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# INHIBITION OF MEMBRANE-BOUND ADRENODOXIN-ADRENODOXIN REDUCTASE ACTIVITY BY PUROMYCIN AND CYCLOHEXIMIDE, WHICH ANTAGONIZE THE ADRENAL STEROIDOGENIC ACTION OF ACTH

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Inhibition of NADPH-dependent adrenodoxin reductase activity in adrenocortical mitochondria by puromycin and cycloheximide was observed at 0.1 mM and 1 mM, respectively. NADPH-dependent reduction of cytochrome P450 was also inhibited at similar concentrations, while solubilized enzymes were unaffected by the antibiotics. Further, puromycin induces high-to-low spin spectral changes with dissociation constants of 0.2 mM and 1.0 mM for membrane-bound and soluble cytochrome P450, respectively. Cycloheximide caused no spin changes.

## INTRODUCTION

Some 20 years ago Ferguson reported that adrenocortical steroidogenic action of ACTH* and cyclic AMP were antagonized by puromycin and he suggested that protein synthesis may be involved in the hormonal actions (1). Subsequently, Garren and his colleagues (2) studied the action of protein synthesis inhibitors *in vivo*. They found that the administration of cycloheximide to hypophysectomized rats maximally stimulated with ACTH resulted in a rapid decline in corticosterone production. They concluded from these results that the labile protein factor involved must be rapidly turned over ($t_{\frac{1}{2}}$ = 8 min). Davis and Garren (3) suggested that the site of action of cycloheximide is the ACTH-stimulated cholesterol side chain cleavage reaction. Since then, many investigators confirmed these observations, leading us to believe strongly the involvement of a so-called labile protein in the action of ACTH on adrenal steroidogenesis.

In 1964, Hechter and Halkerston suggested that the inhibition caused by puromycin may be due to a mechanism other than protein synthesis (4). More recently, Sun and Crane (5) reported that puromycin and actinomycin D inhibit plasma membrane-bound flavin dehydrogenase activities. Their conclusions prompted us to examine whether or not the electron transfer reaction of adrenal cortex mitochondrial steroidogenesis mediated by NADPH, adrenodoxin reductase

*ABBREVIATIONS: ACTH, adrenocorticotropic hormone; EDTA, ethylenediaminetetraacetic acid; DCPIP, 2,6-dichlorophenolindophenol; P450, cytochrome P450; $P450_{scc}$ cytochrome P450 specific for cholesterol side chain cleavage, $P450_{11\beta}$, cytochrome P450 specific for 11β-hydroxylation of deoxycorticosterone.

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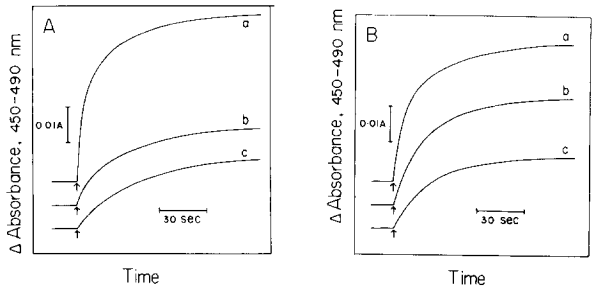



FIGURE 1. Inhibition of NADPH-dependent reduction of P450 by puromycin (A) and cycloheximide (B). Each experiment contained 4.7 mg mitochondrial protein in 3 ml of 10 mM Na phosphate, pH 7.4, and antibiotic, where indicated. CO was bubbled for 1 min before initiation of reduction with 0.25 mM NADPH (arrow) A: a, no antibiotic; b, 0.2 mM puromycin; c, 1 mM puromycin. B: a, no antibiotic; b, 5 mM cycloheximide; c, 10 mM cycloheximide. The reduction was monitored in the dual wavelength mode.

(a flavoprotein), adrenodoxin (an iron-sulfur protein) and P450 may be affected by inhibitors of protein synthesis. Indeed, our present studies unambiguously confirm that the membrane-bound form of adrenodoxin reductase activity is inhibited by both puromycin and cycloheximide at less than 1 mM. Therefore, the actions of these protein synthesis inhibitors on steroidogenesis must be considered in terms of both flavoprotein activity and labile protein synthesis

## METHODS AND MATERIALS

Adrenal cortex tissue was homogenized in 10 mM Na phosphate, pH 7.4, containing 0.32 M sucrose and 2 mM EDTA. Mitochondria were isolated by differential centrifugation and washed thrice in the buffer before being frozen at -20°C. Crude P450 was obtained in the 110,000 x g supernatant following treatment of sonicated mitochondria with 0.5 mg Na cholate per mg mitochondrial protein for one hour. Purified P450 and side chain cleavage activity were performed as described (6). Adrenodoxin and adrenodoxin reductase were prepared as before (7). Protein content was determined by the biuret method (8) in 0.25% (w/v) Na deoxycholate with bovine serum albumin as standard. All solutions were made in 10 mM Na phosphate and neutralized with NaOH to pH 7.4 if necessary.

