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Binding of aldosterone in the toad bladder

Studies have been made of the binding characteristics of aldosterone in the toad bladder by further use of the displaceable binding technique[1], and the binding of aldosterone to electrophoretically and chromatographically separated protein carried out as a preliminary determination of the nature of the binding sites.

All toads *(Bufo marinus)* used in this study were rapidly pithed and the bladders promptly removed after exsanguination. The epithelial layers of the bladders were removed by scraping and homogenised in a tight Potter–Elvehjem homogeniser. Small amounts of this homogenate were subject to agar gel electrophoresis[2] in 0.05 M veronal buffer, pH 8.6, for 25 min at 35 μA. The gels were fixed in 2% acetic acid, and stained with Sudan black after drying down on a microscope slide. The

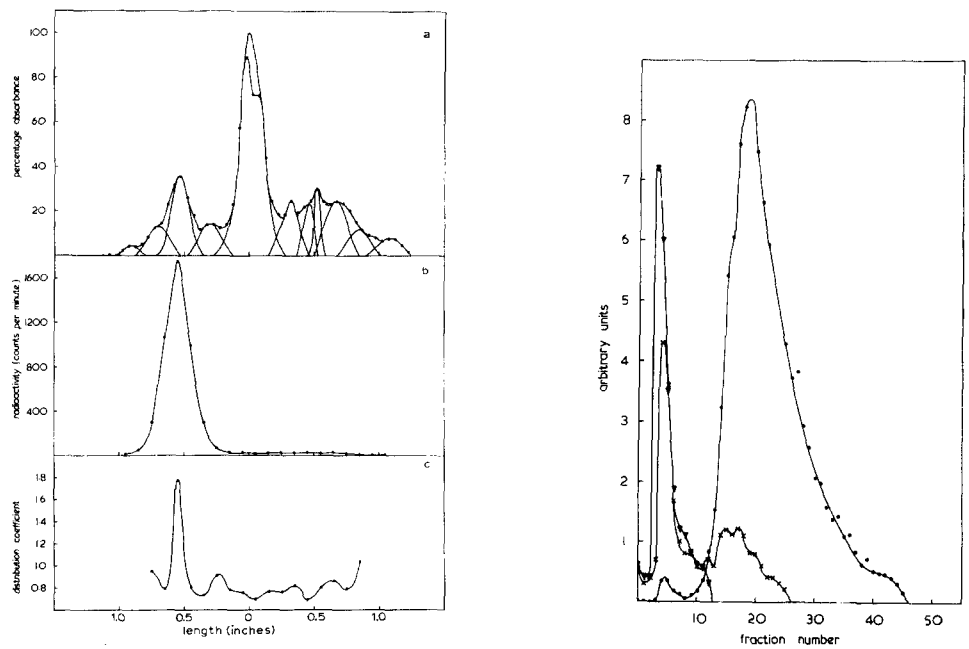

Fig. 1. a. Separation of toad bladder proteins by electrophoresis of homogenates on agar gel (0.05 M veronal buffer, pH 8.6). The gels were run for 25 min at 35 μA after which they were fixed in acetic acid, dried and stained with Sudan black. The reproducible peaks are the result of analysis of the absorption profiles from 6 separations. The left hand side represents the cathode. b. Separation of toad bladder homogenates incubated with [^{3}H]aldosterone in an electrophoretic run corresponding to (a). The gel was cut into 0.1-inch slices, digested and the radioactivity was counted in each section. c. Separation of toad bladder homogenates in an electrophoretic system corresponding to (a), followed by equilibrium dialysis of 0.1-inch slices in Visking dialysis tubing against [^{3}H]aldosterone. The resulting distribution coefficients were determined as the ratio of the number of counts inside to the number of counts outside for 1-ml quantities of solution.

Fig. 2. Chromatographic separation of toad bladder homogenates incubated with [^{3}H]aldosterone, on a Biogel-100 column using neutral 0.05 M phosphate buffer for elution. The protein and nucleic acid peaks (corresponding to 0.720 mg/ml and 0.040 mg/ml, respectively) were identified using a nomograph after spectral examination of the eluted fractions. Some labelled aldosterone is associated with the protein fraction. ●—●, radioactivity; ×—×, nucleic acid; ▼—▼, protein.

density of staining was examined using a Zeiss spectrophotometer in order to determine the characteristic protein bands (Fig. 1a).

Homogenate containing [^{3}H]aldosterone was subject to electrophoresis in the same way. The gel was then cut into 0.1-inch slices and the radioactivity in each slice counted after digestion with conc. HCl. A peak of activity (Fig. 1b) was found on the cathode side. Electroosmosis was excluded by running [^{3}H]aldosterone alone in the gel, after which no peak of activity was detected.

