MECHANISM OF ACTION OF ADRENOCORTICOTROPIN ON THE ISOLATED ADRENAL CELL

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ABSTRACT

Present concepts of ACTH/cyclic nucleotide regulation of steroidogenesis involve stimulation, by either cAMP or cGMP, of a protein kinase which in turn removes an inhibitory protein from a pretranscribed mRNA. A cycloheximide sensitive step between cholesterol and pregnenolone indicates the requirement of a specific protein at this point. The activated mRNA is suggested to translate this protein. The evidence to support these concepts remains to be substantiated. The isolated rat adrenal cell has been used for the assessment of regulatory factors in steroidogenesis. Isolated adrenal cells were prepared by collagenase digestion and steroidogenesis was evaluated by RIA of corticosterone and thin layer chromatography from radioactive precursors. The involvement of RNA and protein synthesis was evaluated by use of the respective inhibitors Actinomycin D and cycloheximide, and the incorporation of ^{3}H-leucine into proteins. Corticosterone production was dose responsive and showed an LD_{50} of 35 μU of ACTH. With transcription inhibited by 12 μg/ml of Actinomycin D, no effect was seen on ACTH stimulated corticosterone production. This tends to support the existence of a pretranscribed mRNA. Cycloheximide inibition of protein translation was dose reponsive(0.1, 1.0, and 10.0 μM) when corticosterone was measured by RIA, however, no dose of cycloheximide was effective when measuring ^{3}H-pregnenolone conversion after ACTH stimulation. This indicates that a protein is required, but the requirement is before pregnenolone. ACTH stimulation of protein synthesis as measured by ^{3}H-leucine incorporation, indicates that ACTH and dbcAMP, but not cAMP and cGMP, stimulated protein synthesis at the doses used. This research supports the present theory of ACTH, but does not clarify the role of cyclic nucleotides in steroidogenesis.
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INTRODUCTION

The mechanism of adrenocorticotropin (ACTH) action on adrenal cells is thought to be through the classic 1st messenger - 2nd messenger pathway first proposed for the action of epinephrine by Sutherland (1), and later for the action of ACTH by Haynes and Berthet (2). After binding to a highly specific plasma membrane receptor, ACTH (1st messenger) activates the membrane bound enzyme, adenylate cyclase, which catalyzes the cytoplasmic conversion of adenosine triphosphate (ATP) to cyclic-3',5'-adenosine monophosphate (cAMP) (2, 3, 4). Cyclic AMP (2nd messenger) activates a specific protein kinase, an enzyme which transfers a phosphate group from ATP to a suggested protein involved in the activation of steroidogenesis (5).

The formation of corticosterone (B) is summarized in Figure 1. The site of ACTH and cAMP stimulation as measured by accumulation of labelled B from radioactive precursors, suggests the rate limiting step of steroidogenesis in the adrenal cell is between cholesterol and 20 α - hydroxycholesterol (6). That protein synthesis is involved in this activation is suggested by experiments using protein inhibitors, i.e. cycloheximide and puromycin. Cycloheximide, a specific inhibitor of