Induced spectra were measured in 3 ml tandem cuvettes. Reduction rates in mitochondrial suspensions were measured on an Aminco-Chance dual wavelength spectrophotometer, while other spectral results were obtained on a Cary 118. DCPIP reductase activity was measured as before (9).

Actinomycin D, bovine serum albumin, cycloheximide, DCPIP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (type XV), NADH, $NADP^+$, NADPH and puromycin were purchased from Sigma. [$^{14}$C]cholesterol was obtained from New England Nuclear.

## RESULTS

Inhibition of NADPH-dependent reduction of mitochondria-bound P450 by puromycin and cycloheximide. The results in Fig. 1 show that puromycin and cycloheximide inhibit the reduction reaction of P450. The initial rates in thawed suspensions of adrenal cortex mitochondria were inhibited 65% and 81% by 5 mM and 10 mM cycloheximide, and 65% and 94% by 0.2 mM and 1 mM puromycin, res-

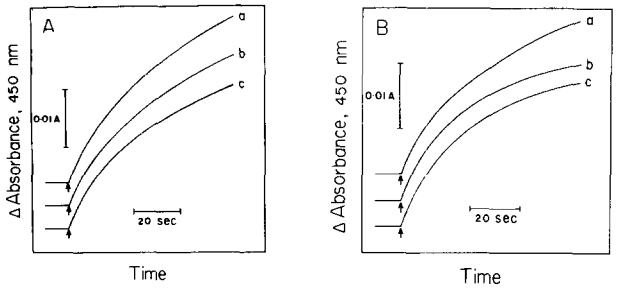



FIGURE 2. Effect of puromycin (A) and cycloheximide (B) on reduction of soluble P450. The reaction mixture ocntained 0.25 M purified P450, 36 nM adrenodoxin, 14 nM adrenodoxin reductase in 10 mM Na phosphate, pH 7.4. Reduction was commenced with 0.25 mM NADPH. A: a, no antibiotic; b, 0.5 mM puromycin; c, 1.2 mM puromycin. B: a, no antibiotic; b, 5 mM cycloheximide; c, 10 mM cycloheximide.

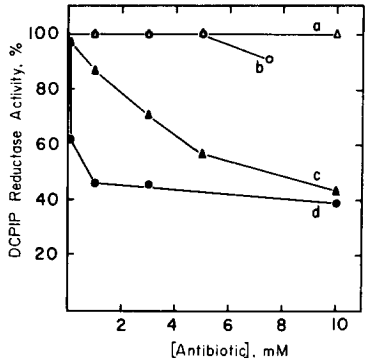
pectively. In addition, the total amount of P450 available for reduction was decreased by 22 and 47 % for the respective cycloheximide concentrations and 54 and 59 % for the respective puromycin concentrations.

Effect of puromycin and cycloheximide on reduction of soluble P450. The NADPH-dependent reduction of the soluble enzyme system was not affected by antibiotics at the same concentrations used in the previous experiment. Nor was the amount of P450 available for reduction appreciably affected (Fig. 2). The mitochondrial membrane, therefore, is necessary for the antibiotics to exert inhibitory actions on the electron transfer system.

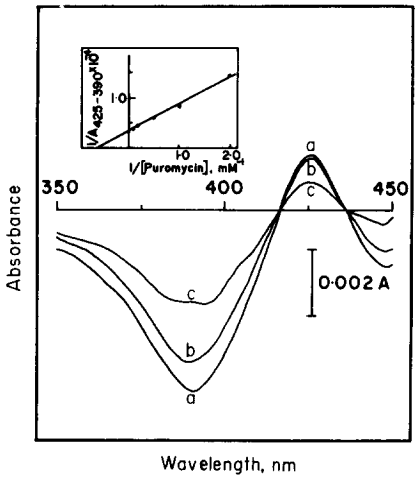
Cholesterol side chain cleavage activity of soluble P450 determined in the prescence of adrenodoxin, adrenodoxin reductase, $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and [$^{14}$C]cholesterol was found to be insensitive to concentrations of up to 10 mM puromycin and 10 mM cycloheximide. These results also support the requirement of the membrane in this system for inhibitory action by these antibiotics.

Inhibition of DCPIP-reductase activity by puromycin and cycloheximide in mitochondria. Fig. 3 shows inhibition of DCPIP reductase activity in thawed mitochondria by puromycin and cycloheximide. Inhibitory behavior of both antibiotics appear to follow a pattern similar to that in the reduction of P450 in mitochondria. Strong inhibition of reductase activity by puromycin was observed at 0.2 mM while cycloheximide showed less inhibition at 1 mM (13% inhibition). The effects of the antibiotics in the soluble system are similar to P450 reductase activity. No inhibition was observed at the concentrations used in the mitochondrial system, confirming the crucial role of the membrane in this action. Reduction by NADH was totally unaffected.