Homogenate without added aldosterone was electrophoretically separated. The gel was cut into 0.1-inch slices, each of which was added to 1 ml water in $^8/_{32}$ inch Visking dialysis tube. These were dialysed overnight against 40-ml quantities of [^{3}H]aldosterone in water (1 μC/l). The gels dissolve and distribution coefficients were determined as the ratio of the number of counts in 1 ml inside to 1 ml outside solution (Fig. 1c). One fraction of gel gives a significantly large distribution of aldosterone and corresponds to that observed in the previous experiment.

An homogenate containing [^{3}H]aldosterone was run through a Biogel-100 column using a neutral 0.05 M phosphate buffer. The protein and nucleic acid peaks (Fig. 2) were identified using a nomograph after spectral examination of the eluted fractions. The radioactivity in each fraction was also measured and some activity is seen to be associated with a protein fraction whereas most comes off as free steroid. These observations have been repeated and confirmed.

The analysis of a SCATCHARD plot[3] for the binding of cortisol to plasma has been described by SANDBERG *et al.*[4], giving characteristic association constants for the specific cortisol-binding protein, transcortin[5] and for the non-specific albumin binding[6]. However, a displaceable binding technique can be used when there is a large set of sites of low affinity that will provide a constant distribution of steroid over a reasonably large concentration range. It is then possible to chase bound, labelled steroid without displacing steroid from this larger set of sites. Separate SCATCHARD plots can thus be obtained for the remaining set of sites. The following procedures

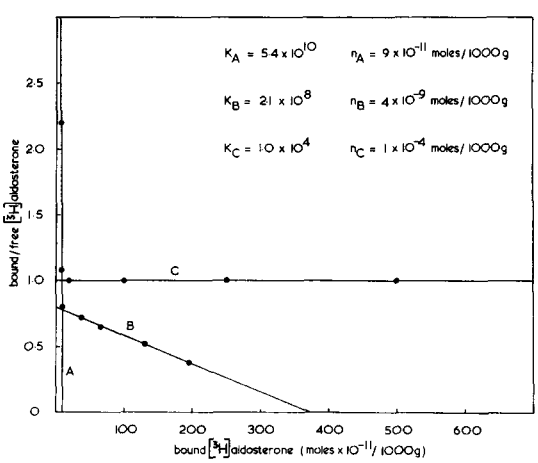

Fig. 3. The binding characteristic of aldosterone in the toad bladder. Points on this graph have been obtained following the method used by SHARP, KOMACK AND LEAF[1]. This graph shows three sets of binding sites with their corresponding association constants and concentration within the tissue.

have therefore been followed. For each concentration of steroid (in the range 10^{-11}–10^{-7} M) 12 half bladders were excised. Each half bladder was cut into 4 smaller pieces. These were paired and one group incubated for 90 min in [^{3}H]aldosterone made up in toad Ringer[1], the other groups were incubated in the same concentration of [^{3}H]aldosterone *plus* a thousand-fold excess of non-radioactive aldosterone for the same period. The pieces of bladder were removed, rinsed in fresh Ringer, dried and weighed. They were then placed in counting vials to which were added 10 ml chloroform–methanol (2:1, by vol.) mixture containing 10^{-4} M deoxycorticosterone, the presence of which increases the efficiency of counting and helps to avoid variability due to adsorption on the sides of the vials, and the steroid extracted from the tissue over a period of 4 h. The tissue was removed and the solvent evaporated off. 10 ml toluene scintillation fluid was added to each vial and counting carried out in a Nuclear-Chicago instrument. The results could then be converted to give values for the bound/free [^{3}H]aldosterone for each set of sites and so obtain the graph (Fig. 3), analysis of which shows that the binding sites may be characterised by three association constants $K_A = 5.4 \cdot 10^{10}$, $K_B = 2.1 \cdot 10^8$, and $K_C = 1.0 \cdot 10^4$ l/mole which correspond to free energies of association ΔG_A −14.4 kcal/mole, ΔG_B −11.2 kcal/mole and ΔG_C −5.4 kcal/mole.

Non-specific adsorption of steroid to protein has been shown[7] to have an association constant of the same order as K_C which is too weak to carry bound steroid through a gel or column. It is therefore supposed that the activity in the electrophoretic and chromatographic systems is moving with a high molecular weight protein possibly corresponding to the second set of binding sites $2.1 \cdot 10^8$ l/mole. This association constant may be compared with that reported for transcortin with cortisol[8–10]. It is therefore considered possible that the active binding sites of the tissue (saturating to the physiologically active range) may correspond to a high molecular weight protein.

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