Protein Synthesis and Adrenocorticotropic Responsiveness*

JAMES J. FERGUSON, JR.

From the Departments of Biochemistry and Medicine, University of Pennsylvania School of Medicine, Philadelphia 4, Pennsylvania

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The mechanism by which adrenocorticotropic hormone stimulates the production of adrenocortical steroids is not fully understood. This paper describes experiments directed toward this problem, and presents evidence compatible with the idea that concomitant protein synthesis is required for adrenocorticotropic responsiveness. A preliminary report of this work has been published (1).

Koritz, Péron, and Dorfman in 1957 (2) examined the possibility that the effects of ACTH might involve protein synthesis in the adrenal. They found that there occurred in vitro no increase in glycine-C14 incorporation into rat adrenal protein in the presence of ACTH. Further, they demonstrated that ACTH was fully effective in stimulating steroidogenesis in the presence of known inhibitors of protein synthesis. However, their experiments did not rule out the possibility that protein synthesis is involved in the mechanism of ACTH action, since (a) incorporation of a minute amount of radioactive amino acid into some key protein might not be detectable in the presence of a considerable basal rate of amino acid incorporation, (b) protein synthesis might not here be a process de novo, but rather the assembly of preformed peptides in the presence of ACTH, and (c) in their inhibitor studies, none of the inhibitors used produced complete inhibition of amino acid incorporation. It is worth noting that several reports have suggested that an increase in labeled amino acid incorporation into protein occurs after exposure to ACTH (3, 4).

In the present study, the antibiotic puromycin has been shown to inhibit both ACTH responsiveness and amino acid incorporation into rat and steer adrenal protein in vitro. Other investigators have utilized puromycin in studying the mechanisms of hormone action. The studies of Mueller, Gorski, and Aizawa (5) indicated that the administration in vivo of puromycin prevents several of the known effects of estradiol on the ovariectomized rat uterus. Cohen, Brenneman, and Toper (6) have recently reported that puromycin in vitro prevents the acceleration of glucose oxidation in rat mammary tissue produced by oxytocin. Puromycin is believed to inhibit protein synthesis by interfering with the assembly of protein molecules on the ribosomal template (7). It has been suggested that puromycin accepts small, preformed peptides from the ribosome surface, releasing them as acid-soluble fragments attached to the puromycin molecule (8). It has recently been reported (9) that puromycin in vivo will cause extreme depletion of hepatic, but not muscle, glycogen in the rat. The mechanism of this effect is not yet known.

EXPERIMENTAL PROCEDURE

Methods—Adrenal incubations in vitro were performed according to the method of Safferan and Schally (10), with a 30-minute preliminary incubation followed by an incubation of variable duration. Adrenal steroid production in vitro was measured either by the tetrazolium method of Elliot et al. (11) or by the acid fluourescence method of Silber, Busch, and Oslapas (12) with corticosterone as a standard; these values are designated "corticosterone" in this paper. The latter method was used for determination of rat serum corticosteroid concentrations. Labeled amino acid incorporation into protein was measured by the technique described by Kral (13). Phosphorylase activity was measured by the method of Sutherland (14). The release of free fatty acid from rat epididymal fat pad was determined by the method of Dole (15). Glucose-6-P dehydrogenase was measured in hypotonic extracts of rat adrenal by the method of Kornberg and Horecker (16). Phosphoglucomutase activity in rat adrenal extracts was estimated in a similar way by measuring spectrophotometrically the rate of TPNH formation in the presence of TPN, glucose-1-P, and excess glucose-6-P dehydrogenase, with magnesium and cysteine present.

Materials—Adult male rats of the Sprague-Dawley strain were used. Hypophysectomized rats were obtained from the Hormone Assay Laboratories. Steer adrenals were obtained from a local slaughterhouse and slices were prepared about 1 hour after death.

