Identification and Properties of Renal Mineralocorticoid Receptors in Relation to Glucocorticoid Binders in Rat Liver and Kidney

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The binding of the natural mineralocorticoid aldosterone and the glucocorticoid corticosterone to macromolecules in rat liver and kidney cytoplasmic fractions was compared by various chromatographic procedures. Equilibration of kidney cytosol with 10nM-aldosterone, either alone or in the presence of a competing steroid, was ideal for ion-exchange chromatography on DEAE-cellulose DE-52, and revealed the presence of four sorts of binding components. One of these, eluted in the 0.001M-phosphate pre-wash, and another, less abundant, forming a peak at 0.006M-phosphate, did not bind corticosterone at equimolar concentrations, and appear to constitute the mineralocorticoid-specific 'MR' receptor in rat kidney. They could not be detected in the liver. Radioactivity eluted in the 0.02 and 0.06M-phosphate regions on DEAE-cellulose DE-52 appears to be due to [³H]aldosterone binding to glucocorticoid-specific 'GR' receptors and to transcortin respectively, since labelling was greater with corticosterone even at 10nM than with the mineralocorticoid at 100nM and since [¹⁴C]corticosterone bound to blood serum transcortin was always co-chromatographed in the 0.06M-phosphate region. These two components appear to be identical with those in the liver and could be labelled maximally only by 100nM-corticosterone. The separation between specific mineralo- and glucocorticoid-binding species was less clear when chromatography was attempted on DEAE-Sephadex A-50 columns, possibly because of disaggregation into subunits in the presence of the high KCl concentrations required for elution. Competitive binding followed by filtration through Sephadex G-200 gels indicated that cellular MR binders, unlike GR receptors, exist mostly as high-molecular-weight aggregates, although both appear to exhibit a comparable monomeric molecular weight of approx. 67000.

The initial step in the mechanism of adrenocortical-hormone action is believed to be the association of the steroid molecule with a protein receptor in the target organ. Both aldosterone (11 β ,21-dihydroxy-3,20-dioxypregn-4-en-18-al; the principal mineralocorticoid) and corticosterone (11 β ,21-dihydroxypregn-4-ene-3,20-dione; the principal glucocorticoid) are bound by receptors in a variety of rat tissues, although this distinction is rather arbitrary, since at higher concentration most 11,21-dihydroxy steroids cross-react with respect to physiological effects (Feldman *et al.*, 1972). Nevertheless, 'specific' mineralocorticoid receptors have been said to exist in the kidney, the parotid, the toad bladder and possibly the intestinal mucosa, whereas 'specific' glucocorticoid-binding molecules (Ballard *et al.*, 1974) are believed to be present in most target tissues. The determination of specificity of these receptors has largely been limited to studies of competitive binding of steroids to the binding proteins, or the chasing of a bound radioactive steroid by an unlabelled structurally related compound. For example, far higher concentrations of corticosterone than of aldosterone are presumably needed to saturate and remove aldosterone from the specific mineralocorticoid receptor.

This approach would not detect possible heterogeneity within a given class of receptors or permit physicochemical characterization of the molecular species in question.

We have previously reported on the partial purification of rat liver corticosterone receptors, and also provided techniques for the physicochemical characterization of these molecules (Agarwal *et al.*, 1970; Agarwal, 1972; Snart *et al.*, 1970, 1972). A few other workers have attempted a similar analysis with rat liver glucocorticoid binders (Beato & Feigelson, 1972; Litwack *et al.*, 1973), but the properties of the mineralocorticoid-specific receptors are still unknown. The present paper summarizes our attempts to establish the physical characteristics of the various populations of mineralo- and glucocorticoid receptors in the kidney, and to compare them with liver glucocorticoid receptors.

Methods

Male Wistar rats (150–200g) were bilaterally adrenalectomized 2–3 days before death in all experiments. Food and 1% NaCl solution were available *ad libitum* at all times after surgery.