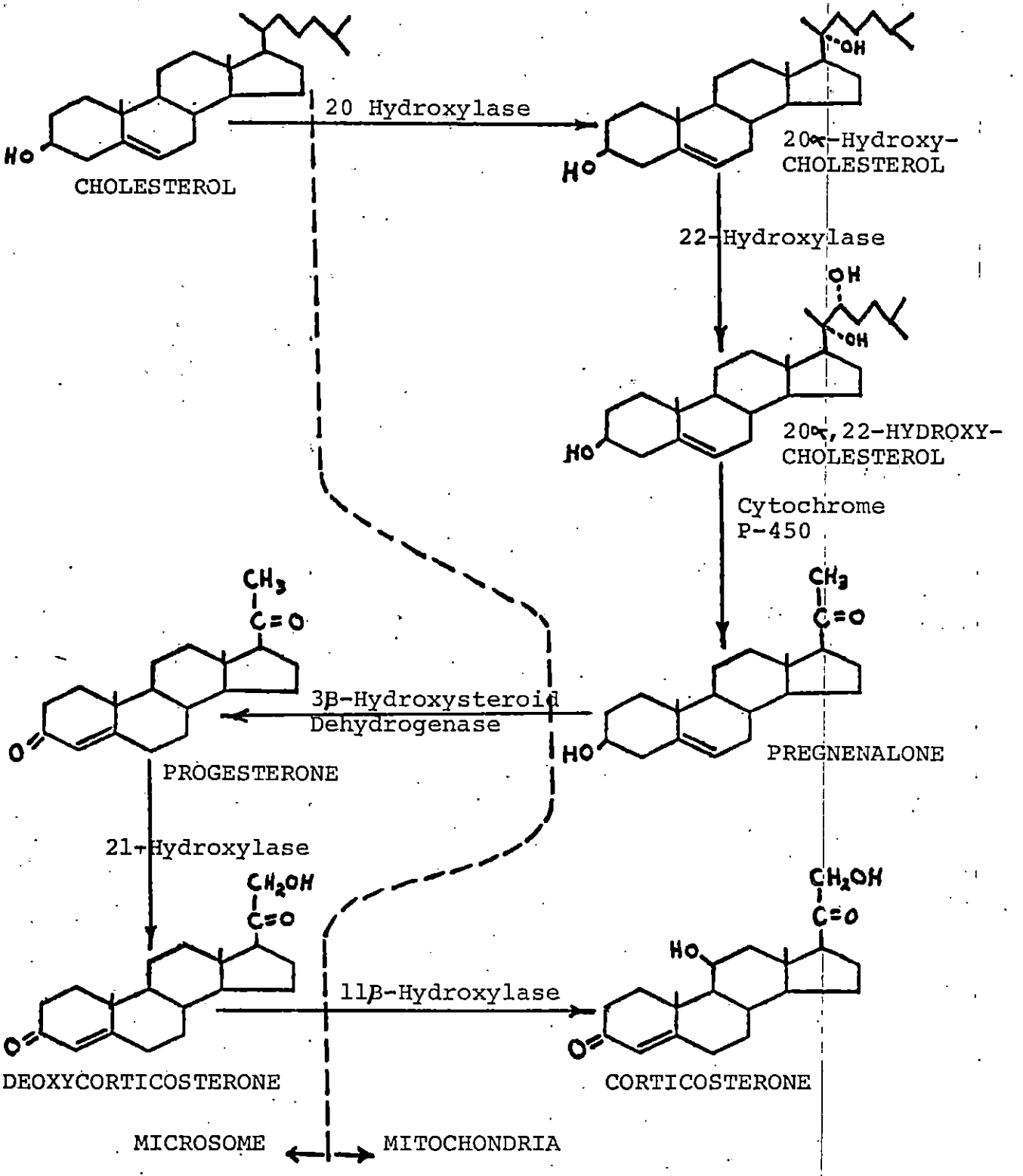

Figure 1. Pathway for glucocorticoid biosynthesis in the adrenal cortex.

protein translation, blocks the stimulation of steroidogenesis by ACTH and cAMP (7). This suggests that ACTH acts via cAMP by stimulating the translation of a protein which acts between cholesterol and 20α-hydroxycholesterol. Furthermore, this protein has been suggested to be derived from a preexisting messenger RNA (8), and possess a half-life of eight minutes (9).

Recently, cyclic-3', 5' guanosine monophosphate (cGMP) has been implicated as the 2nd messenger of ACTH (10, 11). At very low levels of ACTH, which stimulated steroidogenesis but did not increase cAMP, cGMP levels were increased. This evidence suggests two theories for mechanism of ACTH action. One, that ACTH is mediated at low and high levels by two different cyclic nucleotides. Additionally cAMP may be the pharmacological mediator.

This study proposes to clarify the intracellular molecular mechanisms through which ACTH acts upon the isolated rat adrenal cell. Specifically, an understanding of cyclic nucleotides, the extent of protein synthesis involement in steroidogenesis and the time interval between stimulation and steroid release will be studied.

MATERIALS AND METHODS

Animal Care

Female rats of the Sprague-Dawley strain, 150-180 days of age, were maintained in animal quarters under a lighting regimen of 12 hours light: 12 hours dark. They were given Purina Lab Chow (Ralston-Purina Co.) ad libitum. An antibiotic drinking solution (Sulmet Drinking Solution, American Cyanamid Co.) was added to their drinking water at a dosage of 30 ml/3.8 liters of water, given alternately with tap water at every other watering period.

Rats were stunned, decapitated and exsanguated within one minute after their removal from the animal room. The adrenals were removed and placed on saline moistened filter paper in a petri dish on ice. After all adrenals had been collected, they were brought to the lab where they were placed on a chilled glass plate, trimmed of excess fat with a razor blade, and cut into eights. Pooled adrenals were rinsed with 10 ml of cold Krebs Ringer Bicarbonate buffer to which 200 mg% glucose was added (KRBG).

Isolation of Adrenal Cells

All glassware used in the isolation procedure was silicontreated (Siliclad, Clay-Adams). Double distilled water and redistilled organic solvents were used throughout the procedure.