Effect of puromycin on the spin state of P450. Low spin induction of mitochondrial membrane-bound P450 by puromycin is illustrated in Fig. 4. Isosbestic points were observed at 416 nm and 436 nm, and a maximum and minimum at 425 nm and 390 nm, respectively. The Lineweaver-Burke plot of the binding




(3)




(4)

FIGURE 3. Comparison of inhibition of NADPH-dependent DCPIP reductase activity by puromycin and cycloheximide in mitochondria and soluble systems. Each assay contained the respective antibiotic and either 0.16 mg mitochondrial protein and 13 μM DCPIP or 19 μM adrenodoxin reductase and 13 μM DCPIP. Reduction was initiated with 50 μM NADPH and monitored at 600 nm. Δ, cycloheximide and soluble enzyme; ○, puromycin and soluble enzyme; ▲, cycloheximide and mitochondria-bound enzyme; ●, puromycin and mitochondria-bound enzyme.

FIGURE 4. Difference spectra of mitochondria-bound P450 in the presence of puromycin. Increasing amounts of puromycin were added to mitochondria (0.16 mg protein) contained in 1.2 ml 10 mM Na phosphate, pH 7.4 in tandem cuvettes. Corrections were made for dilution with each addition. The inset shows the double reciprocal plot for the spectral data. a, 1.0 mM puromycin; b, 0.5 mM puromycin; c, 0.2 mM puromycin.

data is shown in the inset. The spectroscopic dissociation constant, $K_S$, was 0.2 mM. Cycloheximide up to 10 mM was not effective in inducing any detectable spectral change.

Puromycin was also capable of inducing a low spin change in solubilized P450. Isosbestic points were observed at approximately 372 nm and 405 nm, and a maximum and minimum at 425 nm and 390 nm, respectively. A $K_S$ value of 1.0 mM was calculated from the spectral data.

Actinomycin D, an inhibitor of protein synthesis (10), was also tested on NADPH-dependent side chain cleavage activity and DCPIP reduction in the soluble system with no inhibitory effects up to 1 mM. 42% inhibition of DCPIP reduction in mitochondria was observed at 0.5 mM and appeared to be concentration-dependent.

## DISCUSSION

Our present results clearly show that the membrane-bound NADPH-DCPIP reductase activity catalyzed by adrenodoxin reductase and adrenodoxin is inhibited by both puromycin and cycloheximide. The inhibition was observed at 0.1 mM and 1 mM for puromycin and cycloheximide, respectively. Inhibition of

ACTH-stimulated steroidogenesis has been observed at 1 mM puromycin (1) and 10 μM cycloheximide (11). These results lead us to claim the inhibitory effects of these protein synthesis inhibitors on the electron transfer reactions of the P450-dependent mitochondrial steroid hydroxylases. As adrenodoxin and adrenodoxin reductase provide electrons to both $P450_{scc}$ and $P450_{11\beta}$ in adrenal steroidogenic reactions, these inhibitors should affect both side chain cleavage and 11β-hydroxylation reactions. On the other hand, the short term action of ACTH on the adrenal cortex is specific to the conversion reaction of cholesterol to pregnenolone but not to the 11β-hydroxylation reaction (3). Thus, the action of puromycin and cycloheximide would be expected to be specifically inhibitory of the cholesterol side chain cleavage reaction (3), and 11β-hydroxylation of deoxycorticosterone was, indeed, not inhibited by cycloheximide (12).

Our results, together with results in the literature, suggested that the inhibitory effects of puromycin and cycloheximide on adrenal steroidogenesis should be interpreted as the actions on both flavoprotein activity and protein synthesis. Thus, previous experiments using these inhibitors by in vivo administration to animals may be the result of these dual effects. This could also be extended to the inhibitory action of puromycin and cycloheximide on testosterone production in Leydig cells, where the existence of a 'labile protein' was suggested (13).

In this study we found significant inhibition of the membrane-bound electron transfer system to P450, but no inhibition was observed with the purified, membrane-free system, indicating that the inhibition requires the presence of a membrane. The spectral induction of P450 by puromycin occured in both membrane-bound and membrane-free samples. This phenomenon may, however, not contribute to the inhibition because this effect was uninfluenced by the absence of a membrane. The action of puromycin on ribosomal protein synthesis is due to the formation of peptidyl puromycin by interrupting peptide-chain elongation. Cycloheximide inhibits protein synthesis by 80S eukaryotic ribosomes but not by 70S prokaryotic ribosomes and prevents reassembly of polysomes (15, 16), implying some action on ribosomal proteins. The role of protein synthesis is precluded from these enzymatic reductions based on the time scale of these experiments and the use of isolated mitochondria, so puromycin and cycloheximide must exert their influence on the individual or collective electron transfer proteins involved. In this context, we speculate here that the inhibition by these reagents may be due to interactions between inhibitor and membrane-associated enzymes.

ACKNOWLEDGEMENT

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