Animals were killed by exsanguination under ether anaesthesia, the desired organs were perfused with the starting buffer (according to the chromatography to be performed), and the tissues were homogenized in an equal volume of the same buffer. The 105000g supernatants were equilibrated with the desired concentrations of the steroid for 60 min at 4°C with occasional agitation. To remove unbound radioactivity, activated charcoal (100 mg/ml solution) was added to the cytosol and left for 10 min at 4°C. After removal of the charcoal by centrifugation at 3000g and passage through glass wool, the cell sap was loaded on to the chromatographic resin. In certain experiments, the free steroid was removed by passage through a Bio-Gel P-10 column (1 cm × 25 cm), rather than adsorption by charcoal. The charcoal technique was finally adopted, since passage through Bio-Gel P-10 or Sephadex G-50 enlarged the volumes.

Serum was prepared from rat blood obtained by aortic cannulation. The blood was allowed to clot at 37°C (30 min), then at 4°C (60–90 min), and finally centrifuged at 3000g.

For chromatography on DEAE-Sephadex A-50, 90 cm × 10 cm columns (Pharmacia) were equilibrated with 0.05 M-Tris/HCl, pH 7.5, and eluted at 5–6 ml/h at 4°C. Fractions (3 ml) were collected, of which 1 ml was processed for radioactivity and the remainder for E_{280} . Further details are given in the Figure legends.

Ion-exchange chromatography was performed on DEAE-cellulose DE-52 columns (1 cm × 25 cm) equilibrated with 0.001 M-sodium phosphate, pH 7.5, at a flow rate of 60 ml/h at 4°C (Agarwal *et al.*, 1970; Snart *et al.*, 1972; Agarwal, 1975). The cell sap was loaded on to the resin and after an initial pre-wash with 70–80 ml of the initial buffer (fraction volume 6–7 ml), protein was eluted with a linear gradient between 60 ml each of 0.001 M- and 0.2 M-sodium phosphate, pH 7.5. Fractions (3.5–4.0 ml) were collected, and 1 ml samples were processed for radioactivity determinations. The E_{280} of the eluate was monitored on a Sèvre recorder. Modifications of this standard form of gradient elution are given at appropriate places in the paper.

For estimation of the molecular weights, Sephadex G-200 columns (1 cm × 130 cm) were equilibrated and eluted with 0.01 M-sodium phosphate buffer, pH 7.4, containing 0.1 M-NaCl, at a flow rate of 3–4 ml/h, at 4°C. The cell sap was layered on (usually without removal of free steroid), and fractions (1.1–1.5 ml) were collected, of which 1 ml was used for determination of radioactivity. The E_{280} was monitored continuously. These experimental conditions were finally adopted after numerous modifications in the elution procedures (Agarwal *et al.*, 1970; Snart *et al.*, 1970, 1972; Agarwal, 1975).

In all cases the samples were mixed in 10 ml of

Unisolve (Koch–Light Laboratories, Colnbrook, Bucks., U.K.), and the radioactivity was measured in a Packard Tri-Carb scintillation spectrometer, equipped with automatic background subtraction and external standardization. Corrections for quenching and discrimination between ^{14}C and ^3H were carried out as described previously (Agarwal *et al.*, 1969; Snart *et al.*, 1972).

[1,2- ^3H]Corticosterone (batch 57072; 26 Ci/mmol), [4- ^{14}C]corticosterone (batch 467–248; 57 mCi/mmol) and [1,2- ^3H]aldosterone (batch 1172; 55 Ci/mmol) were obtained from Saclay, Gif-sur-Yvette, France.

DEAE-cellulose DE-52 was a product of Whatman Biochemicals, Maidstone, Kent, U.K.; DEAE-Sephadex A-50, Sephadex G-200 and G-50 were purchased from Pharmacia, Uppsala, Sweden; Bio-Gel P-10 was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Activated charcoal (lot 93C-0360) was prepared by Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were high-purity reagent grade.

