STEROID BIOCHEMISTRY GROUP COLLOQUIUM ON 'THE ACTION OF ADRENOCORTICOTROPHIN'

The Location of Adrenocorticotrophin Receptors in the Adrenal Cortex

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Significant changes are seen in the cells of the human adrenal cortex within 72h of ACTH‡ administration (Symington *et al.*, 1956). The clear cells of the zona fasciculata acquire the morphological characteristics of the compact cells of the zona reticularis (Grant *et al.*, 1957). Griffiths & Glick (1966) showed that 11 β -hydroxylase activity in the rat adrenal was stimulated by ACTH treatment, the increase in enzyme activity being marked at the border between the zona fasciculata and the zona reticularis. This area has therefore been considered as a major site of ACTH action in the cortex (Symington, 1969). Taunton *et al.* (1967a) showed that the first step in ACTH action, both in fat-cells and in adrenals, may be the fixation of the hormone at specific receptor sites on the plasma membrane. The addition of ACTH *in vitro* to adrenal tissue caused an accumulation of cyclic AMP (adenosine 3':5'-cyclic monophosphate) in the adrenal cortex (Haynes, 1958). This effect has also been observed *in vivo* (Grahame-Smith *et al.*, 1967). Stimulation of adenylate cyclase by ACTH seems to be responsible for the accumulation of adrenal cyclic AMP (Grahame-Smith *et al.*, 1967; Taunton *et al.*, 1967b; Lefkowitz *et al.*, 1970).

Lefkowitz *et al.* (1970) demonstrated that membrane preparations from the adrenal cortex will bind ¹²⁵I-labelled ACTH and that the binding is associated with stimulation of adenylate cyclase activity. Sonenberg *et al.* (1951) and Cats & Kassenaar (1957) found that after the injection of ¹³¹I-labelled ACTH into rats the liver and kidney accumulated the greatest concentrations of radioactivity. In these studies relatively large doses of crude preparations of ACTH were employed, but the findings have subsequently been confirmed by the use of 'physiological' doses of ¹³¹I-labelled α -ACTH-(1-24)-tetracosapeptide (Golder & Boyns, 1971). The distribution of radioactivity in serial sections of the adrenal cortex suggested a preferential concentration of iodinated hormone by glomerular and fascicular border tissue and the zona reticularis (Golder & Boyns, 1971). Lowering of the concentration of endogenous ACTH by dexamethasone treatment significantly increased uptake of radioactivity in the border region and in the zona reticularis (Golder & Boyns, 1972). These studies were performed with preparations of

¹³¹I-labelled α -ACTH-(1-24)-tetracosapeptide that, although immunologically active, were of doubtful biological activity.

There is a close structural relationship between the binding site for protein hormones and the enzyme adenylate cyclase (Robison *et al.*, 1969). Therefore we have attempted to determine the distribution of ACTH-sensitive adenylate cyclase activity in the guinea-pig adrenal cortex as an index of the location of ACTH receptors. Adenylate cyclase was assayed by measuring the conversion of [α -³²P]ATP into cyclic [³²P]AMP in whole homogenates and serial sections of the guinea-pig adrenal at 37°C (Taunton *et al.*, 1967b). The cyclic AMP formed was separated from other nucleotides by a batch method that allowed the processing of large numbers of samples. α -ACTH-(1-24)-tetracosapeptide, porcine ACTH, human luteinizing hormone and human growth hormone stimulated adenylate cyclase activity in whole adrenal homogenate, whereas prolactin had no effect. Frozen glands were sectioned by the method of Grunbaum *et al.* (1956) and enzyme activity was measured in each of the cortical zones. Adenylate cyclase activity was detectable throughout the gland. The addition of α -ACTH-(1-24)-tetracosapeptide *in vitro* enhanced enzyme activity in all zones and to a significant extent only in the glomerular-fascicular border region and in reticular tissue. Comparable findings were obtained in adrenals from animals that had been injected with α -ACTH-(1-24)-tetracosapeptide before removal of the adrenal for assay.

ACTH-stimulated adenylate cyclase was also studied in adrenals of hypophysectomized rats. Enzyme activity was undetectable 48h after hypophysectomy. However, the addition of ACTH *in vitro* enhanced enzyme activity. The sensitive regions were again the glomerular-fascicular border region and in the reticular zone. These results provide further evidence that receptors for ACTH are located in specific areas of the adrenal cortex.

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‡ Abbreviation: ACTH, adrenocorticotrophin.