Isolated adrenal cells were prepared by a modification of the collagenase method of Kloppenborg (1968). The pooled adrenal eights were transfered to a 25 ml erylenmeyer flask containing a digesting medium consisting of KRBG, 40 mg (4%) of bovine serum albumin (BSA, Sigma Biochemicals, Inc.) 5 mg of collagenase (Worthington Biochemical Corp.), and 0.05 mg DNAase (Sigma Biochemicals, Inc.). The flask was equilibrated with a 95% oxygen - 5% carbon dioxide gas mixture, stoppered, and incubated at 37°C for 45 minutes in a Dubnoff Metabolic Shaking Incubator (Precision Instruments). Cells were dispersed with repeated excursions with a Pasteur pipet. The final digestant was filtered through a stainless steel mesh screen, pore size 133μ, into a 15 ml polystyrene conical centrifuge tube. The incubation flask was rinsed twice with a 0.5 ml portion of KRBG to remove adhering cells and filtered as above. The filtered digestant was chilled on ice for 5 minutes and centrifuged for seven minutes at 1820 rpm in a clinical centrifuge. The supernatant was discarded

and the pellet was washed twice by redispersion in an equal volume of KRBG containing 0.1% BSA (KRBGA), with centrifugations between each wash as described above. The final pellet was dispersed in KRBGA to a concentration of 1 ml per adrenal equivalent. An aliquot of suspended cells was stained with methylene blue and counted in a hemocytometer.

Incubations

The cell suspension was midly vortexed between each addition of 1 ml aliquots to 25 ml erlenmeyer incubation flasks. The final incubation volume was brought to 2 ml with KRBGA. All incubations were carried out in a Dubnoff Metabolic Shaking Incubator at 37°C under an atmosphere of oxygen-95% and carbon dioxide - 5%, with 40 shaking oscillations per minute. Incubations were for 2 hours unless time course experiments were performed. Adrenocorticotropic hormone (ACTH, Acthar Injectible Solution, 40 units, Armour Pharmaceuticals) and the metabolic inhibitors cycloheximide and actinomycin D (Sigma Biochemicals, Inc.) were made up in 0.9% NaCl and added directly to the incubates. A ten minute preincubation period was allowed for inhibitors prior to ACTH stimulation.

To determine stimulation of steroidogenic pathways, 25λ ^{3}H pregnenolone (New England Nuclear, Specific Activity = 17.2 Ci/mole) in absolute methanol was added to the incubation flasks with or without the presence of the inhibitors, cycloheximide or actinomycin D.

To ascertain the identity of the intracellular mediator that induces protein synthesis, isolated adrenal cells with or without cycloheximide were incubated with 2λ each of [4-^{14}C] - cholesterol (New England Nuclear, Specific Activity = 54 mCi/mmole) in absolute methanol and L-[3,4,5-$^{3}H(N)$] - leucine (New England Nuclear, Specific Activity 110 Ci/mmole) in 0.01 N HCl. Two doses of ACTH, 500μU and 10μU; dbcAMP (Sigma Biochemicals, Inc.), 10 mM and 73.46 ng, were added to the incubations.

All incubations were terminated by the addition of 2 ml of reagent grade dichloromethane. The mixture was swirled and poured into a 12 ml glass conical centrifuge tube. Each incubation flask was rinsed with an additional 2 ml of dichloromethane and the washes were added to the original extracts. The centrifuge tubes were vortexed for two ten second periods and centrifuged at 1820 rpm for 10 minutes. The aqueous layer was saved for protein studies. An aliquot of the organic phase was transferred to a 5 ml round bottom culture tube and assayed immediately for precursor

conversion by thin layer chromatography or for corticosterone by radioimmunoassay (RIA) or capped and stored at 4°C until assayed.

Radioimmunoassay

The RIA procedure was modified from that described by Endocrine Sciences (Plasma Corticosterone Radioimmunoassay Procedure, 1972). Briefly, a dilute antiserum was prepared by adding 10 ml of borate buffer, pH 8.0, 15λ (1, 2 - ^{3}H (N) - corticosterone (New England Nuclear, Specific Activity = 60 Ci/mmole), 200λ 0.025% bovine gamma globulin (Sigma Biochemical Inc.) 200λ 10% bovine serum albumin (Sigma Biochemical Inc.) and 150λ corticosterone antiserum (Endocrine Sciences - lot #4-4). One hundred microliters of corticosterone standards and the dichloromethane extracted samples were dried down in 5 ml disposable round bottom culture tubes (12 x 75 mm, Fisher Scientific Co.) in a vacuum oven at 60°C. to each of these RIA tubes 250λ of dilute antiserum was added, vortexed for 10 seconds and incubated at room temperature for 2 hours. Duplicate 250λ of the dilute antiserum was also added to 20 ml scintillation vials (Fisher Scientific Co.) to determine the radioactive counts per antiserum aliquot. Proteins were precipitated by addition of 250λ of saturated ammonium sulfate

followed by vortexing for 5 seconds and centrifuging at 4000 rpm for 13 minutes. A 400λ volume of supernatant was removed and added to scintillation fluid formulated by the addition of 19.0 gm 2,5-diphenyl-oxazole (PPO-scintanalyzed, Fisher) and 1.9 gm 1,4-bis (5-phenyloxazolyl) benzene (POPOP-scintanalyzed, Fisher) to 3.8 liters of toluene. Each vial was vortexed and counted in a liquid scintillation counter (Packard Model 2009) for a 10 minute period with counts per minute recorded.