Results

Chromatography on DEAE-cellulose

In a previous paper (Agarwal, 1975) it was shown that, on a DEAE-cellulose DE-52 column, the mineralocorticoid-binding molecules are resolved as a more abundant peak in the low-ionic-strength pre-wash (0.001 M-phosphate) followed by a less abundant peak eluted between 0.004 and 0.006 M-phosphate; the glucocorticoid receptors appear to be eluted between 0.01 and 0.02 M-phosphate in both the kidney (Agarwal, 1975) and the liver (Snart *et al.*, 1972), whereas 0.05–0.06 M-phosphate is required to elute the acidic transcortin, which binds tightly to the DE-52 column. The nature of the component eluted in the 0.001 M pre-wash with high concentrations of corticosterone was not defined, although by analogy with liver, some of it could represent the glucocorticoid receptors (Snart *et al.*, 1972; Agarwal, 1975).

Results in Fig. 1 confirm and extend these observations in the kidney. The bound [^3H]aldosterone giving a peak at 0.006 M (peak B) represents mineralocorticoid receptors ('MR') exclusively, since: (i) it was not diminished by unlabelled corticosterone in equimolar (10 nM) concentrations (Fig. 1a); (ii) [^3H]corticosterone (10 nM) alone (Agarwal, 1975) or in the presence of non-radioactive aldosterone (Fig. 1b) does not label proteins in this region; (iii) the elution profile was less clear with 100 nM-aldosterone (Fig. 1c), since the steroid begins to saturate the glucocorticoid receptors at this concentration [although even at 100 nM, corticosterone does not completely saturate the proteins eluted in the 0.006 M region (Fig. 1d)]; (iv) this component is absent from rat liver (see below); (v) this peak is still evident when non-radioactive

Fig. 1. Demonstration of mineralocorticoid-specific receptors in rat kidney

A DEAE-cellulose DE-52 column was prepared, packed and equilibrated as described previously (Agarwal *et al.*, 1970; Snart *et al.*, 1972) and in the Methods section. Kidney cytosol was treated as follows: 8 ml was equilibrated with 10 nM- ^3H aldosterone in the presence of unlabelled 10 nM-corticosterone (Fig. 1a); 6 ml was incubated with 10 nM- ^3H corticosterone in the presence of non-radioactive 10 nM aldosterone (Fig. 1b); 4 ml was treated with 100 nM- ^3H aldosterone (Fig. 1c); 3 ml was mixed with ^3H corticosterone (final concn. 100 nM) (Fig. 1d). After removal of free radioactivity, the protein was washed with 0.001 M-phosphate on the column, and then eluted with 60 ml each of 0.2 M (in the reservoir) and 60 ml of either 0.001 M- (Figs. 1a, 1d) or 0.002 M- (Figs. 1b, 1c) sodium phosphate in the mixing chamber. The higher concentration (0.002 M) was used in an attempt to spread separation over a wider range of fractions (since bound receptors do not begin to be eluted before 0.004 M). However, in these and other experiments, this procedure did not result in clearer resolution, although the bound radioactivity started to be eluted earlier than with 0.001 M-phosphate, pH 7.5. The arrow indicates where gradient elution was begun. In all cases, 1 ml fractions were processed for determination of radioactivity as described in the text. For further details see the text. ●, Radioactivity; ----, E_{280} .

Fig. 2. Absence of mineralocorticoid-specific receptors from rat liver

Liver cytosol (5 ml) was equilibrated with 10 nM-[^3H]aldosterone in the presence of equimolar unlabelled corticosterone (Fig. 2a); 5 ml of liver cytosol incubated with 10 nM-[^3H]aldosterone and 2 ml of blood serum treated with 0.5 μCi of [^{14}C]corticosterone were processed separately for removal of free radioactivity and then mixed together (Fig. 2b). All other details are as described in the legend to Fig. 1 and the text. $\bullet$, ^3H ; $\circ$, ^{14}C ; ----, E_{280} .

corticosterone at 100 nM is allowed to compete with as little as 10 nM-[^3H]aldosterone (results not shown).

