required for accumulation within the cell of sufficient cyclic AMP to actuate the steroidogenic machinery. This was further militated against by kinetic measurements of cyclic AMP production by isolated cells, which showed that the cyclic AMP concentration had more than doubled within 1 min of the addition of ACTH (10^{-2} i.u./ml) and within 3 min had increased 5-fold above basal value. It is suggested that a considerable portion of this 3 min period is required for the accomplishment of processes [e.g. synthesis of specific protein(s)] initiated by cyclic AMP. Since the average transit time for translation of mRNA in some eukaryotic cells has been assessed at 1–2 min (Fan & Penman, 1970; Vaughan *et al.*, 1971), this suggestion is consistent with current concepts of ACTH acting via translational control of protein synthesis (Garren *et al.*, 1971).

Further studies on the kinetics of the steroidogenic response utilized cycloheximide, which at high concentration inhibits protein synthesis by stopping movement of ribosomes along the mRNA strand. At low concentrations the antibiotic results in a decreased rate of translation. In the presence of 10^{-2} i.u. of ACTH/ml, cycloheximide (0.1, 1, 10 and 100 μ g/ml) inhibited protein synthesis by 23, 74, 88 and 94% respectively, and also inhibited steroidogenesis by 23, 66, 89 and 96% respectively. This result showed an ED_{50} of about 0.28 μ g of cycloheximide/ml (i.e. 1 μ M), and this dose was used to investigate further the time-lag before onset of ACTH-stimulated steroidogenesis. It was found that cycloheximide, at a concentration that halved the protein synthesis rate, concomitantly doubled the time-lag before manifestation of the ACTH response. It is concluded that, if indeed there is a role for induced protein in the steroidogenic effect of ACTH, then it plays a direct or obligatory role. No such prolongation of the time-lag would be occasioned if some species of rapidly-turning-over protein had simply a permissive involvement. Further support for this conclusion derives from dynamic superfusion studies with adrenal bisects, where infusion of cycloheximide simultaneous with ACTH infusion allowed no discernible stimulation of steroidogenesis (Schulster *et al.*, 1970).

Steroidogenesis was observed to reach its maximum rate 6 min after ACTH addition to isolated adrenal cells, and this rate remained constant for at least 1 h. When cycloheximide (1 μ g/ml) was added 6 min after the ACTH addition, the capacity for steroidogenesis rapidly decayed, and the decay half-life for the labile protein, invoked as an intermediary in ACTH steroidogenic action, was calculated (Hartwell & Margasanik, 1963) as 2–4 min. This compares with the decay half-life of 7–10 min reported by Garren *et al.* (1965) with the perfused rat adrenal *in vivo* and 45–49 min for the superfused adrenal bisect *in vitro* (Schulster *et al.*, 1970).

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The Effect of Adrenocorticotrophin *in vivo* on Pregnenolone Synthesis by Adrenal Mitochondria

By LAVELL R. JOHNSON and DON H. NELSON
(Departments of Medicine, University of Utah and Latter-day Saints Hospital, Salt Lake City, Utah 84103, U.S.A.)

Administration of 5×10^{-3} i.u. of ACTH* intravenously to 3 h-hypophysectomized female rats promoted a fourfold increase in the rate of pregnenolone (3β -hydroxypregn-5-en-20-one) synthesis from endogenous substrates. The rate of pregnenolone synthesis in mitochondria from the ACTH-treated animals was 0.4 nmol/min per mg of protein with 5 mM-malate as electron donor. When 1% bovine serum albumin (fatty acid-free) was included in the mitochondrial incubation solution, 75% of the pregnenolone was found in the supernatant from mitochondrial preparations from both the control and the ACTH-treated animals. This finding suggests that ACTH did not alter the percentage of pregnenolone passing through the mitochondrial membrane. The stimulating effect of ACTH on pregnenolone synthesis was also observed when 5 mM- β -hydroxybutyrate, 5 mM- α -oxoglutarate and 5 mM-isocitrate were used as electron donors. The generation of reducing equivalents for pregnenolone synthesis from endogenous cholesterol was not affected by the respiratory-chain blockers cyanide and rotenone. The acute ACTH effect seen in these studies appears to be stimulation of a component of the hydroxylation pathway, as it occurs with

* Abbreviation: ACTH, adrenocorticotrophin.

both NAD- and NADP-linked dehydrogenases utilizing a number of different substrates. The effect was lost when the mitochondria were sonicated and pregnenolone synthesis was supported by either NADPH or isocitrate and NADP⁺. In these sonicated mitochondria, pregnenolone synthesis was equivalent to that of whole mitochondria from ACTH-treated animals. The fact that the control sonicated mitochondria produced pregnenolone at the same rate as the non-sonicated mitochondria from ACTH-treated animals suggests the possible release of an inhibition of the desmolase activity after mitochondrial disruption.