Thin Layer Chromatography

A 2.5 ml aliquot of the dichloromethane extracts were dried down under a stream of air, taken up with acetone, spotted on thin layer chromatography (TLC) plates coated with Selica Gel G (type 60) for TLC according to Stahl (EM Laboratories Inc.). Standards (1 mg/ml) were spotted in parallel lanes and the plates were developed in a system of chloroform: ethyl ether, 4:1. Standards were visualized by spraying the TLC plates with 50% H_2SO_4 and heated until charred. Regions corresponding to the standards were scraped with a razor blade into a scintillation vial. Ten milliliters of toluene based scintillation fluid was added and vials were counted as above.

Protein Assay

A 1.5 ml aliquot of the aqueous layer was transferred to a 5 ml conical test tube to which an equal volume of cold 20% trichloroacetic acid was added. Tubes were vortexed and centrifuged at 4000 rpm for 10 minutes. The supernatant was removed and the pellet washed twice with 1.5 ml of cold 5% TCA. The resultant pellet was then digested with 0.2 ml of scintgest (Fisher Scientific Co.) at 50°C for one hour. Two milliliters of toluene based counting fluid was added, and each tube was vortexed and transferred to a counting vial. The test tube was washed twice with 2 ml of counting fluid. Final volume of scintillation fluid in each cuonting vial was brought up to 10 ml.

Statistical Analysis

Data were evaluated by two-paired-sample *t* test at 95% confidence level (degree of freedom was four).

RESULTS

The isolation of viable adrenal cells after collagenase digestion produced mean cell counts, $434,000 \pm 61,000$. Using the uptake of methylene blue stain as an indicator for cell viability, it was determined that 96% of the cells were viable.

A dose related release of corticosterone from isolated adrenal cells is shown in Figure 2. This figure shows that the log dose for half maximal stimulation (LD_{50}) is 35 μU ACTH.

The effect of the transcriptional inhibitor Actinomycin D on ACTH stimulated steroidogenesis in adrenal cells is depicted in Figure 3. At a concentration of 4.0 μg/ml, Act D inhibits greater than 98% of transcription (4). A dosage of 12 μg/ml Act D produced no significant change in corticosterone production when compared to non-Act D stimulated cells ($t = 0.393$).

The effect of varying doses of the protein translation inhibitor cycloheximide, on steroidogenesis was studies. The values for the basal release of corticosterone without cycloheximide and after cycloheximide addition were 6.05 ng/100,000 cells, and 6.49 ng/100,000 cells respectively. These values were shown not to be significantly different ($t = 0.709$).