Much of the peak (A) in the 0.001 M pre-wash must represent mineralocorticoid receptors since: (i) less radioactivity is eluted with 100 nM-corticosterone (Fig. 1d) than with equimolar aldosterone (Fig. 1c); (ii) the occasional but insignificant binding of 10 nM-corticosterone to components in this region (Agarwal, 1975) is abolished when non-radioactive aldosterone is allowed to compete with the [^3H]glucocorticoid at this same molarity (Fig. 1b); (iii) corticosterone even at 100 nM is only partially effective in competing with 10-fold lower concentrations of [^3H]aldosterone for receptors eluted in this region (results not shown); (iv) labelling in liver is greater with corticosterone than with aldosterone (see below).

The radioactivity eluted in the 0.02–0.06 M-phosphate regions (C) definitely represents binding to glucocorticoid receptors and to transcortin, since: (i) these components are more extensively labelled

with 10 nM-[^3H]corticosterone (Fig. 1b) than with aldosterone in equimolar concentrations (Fig. 1a); (ii) peaks at 0.02 and 0.06 M-phosphate are labelled to a greater extent with 100 nM- than with 10 nM-[^3H]corticosterone (compare Figs. 1b and 1d), in keeping with the association constants (at 0°C) of 1×10^8 and $5 \times 10^8 \text{ M}^{-1}$ for the glucocorticoid receptor and for transcortin (Agarwal *et al.*, 1970; Snart *et al.*, 1970, 1972) respectively; (iii) corticosterone at 100 nM completely eliminates binding of 10 nM-[^3H]aldosterone to these proteins (results not shown).

Mammalian liver is generally not considered to be a target for mineralocorticoid action, and is reported to be lacking in molecules exhibiting high-affinity displaceable binding for aldosterone *in vitro* (Feldman *et al.*, 1972). Conclusive evidence is shown in Fig. 2, which reveals a total lack of bound [^3H]aldosterone peak in the region of 0.006 M-phosphate (where the kidney mineralocorticoid receptor is eluted). Moreover, much less radioactivity was eluted in the 0.001 M pre-wash (peak A) than with equivalent amounts of aldosterone in kidney cytosol (compare Figs. 1a and 2b). Rather, bound aldosterone could be eluted between 0.01 and 0.02 M-phosphate (peak X), indicating attachment to the glucocorticoid receptors; the small peak of radioactivity in the region of higher salt concentration possibly represents limited binding to transcortin.

This was confirmed when liver cytosol labelled with 10 nM-[^3H]aldosterone was chromatographed in the presence of [^{14}C]corticosterone bound to serum transcortin. It is clear that the second component of peak X (eluted in 0.06 M-phosphate) of bound [^3H]aldosterone is eluted with the serum carrier Y (Fig. 2b). Further, labelling of this peak was 10-fold greater with 10 nM-corticosterone than with equimolar aldosterone (Fig. 2), and did not further increase as the concentration of either steroid was raised to 100 nM (M. K. Agarwal, unpublished work).

By contrast, in other experiments, 100 nM-corticosterone was required to saturate the macromolecules eluted between 0.01 and 0.02 M-phosphate (see also Agarwal *et al.*, 1970; Snart *et al.*, 1970, 1972), in keeping with the above association constants. In still other studies, 100 nM-aldosterone was only half as effective as the equivalent quantity of corticosterone in labelling these components giving peaks around 0.02 M-phosphate. Most remarkably, a sharp and well defined peak of radioactivity was eluted in the 0.001 M pre-wash when liver cytosol equilibrated with either 10 nM-corticosterone or -aldosterone was used [but this same peak was totally absent when kidney cytosol was incubated at this same concentration of corticosterone (see Fig. 1b)], and increased by nearly 3-fold as the concentration of either steroid was raised from 10 to 100 nM. These findings suggest that (i) this radioactivity is not an artifact of the washing procedure, as suggested by others (Beato & Feigelson,

Fig. 3. *Differential identification of aldosterone binders in rat kidney*

Liver cytosol (4ml) was incubated with $1\mu\text{Ci}$ of [^{14}C]corticosterone, and kidney cytosol (3ml) with 10M -[^3H]aldosterone. After removal of free radioactivity, separately, the two preparations were mixed and eluted together by a gradient formed from 90ml each of 0.001M - and 0.02M -phosphate. All other details are as described in the legends to Figs. 1 and 2 and the text. ●, ^3H ; ○, ^{14}C ; ----, E_{280} .