Factors Affecting Cholesterol Esterase and Cholesterol Side-Chain-Cleavage Activities in Rat Adrenal

By E. R. SIMPSON, W. H. TRZECIAK, J. L. MCCARTHY, C. R. JEFCOATE and G. S. BOYD (*Department of Biochemistry, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.*)

ACTH* interacts with adrenal-cortical cells to produce a number of well-defined responses leading to increased steroidogenesis. Stone & Hechter (1954) deduced that ACTH produced an activation of the first step in steroidogenesis, namely the conversion of cholesterol into pregnenolone (3 β -hydroxypreg-5-en-20-one). This response to ACTH is blocked by the inhibitors of protein biosynthesis puromycin and cycloheximide (Ferguson, 1963; Garren *et al.*, 1965).

In rat adrenal cortex cholesterol constitutes a large part of the intracellular lipid droplets where a major portion of the cholesterol is esterified with long-chain fatty acids (Moses *et al.*, 1969). The cholesteryl ester 'pool' may be an important regulatory factor for steroidogenesis as the reservoir supplying precursor, free cholesterol, for side-chain cleavage. Garren *et al.* (1965) found that injection of ACTH into hypophysectomized rats produced a decrease in adrenal cholesterol content and an increase in steroid output. When cycloheximide was administered before ACTH, steroidogenesis was inhibited, although cholesteryl ester hydrolysis was unaffected and free cholesterol accumulated within the lipid droplets (Garren *et al.*, 1971).

Adrenal responses to ACTH are believed to be mediated by cyclic AMP (adenosine 3':5'-cyclic monophosphate), the intracellular concentrations of which are raised by ACTH (Haynes & Berthet, 1957). Although the steps between increased cyclic AMP formation and increased steroidogenesis are not fully understood, it is known that cyclic AMP activates protein kinase in the adrenal cortex by binding to an

inhibitory receptor protein (Gill & Garren, 1971). The 6-*N*,2'-*O*-dibutyryl derivative of cyclic AMP has been shown to substitute for ACTH in activating the hydrolysis of cholesteryl esters in the perfused rat adrenal gland (Davis, 1969), but so far the effect of cyclic AMP on cholesterol esterase activity has not been studied in cell-free systems from the adrenal cortex.

The aim of the present report was to study the influence of ACTH and cyclic AMP on the cytosol cholesterol esterase and mitochondrial cholesterol side-chain-cleavage activities in rat adrenal cortex. The experiments were performed in control (quiescent) rats, in animals in which adrenal steroidogenesis was stimulated by ether stress (known to elevate plasma ACTH concentrations; Matsuyama *et al.*, 1971), in cycloheximide-treated rats and in animals injected with cycloheximide followed by ether stress.

The activity of cholesterol esterase (sterol ester hydrolase, EC 3.1.1.13) of rat adrenal-cortex 105000g supernatant was studied by following the release of free cholesterol from labelled cholesteryl oleate (Chen & Morin, 1971). The ester hydrolase was stimulated by 1 μ M concentrations of cyclic AMP in the presence of Mg²⁺, ATP and theophylline.

The specific activity of cholesterol esterase was significantly higher in stressed rats than in control and cycloheximide-treated animals. This suggests that in stressed animals the native adrenal-cortical cytosol cholesterol esterase has been already stimulated by stressful conditions, which are known to increase the intracellular cyclic AMP concentration (Matsuyama *et al.*, 1971). Prior cycloheximide injection did not prevent cholesterol esterase from being stimulated *in vitro* by cyclic AMP, and this confirmed previous observations (Davis & Garren, 1966) that stimulation of cholesteryl ester hydrolysis does not require protein synthesis (Davis & Garren, 1968).

The activating effect of cyclic AMP was further enhanced by the presence of ATP, which implies that the activation of cholesterol esterase by cyclic AMP requires a protein factor, presumably a protein kinase, which might phosphorylate a protein or an enzyme such as cholesterol esterase. A similar mechanism of activation of a hormone-sensitive adipose-tissue lipase by cyclic AMP has been proposed (Huttunen *et al.*, 1970).

Cholesterol esterase activation by cyclic-AMP-dependent protein kinase might provide an ACTH-induced mechanism of free cholesterol supply for the mitochondrial 'side-chain-cleavage' system.

Cholesterol side-chain cleavage is a mitochondrial mixed-function oxidase reaction that requires cytochrome *P*-450 as the terminal oxidase (Simpson & Boyd, 1967). Steroid 11 β -hydroxylase also occurs in the mitochondria, and work in this laboratory has shown that the cytochrome *P*-450 fraction catalysing cholesterol side-chain cleavage can be separated from

* Abbreviation: ACTH, adrenocorticotrophin.