Action of Adrenocorticotrophin on a Preparation of Isolated Cells from Rat Adrenals

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Several methods have been described for the preparation of adrenal cell suspensions (Kloppenborg, Island, Liddle, Michelakis & Nicholson, 1968; Swallow & Sayers, 1969; Halkerston & Feinstein, 1968). We now report results obtained by a modification of the method proposed by Kloppenborg *et al.* (1968).

Decapsulated glands were quartered and digested with collagenase (Sigma type I; 5 mg/ml) in Krebs-Ringer bicarbonate buffer, pH 7.4 containing glucose (0.2%) and albumin (3%, w/v). The preparation was filtered through nylon gauze (mesh 61 μ m) and washed twice by centrifugation (5 min at 200g) and resuspension. The equivalent of one adrenal yielded 600 000–1 000 000 cells. After incubation corticosterone content of cells plus medium was assayed by a specific and sensitive protein-binding technique adapted to assay corticosterone over the range 0–10 ng. With mouse serum (1%) as the binding protein source and correction for incomplete removal of unbound corticosterone by the adsorbing agent (Florisil), a linear relationship was obtained between unbound/bound steroid and total steroid (Schulster, Tait, Tait & Mrotek, 1970).

Before incubation each sample (1 ml, equivalent to the cells derived from one gland) contained 27.7 ± 3.2 ng of corticosterone (mean $\pm$ S.E.M. of seven experiments); in the absence of adrenocorticotrophin the value obtained after incubation for 1 h was 36.3 ± 7.0 ng of corticosterone (mean $\pm$ S.E.M. for six experiments). With increasing amounts of adrenocorticotrophin (Third International Standard: Bangham, Mussett & Stacke-Dunne, 1962), 1 h incubations resulted in a sigmoid log (dosage)–response curve. The minimum effective dose, ED₅₀ and the minimum dose for maximum response (25–60 ng/gland per min) were 10^{-6} , 10^{-4} and 10^{-3} i.u./ml respectively. The log (response) was proportional to log (dose of adrenocorticotrophin) in the range 10^{-6} – 10^{-3} i.u./ml (index of precision 0.13).

Previous experiments *in vivo* have shown that adrenocorticotrophin achieves its maximum steroidogenic response within 10 min (Garren, Ney & Davis, 1965), whereas a longer time-period (30 min) has been reported for incubations *in vitro* of cell suspensions prepared by using trypsin (Kitabchi, Thompson & West, 1970). Our preparations, however, showed a rapid steroidogenic response to adrenocorticotrophin (2×10^{-2} i.u./ml);

corticosterone production rate became constant certainly within 10 min after adrenocorticotrophin addition.

The rapidity, sensitivity and magnitude (up to 300-fold stimulation over basal production) of the response elicited by adrenocorticotrophin make the adrenal-cell suspension a useful system for studying the biochemistry of the steroidogenic response.

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Ribonucleic Acid Polymerase from Rat Prostate Nuclei and Nucleoli

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Much insight has been gained recently into the metabolism and binding of testosterone and dihydrotestosterone within rat prostate cells. It would appear that one result of these activities could be to bring about both qualitative and quantitative changes in RNA synthesis. Consequently studies were initiated on the solubilized RNA polymerase(s) of rat prostate gland. RNA polymerase enzyme was extracted by a modified version of the method developed originally by Roeder & Rutter (1969) for extraction of RNA polymerase activity from sea-urchin oocyte nuclei and rat liver nuclei. The method involves solubilization of the enzyme activity from purified nuclei by ultrasonication at high ionic strength, decrease of the ionic strength and removal of chromatin by centrifugation, followed by ammonium sulphate fractionation.

Enzyme activity isolated by this method from rat prostate nuclei showed it to be dependent on added DNA, required all four ribonucleoside triphosphates and was greater in the presence of Mn²⁺ and denatured DNA than with Mg²⁺ and native DNA. On 15–30% glycerol gradients the

activity sedimented at about 20S, a value similar to that reported by Seifart & Sekeris (1969) for rat liver soluble enzyme but at variance with that reported by Liao, Sagher, Lin & Fang (1969) of 13–16S for rat prostatic enzyme prepared by a different method than the one used here. However, after overnight dialysis the activity sedimented in both low-ionic-strength and high-ionic-strength glycerol gradients at about 13S.

Enzyme isolated from animals 24 h after bilateral orchidectomy showed a much diminished Mg^{2+} -dependent activity with both native and denatured DNA.

When subjected to DEAE-cellulose column chromatography (Whatman DE52) the activity isolated from normal and castrated animals separated into two distinct peaks. Chromatography of nucleolar enzyme revealed only one peak. This activity had a sedimentation coefficient of about 13S when analysed on 15–30% glycerol gradients.

The toxin α -amanitin, reported to selectively inhibit one of two DNA-dependent RNA polymerase activities from calf thymus (Kedinger, Gniazdowski, Mandel, Gissinger & Chambon, 1970), was found to inhibit the RNA polymerase activity of rat prostate nuclei when assayed in the presence of Mn^{2+} and 0.3M-ammonium sulphate but not in the presence of Mg^{2+} alone.

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Fluorescence Technique for Studying the Binding of Sulphonamide and other Inhibitors to Carbonic Anhydrase

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Bovine carbonic anhydrase shows fluorescence due to the seven tryptophan residues present in the molecule. Some sulphonamide inhibitors quench this fluorescence when they combine with the enzyme. In particular, hydrochlorothiazide gives 58% quenching, chlorothiazide 70%, benzthiazide 84% and ethoxzolamide 58%. Equilibrium constants for the binding of the first two of these inhibitors can be determined from the degree of

quenching produced by various concentrations of added inhibitor. This direct method does not work with the other two, which have such high affinities for the enzyme that substantially all added inhibitor is bound if any binding sites remain unoccupied. However, an indirect method is available, not only for these sulphonamides, but for others that do not quench the fluorescence and for inhibitors like cyanide that compete with sulphonamides.

When a non-quenching inhibitor is added to a solution of enzyme that contains sufficient quenching inhibitor to give maximal quenching, the quenching is reversed if the first inhibitor displaces the second. Fluorescence measurements in such systems allow the relative affinity of the enzyme for the two inhibitors to be determined. If the dissociation constant for the binding of one is known, then that of the other may be calculated.

Dissociation constants for seven of the eight sulphonamides studied by Maren, Parcell & Malik (1960) were determined at 25°C in 20mM-phosphate buffer, pH 7.5. The constants, expressed in pK_i units, found by the direct method were 5.76 for hydrochlorothiazide and 7.03 for chlorothiazide. Acetazolamide and methazolamide, neither of which quenched, both gave values of 8.12, determined by displacement of chlorothiazide. Benzthiazide and ethoxzolamide gave values of 8.85 and 9.70 respectively in competition with acetazolamide or methazolamide. Dichlorphenamide, which did not quench, gave a value of 9.02 when displacing benzthiazide or ethoxzolamide. The technique was unsuitable for the eighth inhibitor, sulphanilamide, which showed rapid photodecomposition in the spectrofluorometer cell and is itself fluorescent.

Identity of dissociation constants with K_i values was found under conditions where both fluorescence and inhibition measurements could be made. However, the fluorescence technique is much more rapid and convenient and less limited in the range of physicochemical conditions that it allows.

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