ACTH was kindly provided by Armour as either Aethar or "corticotropin" supplied at a concentration of 2.5 units per mg of solute. The manufacturer's assays were accepted without further verification. Weighed samples were diluted with 0.154 M NaCl and used immediately; a final concentration of 0.1 unit per ml was used in all incubations in vitro. 3',5'AMP was obtained from Sigma Chemical Company; it was neutralized with KOH and diluted with 0.154 M NaCl. Puromycin, as the dihydrochloride pentahydrate, was the generous gift of Dr. Leon Goldman of Lederle Laboratories, and was used without further purification. A stock solution was prepared by titration with KOH to about pH 6.8 and dilution to 0.02 M with 0.154 M NaCl. This opalescent solution was partially clarified by warming, and was completely soluble on dilution. Stock solutions were kept frozen, and did not seem to lose potency on repeated thawing. Puromycin aminonucleoside, kindly provided by Dr. David

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1 The abbreviation used is: ACTH, adrenocorticotropic hormone.

2 9-[3-Deoxy-3-(p-methoxy-L-phenylalanylamo)-β-D-ribofuranosyl]-6-dimethylamino-β-purine.

3 9-[3-Amino-3-deoxy-β-D-ribofuranosyl]-6-dimethylamino-β-purine.
Drabkin, was handled in the same way. The d- and l-phenyl-
alanyl derivatives of puromycin, also provided by Dr. Goldman,
were similarly handled, except that stock solutions were slightly
more dilute. dL-Leucine-1-C\textsuperscript{14} was a product of Volk Radio-
chemical Company, with a stated specific activity of 12 mc per
mmole.

**Experimental Results**

Studies in Vitro with Rat Adrenal Quarters—Quartered rat
adrenals, incubated in Krebs-Ringer-bicarbonate with added
glucose, are known to respond to added ACTH by the elabora-
tion of corticosteroid into the medium at a rate 2 to 5 times the
rate of untreated gland quarters (10). If puromycin was added
to such a preparation at a final concentration of 1 mm, the re-
sponse to ACTH was completely abolished, whereas the basal
elaboration of corticosteroid continued. Addition of puromycin
alone did not alter the basal rate of steroid production. Similar
results were also observed in Krebs-Ringer-phosphate with glu-
cose. If, after incubation, gland fragments were homogenized in
13% ethanol and the tissue corticosteroid content was meas-
ured by the tetrazolium assay method, it was found that ACTH in-
creased the amount of corticosteroid in the gland as well as in
the medium, indicating an increased synthesis rather than mere
release of preformed steroid. In the presence of puromycin,
ACTH did not produce this increase in steroid content of the
adrenal cortex, indicating that puromycin blocked the increase in syn-
thesis induced by ACTH, rather than simply depressing the re-
lease of steroid from the gland. Representative experiments
illustrating these several results are shown in Table I. In Table
II are shown the results obtained when puromycin and several
of its structural analogues were tested, with simultaneous meas-
urement of corticosteroid release into the medium and leucine-
1-C\textsuperscript{14} incorporation into adrenal protein. At an antibiotic con-
fcentration of 1 mm, puromycin and l-phenylalanyl puromycin
completely inhibited both steroid production in response to
ACTH and also isotope incorporation. Puromycin aminonu-
ucleoside and the n-phenylalanyl derivative of puromycin did not
affect isotope incorporation, nor did they significantly alter the

**Table I**

**Steroidogenic response to ACTH in presence of puromycin**

Figures represent mean of duplicate determinations on dupli-
cicate flask ± variations from the mean. Each flask contained
the equivalent of two rat adrenals in Krebs Ringer bicarbonate
medium plus glucose; final volume, 2.0 ml. Additions (each in
0.1 ml) were: ACTH, 0.2 unit; puromycin, 2 \( \mu \text{moles.}