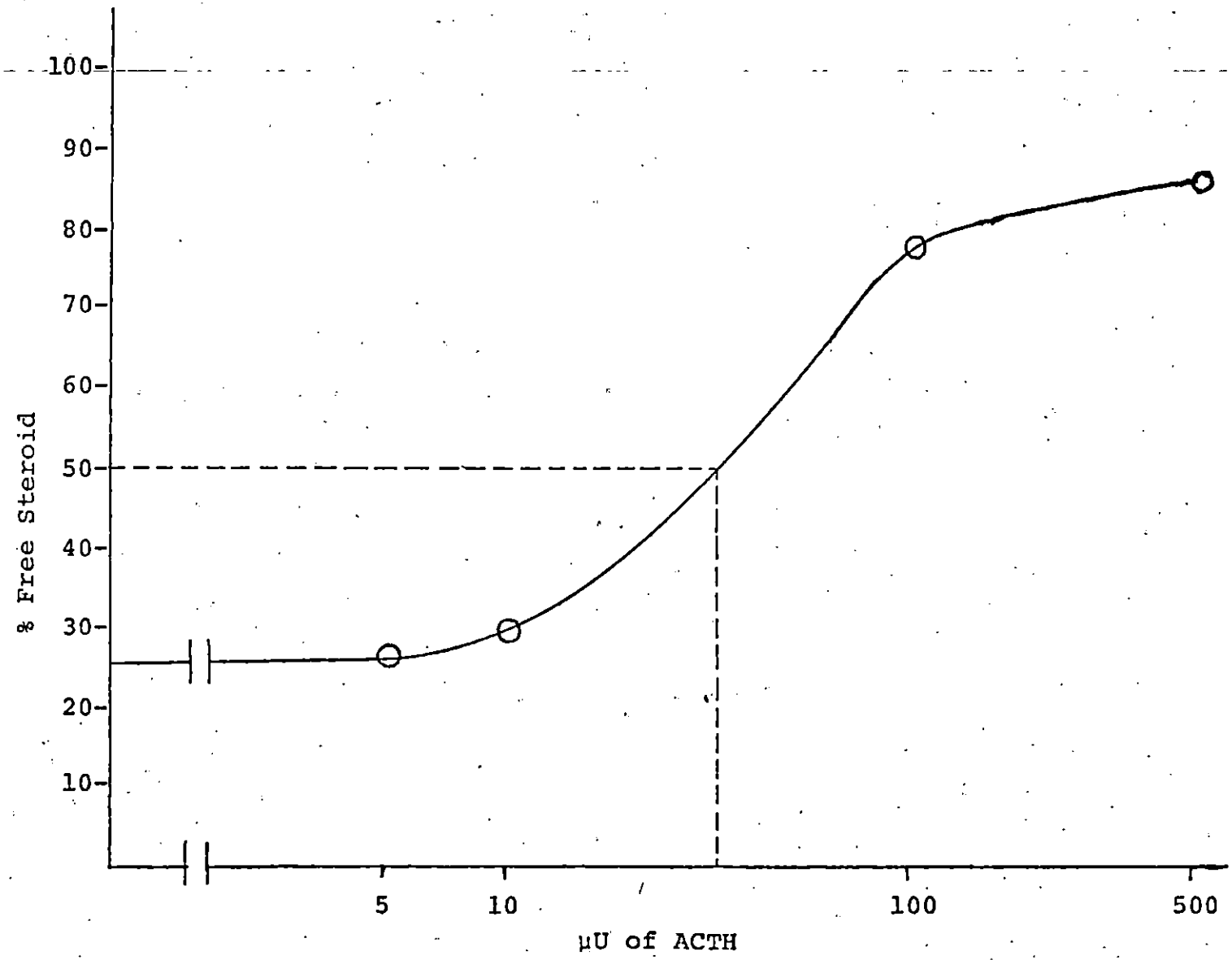

Figure 2. Dose-Response curve of corticosterone production to ACTH stimulation

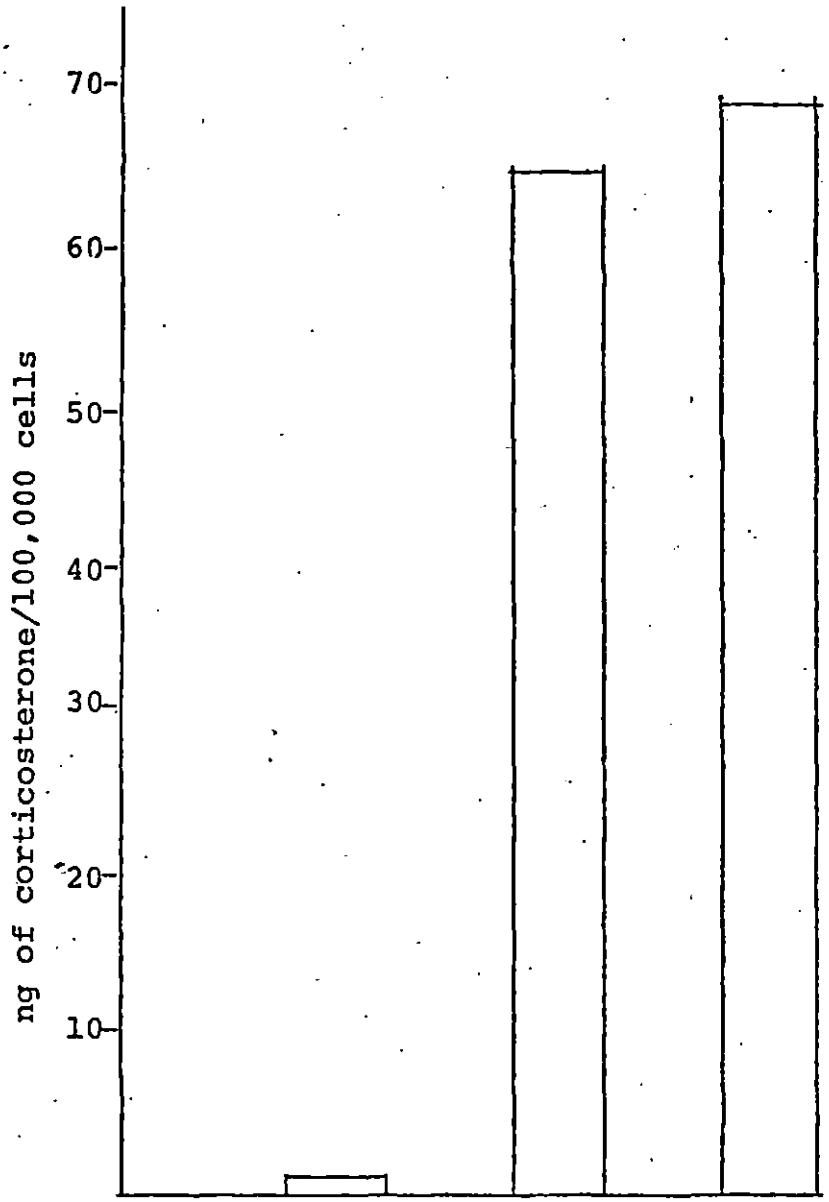

Figure 3. Long term effects of Actinomycin D on corticosterone production. Dosages used were 500μU of ACTH and 12μg/ml of Act D.

Cycloheximide inhibition of ACTH stimulated steroidogenesis is shown in Figure 4. It can be seen that concentrations 0.1 μM, 1.0 μm, and 10 μm of cycloheximide produced respective inhibitors of 16%, 35%, and 84% of a 5000 μU dose of ACTH.

To determine the site of cycloheximide inhibition, ^{3}H-pregnenolone (see Figure 1.) was incubated in an adrenal cell incubation in the presence of 10.0 μM cycloheximide and 500 μU of ACTH. As illustrated in Figure 5 and neither ACTH alone nor ACTH plus cycloheximide affected the conversion of pregnenolone to corticosterone.

To determine activation of protein synthesis by ACTH and cyclic nucleotides, incubations were prepared of isolated adrenal cells with ^{3}H-leucine and ACTH, cAMP, dbcAMP, and cGMP (Table 1). These experiments showed significant incorporation of amino acids into protein using 500 μU ACTH ($t = 0.990$). At physiological levels of 10 μU ACTH and 2 μM cyclic nucleotides, no amino acid incorporation was seen.

