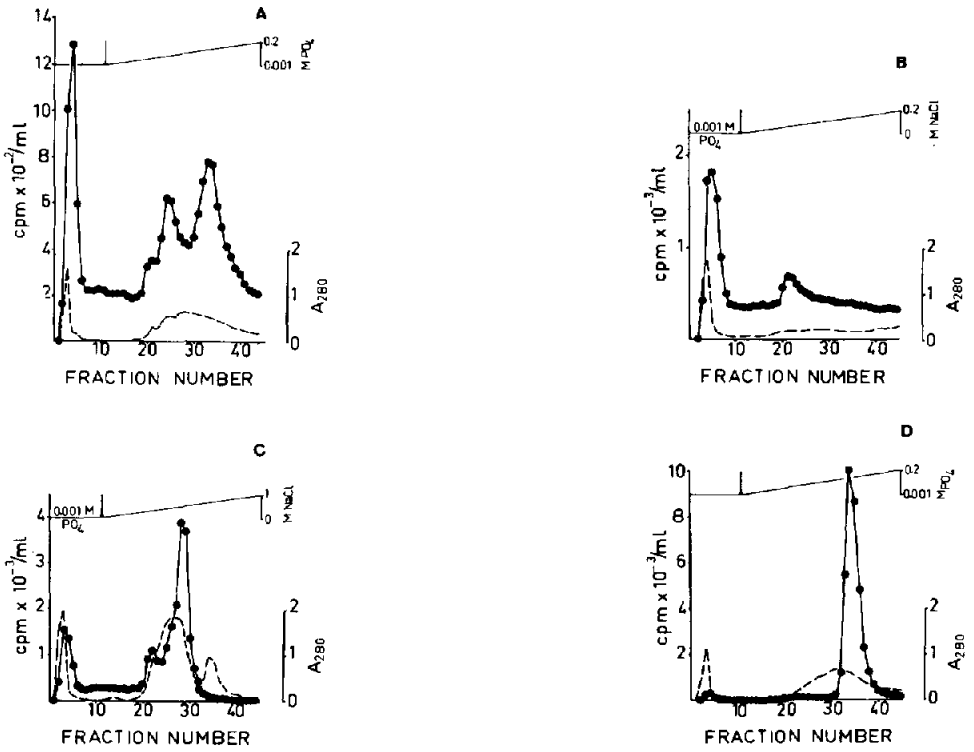

Fig.2. Demonstration of aldosterone specific binders in rat kidney as a function of elution conditions during ion exchange chromatography. A 40% kidney cytosol (6 ml) was equilibrated with 10^{-8} M [^{3}H]aldosterone and chromatographed under the various conditions specified in legend to fig.1. For fig.1D, 4 ml cytosol was equilibrated with 2×10^{-8} M [^{3}H]corticosterone and chromatographed under the standard conditions (fig.1A).

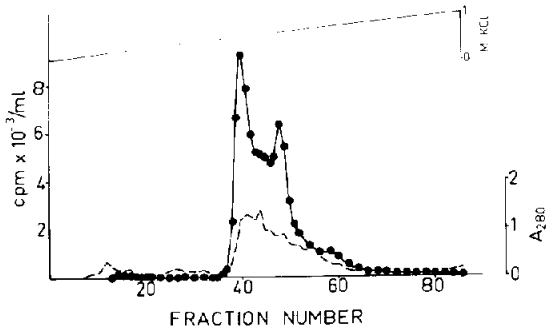

Fig.3. Chromatography of aldosterone binding proteins based on mol. wt. and charge density. The DEAE-Sephadex-A-50 column (1.5 × 90 cm) was equilibrated with 0.05 M Tris· HCl, pH 7.5, and loaded with 5 ml kidney cytosol which had been incubated in presence of 2×10^{-8} M [^{3}H]aldosterone and then treated with charcoal. Protein was eluted by a linear ionic gradient between 150 ml initial buffer, and 150 ml of this buffer containing 1 M KCl. Fractions of approximately 3 ml were collected at a flow rate of 6 ml/h and the samples were analyzed for radioactivity as before.

was not possible under these conditions, probably because high KCl concentrations favour dispersion into monomeric units with comparable molecular dimensions. Some radioactivity consistently came off at 0.18 M KCl and may consist of receptors, ligandin [3], metabolite binders [3], or the A protein [2]. In fact, a number of so-called binders [3] may indeed be explained as polymorphs of a basic unit generated as a function of exposure to high salt buffers.