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Addition</th>
<th>Corticosteroid</th>
<th>Protein specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>Tissue</td>
</tr>
<tr>
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<td>None</td>
<td>4.1 ± 0.2</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>10.1 ± 1.1</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>ACTH + puromycin</td>
<td>3.5 ± 0.1</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Puromycin</td>
<td>3.0 ± 0.2</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>11.7 ± 0.1</td>
<td>8.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>28.2 ± 0.8</td>
<td>14.0 ± 0.2</td>
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<tr>
<td></td>
<td>ACTH + puromycin</td>
<td>13.0 ± 0.2</td>
<td>7.6 ± 0.3</td>
</tr>
</tbody>
</table>

Steroidogenic response to 3',5'-'AMP and TPNH

Conditions and figures were as described in Table I. Additions
(each in 0.1 ml) were: puromycin, 2 \( \mu \text{moles; 3',5'-AMP, 5 \( \mu \text{moles;}
TPN, 2 \( \mu \text{moles; glucose-6-P, 8 \( \mu \text{moles. One-hour incubation and the}
tetrazolium assay were used.}

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Addition</th>
<th>Corticosteroid</th>
<th>Protein specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3',5'-AMP</td>
<td>3',5'-AMP + puromycin</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>14.5 ± 0.1</td>
<td>16.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>3',5'-AMP</td>
<td>42.2 ± 1.7</td>
<td>63.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>3',5'-AMP + puromycin</td>
<td>43.5 ± 3.5</td>
<td>1695 ± 35</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>18.3 ± 0.5</td>
<td>43.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>TPN + glucose-6-P</td>
<td>43.5 ± 3.5</td>
<td>1695 ± 35</td>
</tr>
<tr>
<td></td>
<td>TPN + glucose-6-P + puromycin</td>
<td>43.5 ± 3.5</td>
<td>1695 ± 35</td>
</tr>
</tbody>
</table>

It is worth noting that the addition of puromycin did not produce
an increase in net amino acid C\textsuperscript{14} incorporation into adrenal pro-
tein, in agreement with the findings of Koritz et al. (2). Rather,
there was observed a tendency toward diminished C\textsuperscript{14} incorporation,
first evident 60 to 90 minutes after the addition of ACTH.
It can also be concluded that inhibition by puromycin is not simply the direct inhibition of an enzyme of corticosteroid biosynthesis, since puromycin has no inhibitory effect added glucose-6-P plus TPN.

Experiments were performed to determine the time at which puromycin must be added to these incubations in vitro to be effective. These are illustrated in Fig. 1. If puromycin was added at zero time, i.e. after the preliminary incubation period and simultaneously with ACTH, the ACTH was without effect on total steroid production during a 90-minute incubation. If the puromycin was added 30 minutes after ACTH, the ACTH was fully effective, with a continued high rate of steroid production throughout the period of incubation. If puromycin was added at times between 0 and 30 minutes, the ACTH had intermediate effectiveness. Thus the gland fragments gradually gained "resistance" to the inhibitory effect of puromycin if allowed an exposure to ACTH for a sufficient length of time. This would suggest that ACTH causes the accumulation of some material (e.g. cofactor, substrate, enzyme, etc.), that puromycin added early prevents the elaboration of this material, and that once the material is formed, puromycin is no longer inhibitory.

In all the experiments described to this point, puromycin was present in a final concentration of 1 mM. In order to determine the concentration of puromycin required to inhibit both ACTH responsiveness and amino acid incorporation into adrenal proteins, the experiments depicted in Fig. 2 were performed. In very gross terms, both processes were 50% inhibited at about the same puromycin concentration, i.e. 10 μM. There was considerable scatter about this point, possibly because of the several technical and theoretical problems in this type of experiment.