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The Mode of Adrenocorticotrophin Action in Stimulating Steroidogenesis: the Obligatory Role of Adenosine 3':5'-Cyclic Monophosphate and the Involvement of Rapidly-Turning-Over Protein

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Current concepts for the complicated mechanism whereby ACTH* stimulates corticosteroidogenesis involve the following aspects: ACTH binding at cell-surface receptors, activation of adenylate cyclase and increased production of intracellular cyclic AMP (adenosine 3':5'-cyclic monophosphate), activation of protein kinases with increased phosphorylation of (ribosomal?) protein, induction of labile protein(s) implicated in the rate-limiting step of steroidogenesis and intracellular translocation of steroid intermediates that influence cholesterol conversion into pregnenolone (3β -hydroxypregn-5-en-20-one) (Garren *et al.*, 1971). Two of these aspects, namely the role of cyclic AMP and the involvement of rapidly-turning-over protein, are the subject of this report.

The cyclic AMP content of collagenase-disaggregated rat adrenal cells (Richardson & Schulster, 1970) incubated with ACTH was assayed after Dowex chromatography (Montague & Cook, 1971) by a modification of the saturation-analysis procedure of Brown *et al.* (1971). A sigmoid log(dosage)-response curve was obtained (ED_{50} 5×10^{-3} i.u. of ACTH/ml) that was steepest over the range 5×10^{-4} – 10^{-2} i.u. of ACTH/ml; over this range a 13-fold increase in cyclic AMP (83% of maximum response) was apparent. By contrast, in the identical incubation samples, increasing ACTH concentrations from 10^{-5} to 10^{-3} i.u./ml led to the greatest change in corticosterone content (about 36-fold stimulation) where-

as higher ACTH concentrations elicited no greater corticosteroidogenic response. The ED_{50} for this sigmoid log(dosage)-response curve was 10^{-4} i.u. of ACTH/ml. Results from three experiments showed the relationship between log([ACTH]) and log([cyclic AMP]) to be linear over the entire range 10^{-5} – 10^{-2} i.u. of ACTH/ml; the lowest concentration that appreciably stimulated cyclic AMP concentrations (approx. 3-fold) was 5×10^{-5} i.u. of ACTH/ml. Since doses of ACTH that stimulated steroidogenesis were associated with increases in cyclic AMP and the two effects were not separable, the findings are consistent with an intermediary role for cyclic AMP in the ACTH-stimulated steroidogenic mechanism. However, there appears to be a vast adrenal reserve capacity for cyclic AMP production, and only a small fraction of the cells' potential to synthesize cyclic AMP need be activated in order to achieve maximum steroidogenesis. Thus at 10^{-3} i.u. of ACTH/ml (maximal for steroidogenesis) cyclic AMP concentrations were only 12% of maximum. This finding, obtained with cell suspensions, correlates well with that obtained *in vivo*, but differs markedly from that observed *in vitro* with adrenal quarters, where high concentrations of ACTH (0.5–1.0 i.u./ml) were found to be necessary to maximize steroidogenesis (Grahame-Smith *et al.*, 1967). This may be attributed to the relative inaccessibility of medium components to cells within the tissue of quartered glands, compared with isolated cells or perfused glands *in vivo*, where medium or blood components are readily available to each cell.

The effect of exogenously added cyclic AMP also implies an intermediary role for this nucleotide. Similar sigmoid log(dosage)-response characteristics were observed for corticosteroidogenesis in isolated cells stimulated by ACTH or cyclic AMP. Moreover graphs of log([ACTH]) against log([corticosterone]) and log([cyclic AMP added]) against log([corticosterone]) both exhibited linear relationships over the ranges 10^{-7} – 10^{-3} i.u. of ACTH/ml and 0.5–5 mM cyclic AMP (ED_{50} 3 mM).

Studies *in vitro*, with adrenal fragments to ascertain the time-lag between ACTH addition and onset of stimulated steroidogenesis (Grahame-Smith *et al.*, 1967), were complicated by the time required for ACTH to penetrate the tissue mass and for corticoid to diffuse out. Use of isolated cell suspensions eliminates these problems, allowing immediate access of added stimulants to each cell. However, despite these conditions increased corticoid production was undetectable until 3 min after ACTH addition (10^{-3} i.u./ml). A similar time-lag after ACTH infusion has been observed during perfusions *in vivo* (Urquhart & Li, 1968; Beaven *et al.*, 1964). Further, a 2–3 min time-lag followed exogenous cyclic AMP (5 mM) addition before steroidogenic stimulation was detectable, thus making it improbable that this is the time

* Abbreviation: ACTH, adrenocorticotrophin.