1972), and (ii) it represents species of mineralo- and gluco-corticoid receptors in the kidney and the liver respectively. Further experiments are needed to ascertain whether components eluted in 0.001M - and 0.02M -phosphate are different species of glucocorticoid-specific proteins or aggregated forms of a more fundamental unit eluted at the higher molarity.

If the foregoing is true, it should be possible to demonstrate all the components in a single profile on a DEAE-cellulose column. Results in Fig. 3 show the three peaks (A, B, C) of [^3H]aldosterone (with kidney cytosol) eluted at 0.001 , 0.006 and 0.02M -phosphate respectively. This profile is less clear than in earlier experiments owing to greater volumes and longer elution times. The peak of [^{14}C]corticosterone (X) eluted between 0.04 and 0.06M -phosphate most probably represents residual transcortin in the liver, since it was also evident when kidney cytosol (equilibrated with 10nm -[^3H]aldosterone) or liver cytosol (equilibrated with 100nm -[^3H]corticosterone) was chromatographed together with [^{14}C]corticosterone bound to transcortin; owing to low specific radioactivity of the [^{14}C]glucocorticoid, the peak eluted in the 0.001M pre-wash was greatly diminished, and that in the 0.01 – 0.02M region was abolished. These results are consistent with the observation that a great abundance of both mineralo- and gluco-corticoid receptors is eluted in the 0.001M pre-wash under these conditions. Earlier attempts to retain all the receptors on DEAE- or CM-cellulose were unsuccessful (Snart *et al.*, 1972).

Chromatography on DEAE-Sephadex A-50 columns

Data in Fig. 4 show that radioactivity in the 0.015 – 0.018M -KCl region (peak A) was eluted both when kidney cytosol equilibrated with 100nm -[^3H]corticosterone (Fig. 4a) or liver cytosol in the presence of 20nm -[^3H]corticosterone (Fig. 4b) were separated on DEAE-Sephadex A-50 columns, but was absent when liver cytosol equilibrated with 20nm -[^3H]aldosterone was chromatographed on this resin. On the other hand, with kidney cytosol, this peak was evident with both 20nm -aldosterone (M. K. Agarwal, unpublished work) or 100nm -corticosterone (Fig. 4c), but not with 20nm -corticosterone (results not shown). It may represent the position where highly basic mineralo- or gluco-corticoid receptors would be eluted (by analogy with the 0.001M pre-wash on DEAE-cellulose DE-52).

Fig. 4 also shows that a peak (B) of radioactivity was eluted between 0.35 and 0.45M -KCl, both with kidney cytosol at 100nm -[^3H]corticosterone (Fig. 4a) and with liver cytosol at 20nm -[^3H]corticosterone (Fig. 4b) or -aldosterone (Fig. 4c). Thus both the mineralo- and the gluco-corticoid-specific receptors are eluted at nearly the same positions from DEAE-Sephadex A-50 (rather than exhibiting a clear separation as on DEAE-cellulose DE-52). This is in keeping with the known tendency of these proteins to disperse into monomers in the presence of high salt concentrations (Beato & Feigelson, 1972; Litwack *et al.*, 1973), and confirms earlier observations on kidney cytosol (Agarwal, 1975).

Serum transcortin (labelled with [^{14}C]corticosterone) was eluted as peak Y with 0.6M -KCl on DEAE-Sephadex A-50 (Fig. 4a). The limited binding (peak X) to liver cytosol of 20nm -[^3H]aldosterone (Fig. 4c), and a far greater labelling with corticosterone in both the liver (Fig. 4b) and the kidney (Fig. 4a), may represent transcortin trapped in the organ. These results are in good agreement with those on DEAE-cellulose DE-52 noted above.

