STIMULATION OF STEROID TRANSFORMATIONS IN ADRENAL MITOCHONDRIA BY CYCLIC 3',5'-ADENOSINE PHOSPHATE

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Recent investigations have revealed that cyclic 3',5'-adenosine phosphate (3',5'-AMP) stimulates steroid C-11β and C-18 hydroxylase activities in rat adrenal homogenates (Roberts, Creange and Fowler, 1964; Creange and Roberts, 1966). This effect was not dependent upon enhanced glycogen phosphorylation, increased NADPH generation, or the availability of endogenous precursors (Creange and Roberts, 1965; Creange and Roberts, 1966). Stimulation of C-11β hydroxylase activity by the cyclic nucleotide was also observed in purified rat adrenal mitochondria fortified with NADPH (Roberts, Creange and Young, 1965). The present investigations demonstrate that 3',5'-AMP enhances additional steroid conversions in mitochondria, including the transformation of 11-deoxycorticosterone to 18-hydroxy-11-deoxycorticosterone and the production of pregnenolone from exogenous cholesterol.

EXPERIMENTAL

Adrenal glands were obtained under light 'Nembutal' (sodium pentobarbital) anesthesia from young male rats (130-150 g) of an inbred Sprague-Dawley strain. All subsequent procedures prior to incubation were carried out with adrenal preparations kept at 0-4°C. Mitochondria were prepared using a modification (Roberts, Creange and Young, 1965) of the method described by Brownie and Grant (1954). Adrenal tissue was homogenized in 0.25 M sucrose containing

0.8 mM Tris-HCl buffer at a final pH of 7.1. The suspension was centrifuged at 700 g for 10 min. The supernatant was recovered and recentrifuged at 5000 g for 10 min; the supernatant and loosely-packed material from this centrifugation were discarded. The pellet was resuspended in the Tris-sucrose medium and the preceding step was repeated. The pellet was then resuspended in Tris-sucrose and centrifuged at 10,000 g for 10 min and the supernatant was removed. Electron microscopy revealed that the pellet obtained at this stage was composed mainly of intact mitochondria. In incubation studies with added cholesterol-4-^{14}C, the last step was repeated twice to reduce contamination of mitochondria with extra-mitochondrial enzyme systems active in steroid metabolism. Finally, the pellet was resuspended in a buffer composed of 13 parts of 0.154 M $NaHCO_3$ and 37 parts of 0.154 M KCl. Protein was measured in aliquots of the mitochondrial suspension by the method of Lowry, Rosebrough, Farr, and Randall (1951).

The incubation mixture contained 0.1 ml of mitochondrial suspension, 0.5 μmole NaNADPH, 24 μmoles $NaHCO_3$, ^{14}C-labeled steroid in 0.01 ml of 95 per cent ethanol, and other additives, made up to a final volume of 1 ml with 0.154 M KCl. The amount of mitochondrial suspension added to each sample was equivalent to approximately 0.3-0.4 mg mitochondrial protein derived from 50 mg adrenal tissue. Methods employed for incubation, extraction, chromatography on thin-layer plates, and measurement of radioactivity are described in detail elsewhere (Roberts, Creange and Fowler, 1964; Makoff, Roberts and Fowler, 1964; Creange and Roberts, 1966). The chromatography systems employed included methylene chloride:methanol (94:6) as described by Nakamura and Tamaoki (1964), the benzene:ethyl acetate (3:1) system of Avigan, Goodman and Steinberg (1963), and hexane:ethyl acetate (1:1) according to Smith and Foell (1962). All steroidal products were run in at least two chromatography systems. Other identification procedures included acetylation, chromic acid oxidation, and analysis of sulfuric acid spectra (Roberts, Makoff and Fowler, 1964; Creange and Roberts, 1966). Recovery of radio-

active steroids carried through the incubation and extraction procedures was virtually quantitative.

When rat adrenal mitochondria were incubated with 11-deoxycorticosterone-4-^{14}C for 15 min, the major radioactive metabolites formed were corticosterone and 18-hydroxy-11-deoxycorticosterone (Fig. 1). Addition of 2-4 mM 3',5'-AMP markedly enhanced both C-11β and C-18 hydroxylations. Experiments reported earlier revealed that significant stimulation of C-11β hydroxylation of 11-deoxycorticosterone could be obtained with 0.5 mM 3',5'-AMP (Roberts, Creange and Young, 1965).

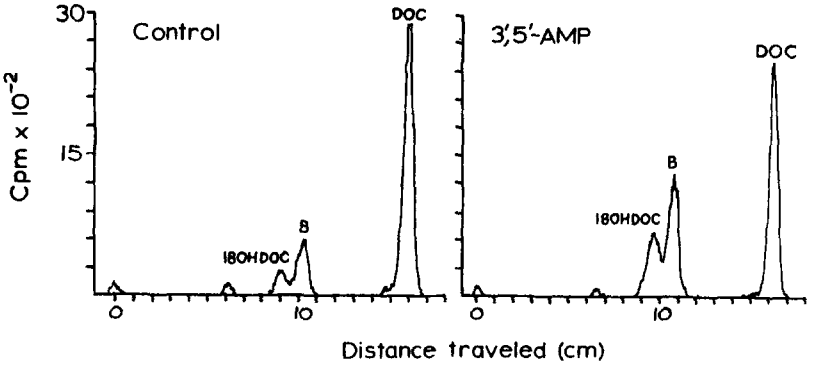

Fig. 1. Radioactivity scanning patterns of thin-layer chromatograms, showing conversion of 11-deoxycorticosterone to corticosterone and 18-hydroxy-11-deoxycorticosterone during incubation with rat adrenal mitochondria for 15 min. The incubation medium (1 ml) contained 0.1 μmole 11-deoxycorticosterone-4-^{14}C (0.05 μC), 0.5 μmole NADPH, and mitochondrial suspension equivalent to approximately 40 mg adrenal tissue; 4 μmoles of cyclic 3',5'-AMP were added where indicated. The chromatography system was composed of methylene chloride:methanol (94:6). DOC, 11-deoxycorticosterone; B, corticosterone; 18 OH DOC, 18-hydroxy-11-deoxycorticosterone.