If the inhibition of amino acid incorporation by puromycin is causally related to the observed inhibition of ACTH responsiveness, then one would have to know whether the synthesis of all species of protein molecule is uniformly inhibited by a particular concentration of puromycin. Any selective inhibition of protein synthesis would limit the validity of the results. Further, if one assumed that ACTH causes the synthesis of an enzyme protein which is rate-limiting for steroid biosynthesis, amino acid incorporation into this protein would be expected to precede the accumulation of the product (here corticosteroid) of the reaction catalyzed by the enzyme being synthesized. However, accepting these reservations, the data indicate that there is at least some correspondence in the concentrations of puromycin needed to produce each of the two effects. It is also worth noting that both effects were regularly and completely inhibited at a puromycin concentration of 1 mM.

When puromycin is used in vivo, animals are known to recover from doses which inhibit hepatic protein synthesis (19). Accepting this as an indication of the reversibility of the acute effects of puromycin, experiments were performed to determine if this reversibility could be demonstrated in vitro. Quartered rat adrenals were incubated for 30 minutes in medium containing puromycin at a concentration of 1 mM. They were rinsed in fresh medium and then resuspended in medium containing ACTH but no puromycin. They were found to regain fully both the ability to incorporate leucine-1-C14 into protein and the ability to respond to ACTH. Recovery from puromycin followed the time course illustrated in Fig. 3. There was regularly observed a delay of nearly 30 minutes before the gland fragments almost simultaneously regained both abilities. Control flasks, containing ACTH but never exposed to puromycin, exhibited no significant delay in the onset of the two processes.

The antibiotic actinomycin D, recently reported to inhibit
selectively the synthesis of "messenger" RNA (20), was found not to inhibit the steroidogenic response to ACTH, at a concentration of 10 μM.

**Effects of Puromycin in Vitro**—It has been observed (21) that administration of puromycin to the living rat causes a decrease of protein synthesis in vivo. This decrease varies in different tissues, being greatest in liver and least in thymus, of the tissues studied; these did not include the adrenal. When administered by the intraperitoneal route to hypophysectomized rats according to a dosage program similar to that used by Mueller et al. (5), puromycin was found not to alter ACTH responsiveness as measured by corticosteroid release into peripheral blood. No attempt was made to determine whether this amount of puromycin altered adrenal protein synthesis in vitro, nor were other routes of puromycin administration tried. There is available no information on the visceral distribution of puromycin. Its known selective depression of hepatic protein synthesis may reflect a concentration of the drug in the liver, rather than a specific sensitivity of the liver to puromycin.

**Puromycin and Phosphorylase Activation**—Haynes and Berthelot (22) demonstrated an increase in phosphorylase activity after the exposure of steer adrenal cortex slices to commercial ACTH. Attempts in this laboratory to reproduce this observation with quartered rat adrenals have consistently failed, as in the recent report of Williams, Johnson, and Field (23). In order to determine whether puromycin was blocking ACTH responsiveness by inhibiting phosphorylase activation, steer adrenal cortex slices were used rather than quartered rat adrenals. Increased phosphorylase activity could readily be shown after exposure of the slices to ACTH in vitro, concomitant with increased corticosteroid production. In order to inhibit amino acid incorporation into cortical protein and block the steroidogenic response to ACTH, a prior incubation of the slices in the presence of puromycin was found necessary. Under these conditions, with amino acid incorporation and steroidogenic responsiveness effectively inhibited, ACTH still produced the expected increase of phosphorylase activity. Thus the steroidogenic and the phosphorylase-activating effects of ACTH could be dissociated by puromycin. Of note is the fact that puromycin regularly (7 experiments) enhanced the activation of phosphorylase by ACTH, whereas in the absence of ACTH, puromycin was without effect on phosphorylase activation. Representative experiments illustrating these results are shown in Table IV.

**Effects of Puromycin on Extra-adrenal Responses to ACTH**—ACTH is known to produce several effects on nonadrenal tissues (24). By using rat epididymal fat pad as a test organ, the stimulatory effect of ACTH on fatty acid release and on phosphorylase activation could readily be demonstrated. Puromycin had no effect on these responses to ACTH.