Filtration through Sephadex G-200 columns

Data in Fig. 5(a) show two sharp peaks (A, B) of radioactivity when kidney cytosol equilibrated with 100nm -[^3H]aldosterone and liver cytosol labelled with [^{14}C]corticosterone were filtered together through Sephadex G-200. The peak of [^{14}C]corticosterone bound to liver cytosol was eluted somewhat later than the second [^3H]aldosterone peak, and this was confirmed in replicate experiments. However, neither peak appeared to be transcortin (Fig. 5b).

To estimate this more precisely, the Sephadex G-200 column was calibrated with markers of known molecular weight and the positions of the various corticoid-binding components were then compared; this could be accomplished with greater accuracy by following the effluent volumes since slight variations

Fig. 4. Separation of corticosteroid binders from rat liver and kidney

DEAE-Sephadex A-50 columns were prepared, packed and equilibrated as described in the Methods section. Kidney cytosol (3ml) was incubated with 100nM-[^3H]corticosterone and 2ml of blood serum with [^{14}C]corticosterone (Fig. 4a); liver cytosol (6ml) was treated with either 20nM-[^3H]corticosterone (Fig. 4b) or an equivalent amount of [^3H]aldosterone (Fig. 4c). After removal of free radioactivity with charcoal, the supernatant was loaded on the column and eluted with 150ml each of the initial buffer (0.05M-Tris/HCl, pH7.5) containing 0 (mixing chamber) or 1M- (reservoir) KCl. Co-chromatography (as described in the legend to Fig. 3) was performed for the experiment in Fig. 4a. Fractions were processed for determination of E_{280} (----) and of radioactivity. Further details are given in the text. ●, ^3H ; ○, ^{14}C .

in the flow characteristics were occasionally observed on this long thin Sephadex G-200 column. With the logarithmic correlation between the Stokes radii and the elution position (Fig. 5c), a mol.wt. of 63000 was calculated for transcortin (Fig. 5b), and this agrees well with values reported previously (Beato & Feigelson, 1972). The [^{14}C]corticosterone (Fig. 5a) seemed to be bound to a mixture of transcortin and the glucocorticoid receptor in the liver (peak X), in keeping with comments made in the section on chromatography on DEAE-cellulose, since the low specific radioactivity of this steroid makes it difficult to reveal either component clearly. In any event, the

[^3H]aldosterone appears to be bound to two proteins (mol.wts. 67000 and 113000), which may represent polymerized forms of the same molecule. Chromatography under various ionic concentrations is required to answer this unequivocally. The mol.wt. (67000) is in the same range as that reported for liver glucocorticoid receptors, where large aggregates of mol.wt. 200000 or more are known to form as a function of ionic strength (Snart *et al.*, 1972; Beato & Feigelson, 1972).

Attempts were made to distinguish between the gluco- and the mineralo-corticoid receptors in the kidney on Sephadex G-200 columns, and to compare