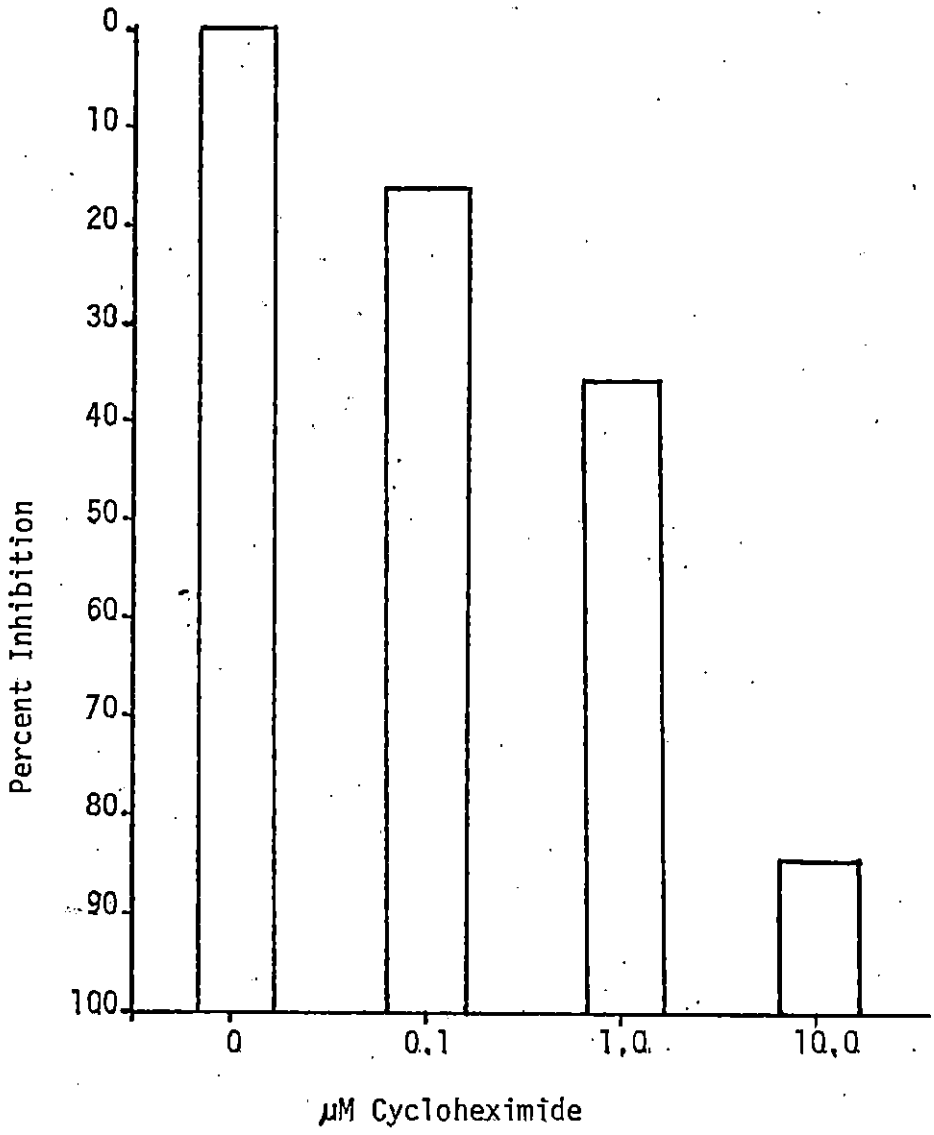

Figure 4. Dose related effect of cycloheximide on the inhibition of corticosterone production in response to 500 μU ACTH.

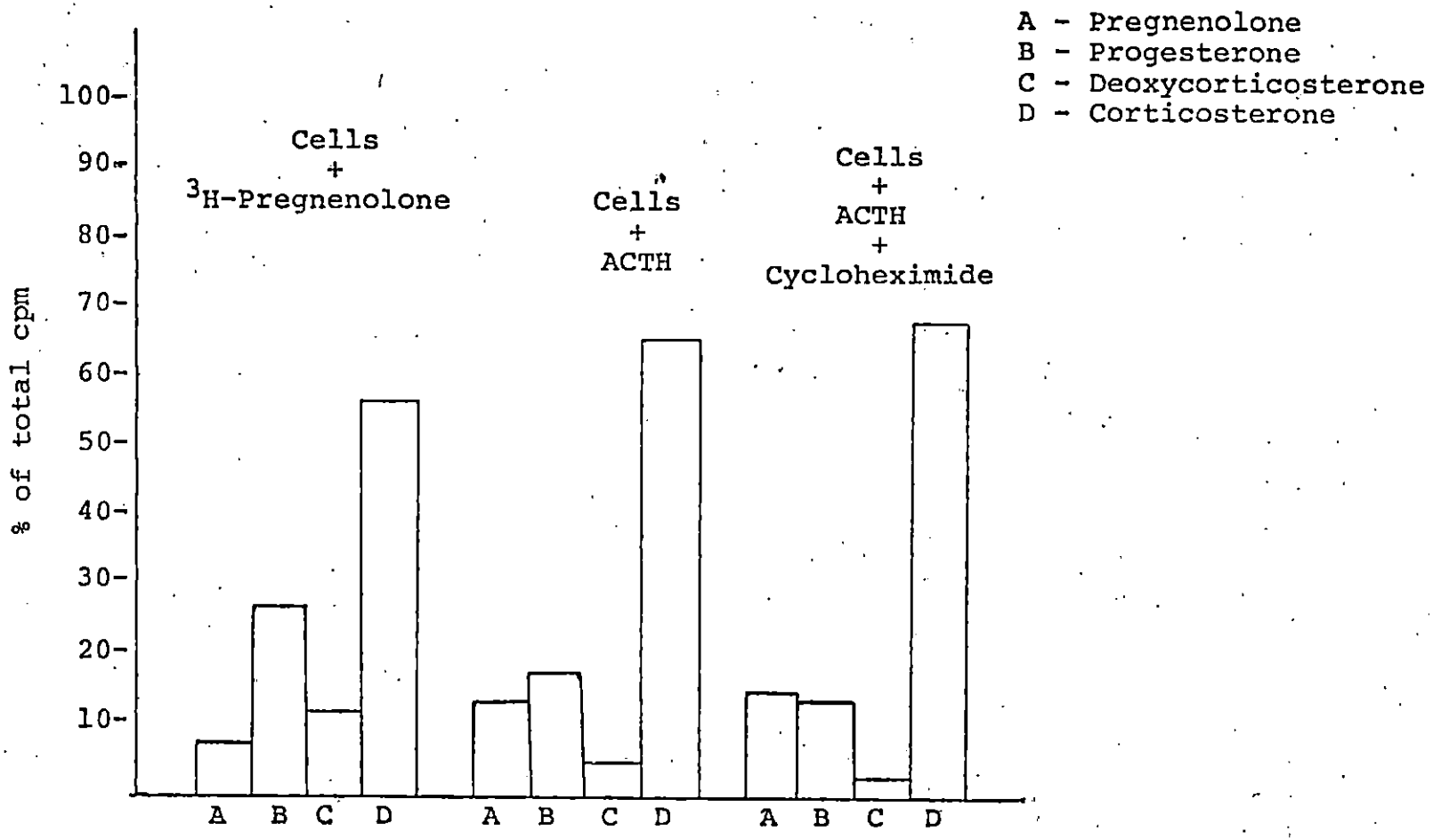

Figure 5. Long term effects of inhibitors on the conversion of ^{3}H-pregnenolone to corticosterone. ACTH was added in the amount of 500μU, and cycloheximide to a final concentration of 10μM.