**Effects of Puromycin on Adrenal Enzymes**—Puromycin at a concentration of 1 mM was found to have no effect on phosphorylase, glucose 6-phosphate dehydrogenase, and phosphoglucomutase activities of hypotonic KC1 extracts of rat and steer adrenal. Likewise, puromycin did not inhibit the ability of a homogenate of rat adrenal to convert endogenous cholesterol to tetrazolium-reacting corticosteroids (25).

**DISCUSSION**

It is at present impossible to describe the precise molecular events involved in the mechanism by which ACTH acts in adrenal steroidogenesis. Haynes has presented an attractive hypothesis based on the observations that ACTH produces a rapid activation of adrenal phosphorylase (22) and an accumulation of 3',5'-AMP (26), which itself can mimic the steroidogenic effect of ACTH (17). Noble and Papageorge (27) described the depredation of adrenal glycogen in rats after cold stress and ACTH, but Vance, Girard, and Cahill (28) could not reproduce this effect of ACTH in hypophysectomized rats or in vitro. Cohen (29) presented histological evidence for rapid, transient glycogen depletion from subglomerulosa layers of normal rat adrenal cortex

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**Table IV**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Addition</th>
<th>Phosphorylase</th>
<th>Corticosteroid in medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>1.9 ± 0.3</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>7.3 ± 2.2</td>
<td>9.0 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>ACTH + puromycin</td>
<td>11.1 ± 0.9</td>
<td>4.7 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>1.8 ± 0.3</td>
<td>3.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>7.2 ± 2.5</td>
<td>13.8 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>ACTH + puromycin</td>
<td>10.0 ± 3.5</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>1.1 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td>3.4 ± 0.9</td>
<td>6.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>ACTH + puromycin</td>
<td>6.9 ± 0.5</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Puromycin</td>
<td>1.0 ± 0.1</td>
<td>3.0 ± 0.1</td>
</tr>
</tbody>
</table>

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*These assays were performed by Mr. Kenneth Lembach.*
after cold stress, which he could not produce in hypophysectomized rats. Greenberg and Glick have described glycogen depletion in rat adrenal cortex after ACTH by means of histochemical analyses (30); they have also demonstrated an increase in adrenal phosphorylase activity by these techniques (31). However, to date there is no direct experimental evidence that the increase of adrenal phosphorylase activity by ACTH, and subsequent glycogenolysis, are causally related to enhanced steroidogenesis, and there is, in fact, some evidence against this idea. With a system in vitro, Field et al. (32) failed to find evidence for increased endogenous glucose-6-P production after exposure to ACTH. A recent report from Field’s laboratory (23) describes an ACTH-induced increase in C14O2 release from glucose-C14 in the medium, which is quite the opposite of what one would grossly expect in the presence of concomitant glycogen breakdown. Vance et al. have demonstrated the same effect (28). In failing to find evidence for a metabolic effect of adrenal glycogen breakdown, experiments of this type at least prompt a re-evaluation of the validity of the “phosphorylase activation hypothesis.” Our finding of normal phosphorylase activation in puromycin-inhibited slices may mean that steroidogenesis in response to ACTH is not dependent on phosphorylase activation.

Koritz (33) has presented evidence that 3'-5'-AMP may cause steroidogenic changes in the adrenal which are not directly related to TPNH production, since 3',5'-AMP further increased steroidogenesis in gland fragments maximally stimulated by glucose-6-P plus TPN. The recent observation of pyridine nucleotide oxidation rather than reduction after ACTH administration in vivo (34), coupled with similar histochemical observations by Greenberg and Glick (35), poses some further questions regarding a functional role for phosphorylase activation. There exists, of course, the real possibility that the adrenal cortex is controlled by ACTH through several regulatory mechanisms which act simultaneously, furnishing multiple levels of regulatory refinement and duration.