Fig. 5. Molecular filtration of aldosterone binders from rat liver and kidney

For Fig. 5(a), Sephadex G-200 column was packed and equilibrated as described previously (Agarwal *et al.*, 1970; Snart *et al.*, 1970, 1972; and the Methods section). Kidney cytosol and liver cytosol (2 ml of each) were incubated with 100 nM- $[^3\text{H}]$ -aldosterone and 1 μCi of $[^{14}\text{C}]$ corticosterone respectively. After removal of free radioactivity, separately, by the charcoal technique, the two supernatants were mixed and co-filtered through the resin, which was developed with 0.01 M-phosphate, pH 7.4, containing 0.1 M-NaCl. Fractions (1.1 ml) were collected, and the E_{280} (----) was recorded continuously. In replicate experiments, the free steroid was removed by passage through Bio-Gel P-10 columns, before molecular sieving through the Sephadex G-200, equilibrated with the same buffer. This enlarged the volumes such that the resolution was less sharp than presented here. $\bullet$, ^3H ; $\circ$, ^{14}C . For Fig. 5(b), blood serum (2 ml) was incubated in the presence of 1 μCi of $[^{14}\text{C}]$ -corticosterone and then loaded (without the charcoal treatment) on the Sephadex G-200 column. Fractions (1.6 ml) were collected, and 1 ml of each was processed for determination of radioactivity. All other details were as for Fig. 5(a). For calibration in Fig. 5(c), the Sephadex G-200 column was equilibrated by passage of macromolecules of known molecular weight. Immunoglobulin (IgG, 156 000), haemoglobin (Hb, 68 000), ovalbumin (Ovalb, 43 500) and cytochrome *c* (Cyt. *c*, 15 600) were passed through the Sephadex G-200 column as above. Their relative positions are marked according to the absorption maxima in the effluent fluid. The excluded and the total volumes were estimated with Dextran Blue 2000 and the free steroid respectively. The positions of the heavy (H) and the light (L) aldosterone binders in rat kidney (Fig. 5a) are indicated on this quantified column.

them with the glucocorticoid receptors in the liver. When liver cytosol was equilibrated with 100 nM- $[^3\text{H}]$ aldosterone, radioactivity was eluted in the 110 000–120 000- and 60 000–70 000-mol.wt. regions on Sephadex G-200 (Fig. 6), confirming the above stated possibility of similar molecular sizes of the gluco- and the mineralo-corticoid receptors. Accord-

ingly, competition studies were performed with kidney cytosol to assess binding as a function of steroid concentration.

In equimolar concentrations, non-radioactive corticosterone competed with 10 nM- $[^3\text{H}]$ aldosterone, resulting in the consequent diminution of the 67 000-mol.wt. peak in kidney cytosol (Fig. 7a). This would

Fig. 6. Analysis by gel filtration of aldosterone-binding proteins from rat liver

Liver cytosol (2ml) was incubated in the presence of 100nM-[³H]aldosterone and loaded (without charcoal treatment) on the Sephadex G-200 column. All other details are as described in the legend to Fig. 5.

suggest that most of the aldosterone receptors are present as higher-molecular-weight complexes at this near-physiological ionic strength and pH. In a reciprocal experiment, the presence of non-radioactive aldosterone essentially abolished the binding of 10nM-[³H]corticosterone to the high-molecular-weight peak (Fig. 7b). In additional studies, [³H]-corticosterone was largely bound to macromolecules in the 60000–70000-mol.wt. range at both 10 and 100nM; the radioactivity eluted in the higher-molecular-weight region was only 1/300 and 1/100 respectively that in the major peaks of low molecular weight (results not shown). These are in good agreement with the above results on DEAE-cellulose, since at 100nM the glucocorticoid begins to saturate macromolecules exhibiting preferential binding for aldosterone at 10nM, as well as many other proteins (Beato & Feigelson, 1972; Litwack *et al.*, 1973). Collectively, these data suggest that specific glucocorticoid-binding proteins most probably exist as low-molecular-weight entities at physiological ionic strength and pH.

Discussion

Contrary to the approach described in this paper, almost all other researchers have usually studied the equilibrium dissociation of steroids bound to crude cell preparations, in the presence of competing molecules. The Scatchard plots obtained can yield confusing information, since binding may occur to various proteins, or different conformations of a single protein, endowed with comparable affinity for the given steroid molecule. Dissimilar binding of a steroid to two distinct classes of macromolecules may also be observed. Erroneous estimations are further possible because charcoal may cause non-equilibrium

Fig. 7. Evidence that mineralocorticoid receptors are probably present as high-molecular-weight complexes in rat kidney

