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

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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.

11 β -hydroxylase cytochrome *P*-450 (Jefcoate *et al.*, 1970).

Cholesterol side-chain cleavage was studied in adrenal mitochondria from rats subjected to the conditions described above. It was shown that pregnenolone formation from endogenous mitochondrial cholesterol followed a biphasic time-course, with an initial rapid rate of formation followed by a much slower phase (Boyd *et al.*, 1971). The effect of stress was to increase the pregnenolone formation 2–3-fold compared with that in cycloheximide-treated or quiescent controls, particularly in the initial rapid phase.

The work of Allmann *et al.* (1970) has pointed to the configurational state of the mitochondria as being a determinant in controlling the rate of steroidogenesis compared with oxidative phosphorylation. Changes in the configuration of the mitochondria are induced by Ca²⁺, Mg²⁺ and free fatty acids. For this reason, these substances were measured in adrenal mitochondria from stressed rats and cycloheximide-treated rats. Although the concentrations of Ca²⁺, Mg²⁺ and free fatty acids were high in rat adrenal mitochondria, there was no significant difference in their concentrations in the adrenal mitochondria of the two groups of rats.

Mitochondria incubated in the standard medium, consisting of 250 mM-sucrose–20 mM-KCl–15 mM-triethanolamine–HCl–10 mM-potassium phosphate–5 mM-MgCl₂, pH 7.2, exhibited no respiratory control. Coupling could be induced in the presence of 0.5% bovine serum albumin and 0.1 mM-EDTA, but only in the absence of Mg²⁺. This finding presumably reflects removal of Ca²⁺ and free fatty acids. Again there was no difference in respiratory-control ratios between mitochondria from stressed and cycloheximide-treated rats.

Cholesterol side-chain cleavage was found to be stimulated by Mg²⁺ and further stimulated by bovine serum albumin and EDTA, whereas Ca²⁺ (4 mM) inhibited side-chain cleavage. Thus there does not appear to be a simple relationship between side-chain cleavage activity and respiratory coupling ability.

Pregnenolone binds to side-chain-cleavage cytochrome *P*-450 to give a type II difference spectrum (Jefcoate & Boyd, 1971). This is believed to be due to displacement of cholesterol from the cytochrome *P*-450 (Harding *et al.*, 1970). The effect of stress pretreatment was to increase the pregnenolone-binding spectrum 2–3-fold compared with cycloheximide treatment (Boyd *et al.*, 1971). This spectral change has been interpreted to mean that the action of stress, mediated by ACTH, is to increase the amount of cholesterol bound in an active high-spin complex to the side-chain-cleavage cytochrome *P*-450. Since the total mitochondrial cholesterol does not change as a result of the stress pretreatment, it is believed that the action of ACTH results in a redistribution of

cholesterol within the mitochondria, such that more is associated with cytochrome *P*-450 and thus is available for side-chain cleavage.

Kan & Ungar (1972) have reported the presence of a cholesterol-binding protein in heated extracts of acetone-dried bovine adrenal mitochondria. This factor could stimulate cholesterol side-chain cleavage in acetone-dried bovine adrenal mitochondria. In preliminary experiments we have confirmed that this factor can stimulate side-chain cleavage in intact adrenal mitochondria from cycloheximide-treated rats. Work is in progress to test various hypotheses about the possible nature and function of the cycloheximide-sensitive component involved in the rate-limiting step in cholesterol side-chain cleavage in adrenal-cortical tissue.

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