Unfortunately, the observation that puromycin inhibits ACTH responsiveness only prompts more questions regarding the mechanism of ACTH action. We do not yet have an incontrovertible explanation for this effect of puromycin, and several obvious possibilities exist. Puromycin might inhibit, competitively or noncompetitively, the activity of some enzyme involved in the production of corticosteroids, either on the biosynthetic pathway between cholesterol and corticosteroids or on some regulatory pathway. Direct inhibition of an enzyme on the biosynthetic pathway seems unlikely, since maximal steroid production can be achieved in the presence of puromycin with added TPN plus glucose-6-P. Without certain knowledge of the mechanism of regulatory reactions, the possibility of puromycin inhibition of these reactions is difficult to test. On the assumption that 3',5'-AMP is involved in ACTH responsiveness, it is possible that puromycin might competitively inhibit its metabolic effects, or activate an enzyme concerned in the removal of 3',5'-AMP. We can rule out the possibility that puromycin interferes with the activation of adrenal phosphorylase. It does not, in adrenal extracts, seem to inhibit enzymes concerned with glucose-6-P production from glycogen and TPNH production from glucose-6-P. Puromycin could remove some other required cofactor, such as calcium ion. But again, the ability of puromycin-treated glands to respond to TPN plus glucose-6-P makes this unlikely. If TPNH availability is really a contributing factor in ACTH responsiveness in the intact cell, puromycin may directly or indirectly inhibit its production from an as yet unrecognized source.

Finally, one can speculate on the possibility that puromycin inhibits ACTH responsiveness because it inhibits protein synthesis. Concomitant protein synthesis might be necessary to maintain cell membrane and the general osmotic and metabolic integrity of the cortical cell. Such a need may be reflected in the repeated observation that broken cell preparations of adrenal do not respond to ACTH, either by increased steroid production or by increased oxygen consumption (36).

However, it is also possible that puromycin inhibits the ACTH-induced synthesis of a specific enzyme molecule which is rate-limiting for the over-all process of steroidogenesis, be it on the biosynthetic pathway or the regulatory pathway. None of the data presented is at variance with this idea. The noninhibition of TPNH responsiveness could indicate that the enzyme synthesized is concerned with TPNH (or TPN) synthesis, in turn required for steroidogenesis. Hilt et al. have reported changes in adrenal 5'-nucleotidase (37) and glucose 6-phosphatase (38) activities after administration of ACTH in vivo. Whether these changes are causally related to steroidogenesis is as yet unknown. It is also unknown whether these changes in enzyme activity represent changes in the absolute amount of enzyme protein present, reflecting a change in rate of protein synthesis.

Unfortunately, this discussion must now remain speculative. Of the several possible explanations for the observed effects of puromycin, the most provocative but most difficult to prove is the idea that a specific protein must be synthesized in order for the adrenal to increase steroid output. Such a proof would entail the isolation of the protein and demonstration of its specific, obligatory role. At the moment there exists circumstantial evidence that ACTH works through such a mechanism.

SUMMARY

Puromycin has been shown to cause, in vitro, parallel inhibitions both of amino acid-C14 incorporation into adrenal protein and of corticosteroid synthesis in response to adrenocorticotropic hormone (ACTH). Both inhibitory effects are reversible, both occur at approximately the same concentration of puromycin, and both can be reproduced by the L-phenylalanyl derivative of puromycin, but not by the n-phenylalanyl derivative or by puromycin aminonucleoside. Puromycin also inhibits the steroidogenic effect of adenosine 3',5'-cyclic phosphate, but not that of reduced triphosphopyridine nucleotide. It does not inhibit ACTH-induced activation of adrenal phosphorylase.

The data reported are consistent with the idea that protein synthesis is necessary for ACTH responsiveness. It is further suggested either that the activation of adrenal phosphorylase is not causally related to enhanced steroidogenesis in response to ACTH or, if it is related, that concomitant protein synthesis is also required.

Acknowledgments—The author is indebted to Drs. Joel Flaks and Gabriel de la Haba for many helpful discussions of this work.

REFERENCES