Table 1. Response of isolated adrenal cells to various factors as observed by ^{3}H-leucine incorporation into proteins.

Treatment	Mean cpm-^{3}H-leucine	% Control	t-level
Control	10,149	—	
+ 500μU ACTH	15,597	154	4.310 *
+ 1.0mM dbcAMP	15,495	153	3.104 *
+ 10mM cAMP	10,880	107	0.040
+ 10mM cGMP	8,644	85	0.990

* Significantly greater from control ($P < .05$).

DISCUSSION

Present concepts of ACTH action are not fully understood. That ACTH binds to adrenocortical cells and induces the formation of an intracellular mediator is strongly proposed. However, whether this mediator is cAMP(2,3,4) or cGMP(10,11) is still to be determined. The action of these cyclic nucleotides is to activate a specific protein kinase which in turn phosphorylates a regulatory protein(13). It has been suggested by Garren(8),that this protein induces the translation of a pretranscribed mRNA. The translated protein is necessary for the stimulated conversion of cholesterol to 20,22-hydroxycholesterol(14). The present study adds support to the hypothesis that ACTH action is prior to the formation of pregnenolone and possesses no apparent stimulatory properties on the conversion of pregnenolone to corticosterone(Figure 5).

Ungar's lab has focused the action of ACTH on stimulating the transport of cholesterol from the cytoplasmic pools into the mitochondria(15). Supporting experiments by Sharma and Sawhney(10) showed that there was no stimulatory effect by ACTH, cAMP or cGMP observed on the conversion of cholesterol to corticosterone in the mitochondria. This proposed,stimulated transport mechanism by ACTH, cAMP and cGMP is shown to

be sensitive to cycloheximide(Figure 4). This investigation showed that Actinomycin D, a transcription inhibitor, did not depress ACTH stimulated steroidogenesis(Figure 3). This evidence adds support to Garren's hypothesis that there is a preformed mRNA which is translated into a protein upon ACTH stimulation(8).

To evaluate the effects of ACTH, cAMP and cGMP on protein synthesis, ^{3}H-leucine incorporation into proteins was studied. It was determined that ACTH and the lipid soluable dbcAMP caused a significant increase in protein synthesis over that of the control incubate(Table 1). However, cAMP and cGMP failed to produce ^{3}H-leucine incorporation into proteins. The stimulation of protein synthesis by dbcAMP but not cAMP may be explained since the dibutyryl derivitive is more permeable to cell membranes than cAMP, and is less likely to be degraded by phosphodiesterase(14).

In summary, this research showed that inhibition of de novo synthesis of mRNA did not prevent cyclic nucleotide or ACTH stimulation of corticosterone formation. Also, the induction of protein synthesis may occur through the unmasking of a preformed mRNA. Finally, pharmacological levels of both cAMP and cGMP failed to induce amino acid incorporation. The dibutyryl derivative was effective. Cycloheximide inhibition of protein synthesis showed that protein synthesis is needed at a site between cholesterol and pregnenalone.

LITERATURE CITED

1. Sutherland, E.W., and Rall, T.W., J. of Amer. Chem. Soc. 79: 3608 (1957).

2. Haynes, R.C., and Berthet, L., J. of Biol. Chem., 225: 115 (1957).

3. Lefkowwitz, J.J., and Roth, J,, Proc. Nat. Acad. Sci., 65: 745 (1970).

4. Shin, S., and Sato, G.H., Biochem. Biophys. Res. Commun. 45: 50] (197]).

5. Sharma, R.K., Ahmed, N.K., and Skazkey, G., Eur. J. of Biochem. 70: 427 (1976).

6. Hall, P.F., and Young, D.G., Endo. 82: 559 (1968).

7. Ferguson, J.J., J. of Biol. Chem. 242: 5535 (1976).

8. Garren, L.P., Gill, G.N., Masui, H., and Walton, G.M., Rec. Prog. Horm. Res. 27: 433 (197]).

9. Koritz, S.B., and Wiesner, R., Proc. Soc. Exper. Biol. Med.]49: 433 (1975).

10. Sharma, R.K., and Sawhey, R.S., Biochem. 17: 316 (1978).

]]. Perchellet, J.P., Shanker, G., and Sharma, R., Science 199: 311 (1978).

12. Greengard, P., Science 199: 146 (1978).

13. Kitbachi, A.E., and Sharma, R.K., Endo. 88: 1109 (1971).

14. Ungar, F., Kan, K.W., and McCoy, K.E., Ann. N. Y. Acad. Sci. 212: 276 (1973).