Enhancement of mitochondrial C-11β and C-18 steroid hydroxylations was also obtained with 5'-AMP, but probably to a lesser extent than with the cyclic nucleotide (Table I). Little or no stimulation was produced with 4 mM 5'-ADP or 4 mM 5'-ATP. The stimulatory effect of 5'-AMP on C-11β and C-18 steroid hydroxylations has also been observed in adrenal homogenates (Creange and Roberts, 1966).

TABLE I

INFLUENCE OF ADENINE NUCLEOTIDES ON STEROID HYDROXYLATIONS IN RAT ADRENAL MITOCHONDRIA

	Hydroxylase activity	
	C-11β	C-18
Control	120 ± 22	58 ± 17
3',5'-AMP	276 ± 15	143 ± 20
5'-AMP	227 ± 15	125 ± 13
5'-ADP	155 ± 5	66 ± 8
5'-ATP	163 ± 14	84 ± 10

Incubation conditions were the same as those described in the legend to Fig. 1. Nucleotides were present in 4 mM concentrations. Results are expressed as mμmoles steroid converted/mg mitochondrial protein/hr. Each value represents the average ± S.E. of 3 determinations.

Evidence has been advanced in support of the hypothesis that 3',5'-AMP stimulates corticosteroidogenesis in adrenal whole-cell preparations at least in part by enhancing the conversion of cholesterol to pregnenolone (Karaboyas and Koritz, 1965). The present investigations supported this view. Adrenal mitochondria were incubated with cholesterol-4-^{14}C for short intervals to minimize the conversion of pregnenolone to other adrenal steroids by extra-mitochondrial enzymes remaining in the washed preparation. After 10 min, little or no radioactive pregnenolone accumulated in the absence of the cyclic nucleotide (Fig. 2). However, small quantities of radioactive progesterone appeared. Addition of 3',5'-AMP markedly enhanced the conversion of exogenous cholesterol to pregnenolone. Progesterone formation appeared to be only slightly stimulated. The rate of utilization of exogenous cholesterol in control incubations was essentially linear for the first hr. Approximately 0.6 μg cholesterol was converted to other products during this period by the mitochondria derived from 100 mg adrenal tissue. Lower rates of conversion have been reported by Halkerston, Eichhorn and Hechter (1961), using bovine adrenal mitochondria. However,

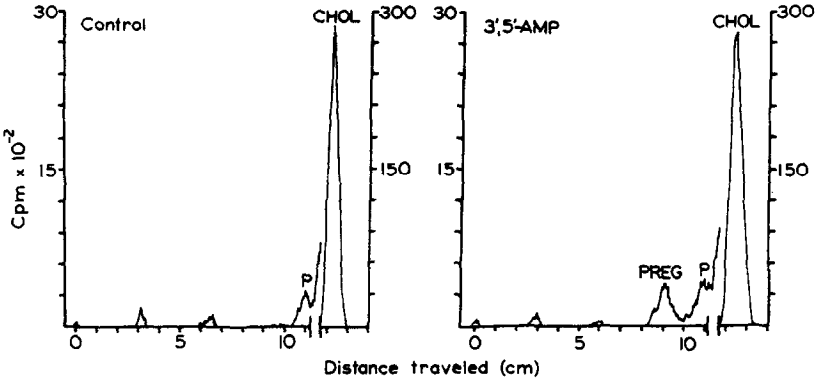

Fig. 2. Radioactivity scanning patterns of thin-layer chromatograms, showing conversion of cholesterol to pregnenolone and progesterone during incubation with rat adrenal mitochondria for 10 min. The incubation medium (1 ml) contained 3.5 μg cholesterol-4-^{14}C (0.5 μC), 0.5 μmole NADPH, and mitochondrial suspension equivalent to approximately 60 mg adrenal tissue; 4 μmoles of cyclic 3',5'-AMP were added where indicated. The chromatography system was composed of benzene:ethyl acetate (3:1). Radioactivity in the cholesterol band was measured at one-tenth the counting sensitivity employed for the remainder of the chromatogram. CHOL, cholesterol; P, progesterone; PREG, pregnenolone.

lack of information regarding the degree of mixing of the exogenous isotope with endogenous cholesterol prevented calculation of the actual rate of utilization in either situation.

The mechanisms involved in the enhancement of mitochondrial steroid conversions by 3',5'-AMP and 5'-AMP remain obscure. One effect may be a direct one on the mitochondrial membrane or on certain mitochondrial enzymes. In addition, indirect stimulation of steroid hydroxylations by 3',5'-AMP probably occurs in the intact cell due to activation of α-glucan phosphorylase and the consequent increase in NADPH generation (Haynes, 1958). Conflicting reports dealing with the site of action of ACTH on adrenal steroid biosynthesis may be resolved by the present observations. Thus ACTH, by enhancing the formation of 3',5'-AMP (Haynes, 1958), may produce a generalized stimulation of steroid transformations in the adrenal cortex, including the conversion of cholesterol to pregnenolone (Stone and Hechter, 1954) as well as later steps in corticosteroidogenesis (Heard et al., 1956).

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