Passage through Sephadex G-200 columns equilibrated at physiological ionic strength and pH (7.4) revealed the presence of two components (fig.4). Comparison with other markers showed a first, less abundant species with a gross mol. wt of 120 000 and another, more abundant, of 60–70 000 daltons. These molecular sizes are comparable to those of corticosterone [4–6], the 'G' [2] or the steroid II [3] receptors, although neither peak corresponds to serum transcortin (62–63 000 daltons). The close similarity in molecular sizes, and the fact that transcortin is present to variable extent in most tissue preparations, obviate the importance of filtration through Sephadex gels; these are also true of sucrose density gradients further unsuitable due to huge dissociation during the lengthy centrifugation period [4–6,11].

Ion exchange chromatography on DEAE-cellulose-52 gels, thus, would appear to be the only method available for characterization of the receptor moities with respect to heterogeneity, specificity, and overall charge density. If one respects the conditions developed here (see also [4–7]), and takes the stated precautions, separation of various macromolecules on DE-52 is clear, reproducibility is excellent, dissociation does not pose a problem, and the total procedure only requires approximately 3 h. By contrast, the usual binding assays are lengthier, more cumbersome, less specific, and yield limited information.

The large width/height column ratio (thereby diminishing depth of ion gradient), use of DE-32 instead of DE-52 (the latter diminishes errors in resin preparation), cortisol or dexamethasone (the latter may bind nonspecifically to a number of components), changes in buffer between homogenization and chromatography with prolonged exposure to pH 8.3 (steroid binding decreases between pH 7.5 and 8 [5]), and presence of glycerol (whose viscosity interferes with ion exchange [12]), or NaCl, or of a number of other ions (all of which may influence ion exchange in an unpredictable manner), may have collectively contributed to baffling results reported by others [2]. The differences in elution with Na phosphate, vs NaCl, are dramatic and seem to be of fundamental

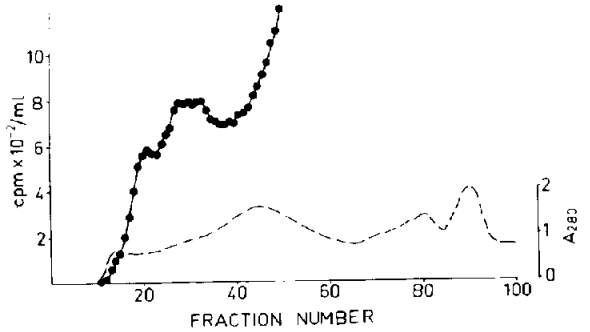

Fig.4. Mol. wt. determination of aldosterone binding proteins. Sephadex G-200 columns (1 × 130 cm) were equilibrated with 0.01 M Na phosphate, pH 7.4, containing 0.1 M NaCl, and then loaded with 2 ml kidney cytosol which had been incubated in presence of 10^{-8} M [^{3}H]aldosterone. The charcoal treatment was not required for this manipulation although this did not alter the elution profile. Fractions of 1.5 ml were collected at a flow rate of 4 ml/h. Further details as before [4–6].

importance; there is no easy way to explain why, on the basis of molarity, nearly 10 times more NaCl is required for elution of the same component under otherwise identical conditions (see figs.1 and 2 and text).

Summarily, these studies form the pioneering evidence to distinguishing mineralo- from gluco-corticoid receptors in the kidney and the liver, at the physicochemical level, and outline the technical considerations that may be fruitfully employed in future analyses of steroid-target organ specificity.

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