Kidney cytosol (2ml) was equilibrated with 10nM-[³H]-aldosterone in the presence of unlabelled 10nM-corticosterone, and then layered on the Sephadex G-200 column (Fig. 7a). For Fig. 7(b), 2ml of cytosol was equilibrated with 10nM-[³H]corticosterone, in the presence of an equimolar concentration of unlabelled aldosterone. All other details were as described in the legend to Fig. 5 and the text.

absorption of steroids, modification of the receptor molecules or transfer of binding components from the cytoplasm to the nucleus in the whole cell suspensions. The few attempts with centrifugation on sucrose gradients (Kaiser *et al.*, 1974) or molecular filtration through gels (Alberti & Sharp, 1969; Schaumburg, 1970) cannot differentiate between the various steroid-specific receptor proteins of similar molecular weights; the former procedure can also lead to steroid dissociation. Ion-exchange chromatography (Snart *et al.*, 1972; Agarwal, 1975) appears to be the method of choice provided that one does not use conditions (Beato & Feigelson, 1972; Litwack *et al.*, 1973) suboptimal in pH, concentration, or choice of ions or resin. Further, this procedure gave essentially similar results when the free steroid was removed by passage through Bio-Gel P-10 or Sephadex G-50 or by charcoal adsorption.

The results summarized in this paper provide the only available physical characterization of the

mineralocorticoid-specific 'MR' receptors present in rat kidney, but absent from the liver, that were apparently eluted in 0.006M-phosphate from DEAE-cellulose DE-52; these may or may not be identical with the high-affinity (K_d at 37°C, 5×10^{-10} M) 'type I' aldosterone-binding sites detected by Scatchard analysis (Feldman *et al.*, 1972). Detailed studies on corticosterone binders (K_d 10^{-10} M) in the liver (Agarwal *et al.*, 1970; Snart *et al.*, 1972) have shown that such glucocorticoid-specific 'GR' receptors from both liver and kidney are eluted between 0.01 and 0.02M-phosphate and are probably identical. It is not clear whether they can be equated with the 'type II' (K_d at 25°C, 5×10^{-9} M) binders in the kidney (Feldman *et al.*, 1972) the 'G' protein (Beato & Feigelson, 1972) or the binder II (K_d 6×10^{-10} M) from rat liver (Litwack *et al.*, 1973), all of which bind dexamethasone with high affinity. However, bound corticosterone eluted at around 0.06M-phosphate from DEAE-cellulose DE-52 appears to represent serum transcortin (Agarwal *et al.*, 1970; Snart *et al.*, 1972), the type III (K_d at 25°C, 3×10^{-9} M) receptors from the kidney (Feldman *et al.*, 1972) or the B proteins from rat liver (Beato & Feigelson, 1972), which is absent from hepatoma HTC cells (Litwack *et al.*, 1973).

The nature of macromolecules from both liver and kidney eluted in the low-ionic-strength pre-wash remains in doubt. In the kidney they are revealed exclusively by aldosterone at 10nM and in the liver by both aldosterone and corticosterone (at either 10 or 100nM). The overall resolution is actually better with aldosterone, since this steroid binds poorly to transcortin (Agarwal, 1975), but exerts glucocorticoid-like action (Feldman *et al.*, 1972). Thus these highly basic proteins could represent receptors adapted to organ-specific function and possibly different from those eluted at higher salt concentrations, at least in the case of the liver. Alternatively elution at low ionic strength suggests that they may be aggregated forms of a more fundamental unit eluted as a peak at higher ionic strength (approx. 0.006M- and 0.02M-phosphate for the mineralo- and the gluco-corticoid respectively). Failure to obtain clear separation by elution with high salt concentrations up to 1M-KCl on DEAE-Sepha-

dex A-50 and DEAE-cellulose DE-52 (M. K. Agarwal, unpublished work) supports the hypothesis of polymorphism and aggregation. Nevertheless, any one class of receptor molecules seems to exist in several forms, which escape detection by the conventional methods of equilibrium dialysis or Scatchard analysis.

It remains uncertain whether the mineralo- or the gluco-corticoid-binding molecules, which are similar in molecular size, represent different proteins or modified conformations of a common protein unit.

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