THE ENERGY LINKED SYNTHESIS OF PREGNENOLONE IN BEEF ADRENAL CORTEX MITOCHONDRIA

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Special interest in the conversion of cholesterol to pregnenolone in the adrenal cortex arises from the observation that both ACTH and 3',5'-AMP stimulate steroidogenesis in this sequence of reactions (Stone and Hector, 1954; Karaboyas and Koritz, 1965). Halkerston et al. (1961) have shown that cholesterol side chain cleavage takes place in the mitochondria of beef adrenal cortex. In their system the reactions were supported by extra-mitochondrial TPNH and, to a lesser extent, by some Krebs-cycle intermediates. The requirement for TPNH is in keeping with the observations that 20α-hydroxycholesterol (Solomon et al., 1956) and 29α, 22ξ-dihydroxycholesterol (Shimizu et al., 1962; Constantopoulos et al., 1962) are intermediates in this transformation and that TPNH and oxygen are required for steroid hydroxylations.

Harding et al. (1965) showed that the 11-hydroxylation involved in the transformation of deoxycorticosterone to corticosterone proceeded much better in adrenal mitochondria in the presence of succinate than with extramitochondrial TPNH. The inhibition of the succinate-linked 11-hydroxylation by amytal suggested to these investigators that reverse electron transport to generate intra-mitochondrial TPNH may be involved. In the present study it has been found that succinate, to a greater extent than any other Krebs-cycle intermediate or extramitochondrial TPNH, supports the formation of pregnenolone from endogenous precusors, and that this is inhibited by seconal, antimycin A and pentachlorophenol, but is not inhibited by oligomycin.

EXPERIMENTAL PROCEDURE

The cortex from bovine adrenals was removed and a 10% homogenate prepared in 0.001 M EDTA-0.25 M sucrose, pH 7.2. The homogenization was carried out with a teflon pestle and no more than two passes were made. The homogenate was centrifuged at 400 x g for 10 minutes to sediment the nuclei and cell debris and the supernatant was centrifuged at 6600 x g for 10 minutes to sediment the mitochondria. The mitochondria were resuspended in the EDTA-sucrose medium by hand homogenization and resedimented at 5600 x g for 10 min. Four such washes were made and after the final wash the mitochondria were suspended in 0.25 M sucrose at a concentration of 9-12 mg protein per ml.

The basic complete incubation mixture contained 0.3 ml mitochondria, 77 μmoles histidine buffer (pH 5.4), 20 μmoles Na_2HPO_4 (pH 5.4), 25 μmoles Na succinate, 10 mg bovine serum albumin (BSA), 10 μmoles $MgCl_2$, 98 μmoles KCl and 0.25 M sucrose to a final volume of 2.0 ml. When TPNH was added it was prepared in advance from a TPNH generating system (TPN, 40 mg/ml; glucose-6-phosphate, 50 mg/ml; and glucose-6-phosphate dehydrogenase). Aliquots were taken so that 1.6 mg TPNH per beaker were present. Incubations were carried out with agitation at 37° in air for 15 min (these represent initial rates) and assayed for pregnenolone (Koritz, 1962). All values have been corrected for zero time controls.

RESULTS

The presence of succinate and Mg^{++} is essential for pregnenolone synthesis (Table I). The omission of both these substances resulted in no pregnenolone synthesis at all. Bovine serum albumin (BSA) and K^{+} augmented the synthesis somewhat. It is of interest the extra-mitochondrial TPNH has little, if any, effect. In other experiments it was found that extra-mitochondrial TPNH, in the absence of succinate and Mg^{++}, did not support pregnenolone synthesis at all. These results with respect to TPNH are similar to those obtained by Harding et al. (1965) with the 11-hydroxylation system.

TABLE I

CONDITIONS FOR PREGNENOLONE SYNTHESIS

System	Complete	No Succinate	No BSA	No Mg^{++}	No K^{+}	No Pi	No TPNH
µg Pregnenolone per beaker	4.2	0.7	3.7	1.8	3.6	3.9	3.8

TPNH was present in the complete system in this experiment. The values are expressed as µg pregnenolone per beaker.

The data of Table 2, experiment 1, show that succinate was superior, by at least a factor of two, to a variety of Krebs-cycle intermediates and other substances known to generate reduced pyridine nucleotides in some types of mitochondria.

TABLE 2

EFFECT OF VARIOUS OXIDIZABLE SUBSTRATES ON PREGNENOLONE SYNTHESIS

Substrate	µg Pregnenolone per Beaker		
	Experiment 1	Experiment 2	
			+ Succinate
Succinate	5.6	5.1	---
L-Malate	2.1	1.8	3.8
α-Ketoglutarate	2.0	0.8	3.5
L-Glutamate	0.4	---	---
Citrate	1.5	0.7	2.8
DL-Isocitrate	1.4	1.2	2.6
DL-β-Hydroxybutyrate	0	---	---
DL-α-Glycerophosphate	0	---	---

Substrates were present at 25 µmoles per beaker except with those in the DL form where 50 µmoles were added. Complete system with the indicated substrates.

The effects of various inhibitors of electron transport and high energy bond formation in the presence of succinate as the oxidizable substrate are recorded in Table 3. It is seen that pregnenolone synthesis is insensitive to oligomycin but largely inhibited by seconal, amytal, antimycin A, and pentachlorophenol. In accord with the report by Weinbach et al. (1963) pentachlorophenol is more effective in the absence of bovine serum albumin (BSA). Antimycin A and seconal will also abolish pregnenolone synthesis when other Krebs-

cycle intermediates replace succinate. It is to be noted that the presence of other Krebs-cycle acids inhibit succinate supported pregnenolone synthesis (Table 2, experiment 2).

TABLE 3

EFFECTS OF VARIOUS INHIBITORS ON PREGNENOLONE SYNTHESIS

System	μg Pregnenolone per Beaker
Complete	7.1
Complete + oligomycin 3.3 μg	7.1
Complete + oligomycin 10 μg	7.1
Complete + antimycin A 3.3 μg	0
Complete + seconal, 1 mM	1.0
Complete + seconal, 5 mM	0
Complete + amytal, 1 mM	4.3
Complete + amytal, 5 mM	0
Complete + pentachlorophenol, 0.02 mM	4.6
Complete + pentachlorophenol, 0.10 mM	0
No BSA	4.7
No BSA + pentachlorophenol, 0.02 mM	0

The requirement for succinate for pregnenolone synthesis and the inhibition by amytal and seconal suggest the involvement of energy dependent reverse electron transport (Chance and Hollunger, 1960) and probably transhydrogenation (Danielson and Ernster, 1963) to generate the TPNH required for the hydroxylation reactions involved in pregnenolone synthesis. The absence of inhibition by oligomycin under these conditions is consistent with this interpretation (Danielson and Ernster, 1963). Since the energy required is provided by succinate oxidation, the inhibition of pregnenolone synthesis by pentachlorophenol and by antimycin A (Ernster et al., 1963) is not unexpected.

The decreased synthesis of pregnenolone when succinate is replaced by other Krebs-cycle acids is in keeping with the observation of Harding et al. (1965) that malate was less effective than succinate in adrenal mitochondrial 11-hydroxylation. In liver mitochondria, Azzone et al. (1963) have found that succinate was superior to other Krebs-cycle acids in the removal of acetoacetate. The possibility should also be considered that succinate-linked pyridine nucleotide reduction involves a special pool, as has been suggested by Chance

and Ito (1963), and that this pool is involved in pregnenolone synthesis.

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