REQUIREMENT FOR PROTEIN SYNTHESIS IN ADRENOCORTICOTROPIN STIMULATED STEROIDOGENESIS OF ISOLATED RAT ADRENAL CELLS

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ABSTRACT

REQUIREMENT FOR PROTEIN SYNTHESIS IN ADRENOCORTICOTROPIN STIMULATED STEROIDOGENESIS OF ISOLATED RAT ADRENAL CELLS

The mechanism of action of adrenocorticotropin (ACTH) and cyclic nucleotides was studied on isolated adrenal cells prepared from collagenase digestion of female rat adrenals. This study was designed to investigate the specific mechanism in which ACTH stimulates the conversion of cholesterol to pregnenolone, the rate limiting step in the biosynthetic pathway leading to corticosterone. Results were obtained by radioimmunoassay of corticosterone, conversion of radioactive precursors, and the use of inhibitors of DNA translation and protein synthesis.

It was concluded that ACTH and dibutyryl cyclic 3', 5'-adenosine monophosphate (dbcAMP) stimulate protein synthesis as well as steroidogenesis. This stimulation is inhibited by cycloheximide but shows an insignificant decrease in protein synthesis in response to actinomycin D. Furthermore, it was determined that the site of cyclohexmide inhibition is between cholesterol and pregnenolone.

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INTRODUCTION

It is well substantiated that adrenocorticotropin (ACTH) maintains the structure and enzyme activity of the adrenal cortex (Mostafapour and Tchen, 1971; Kolanowski et al, 1977; Mazzocchi et al, 1976; Armato, Andreis and Dragni, 1977). ACTH specifically stimulates the zona fasciculata and zona reticularis of the adrenal cortex to produce the glucocorticoids cortisol (primates) or corticosterone (rodents) (Mostafapour and Tchen, 1971). However, the mechanism of ACTH stimulation of cortical cells is not completely understood.

The stimulation of glucocorticoids by ACTH is not a release mechanism but an activation of steroidogenic pathways (Peron and Koritz, 1958). It is generally accepted that the mechanism of ACTH action is by the first messenger-second messenger hypothesis of Sutherland and Rall (1957). The first messenger, ACTH, has been shown to bind to a pronase sensitive membrane receptor (Muraki, Saito, and Ichikawa, 1976). Receptor binding is followed by the activation of the membrane bound enzyme, adenylate cyclase, both in intact preparations (Grahame-Smith et al, 1967) and in cell free membrane fractions (Grahame-Smith, et al, 1967; Taunton et al., 1969). Adenylate cyclase activation is followed by the

formation of the internal second messenger cyclic-3', 5'-adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) (Haynes and Berthet, 1957; Lefkowitz et al., 1970; Shin and Sato, 1971). At doses of 5×10^{-6}M, cAMP mimicks the action of ACTH on steroidogenesis (Grahame-Smith et al., 1967; Shin and Sato, 1971; Sharma, 1973). Cyclic AMP appears to activate a protein kinase, an enzyme which transfers a high energy phosphate group from ATP to a protein molecule involved in steroidogenic activation (Sharma et al., 1976).

A recent theory has been proposed that has implicated cyclic-3', 5' guanosine monophosphate (cGMP) as the second messenger of ACTH in the adrenal cell (Sharma and Sawhney, 1978; Perchellet et al., 1978). It has been reported that physiological levels of ACTH in an isolated rat adrenal cell preparation, stimulated corticosterone production without increasing cAMP levels (Beall and Sayers, 1972). However, cGMP levels were increased at these concentrations (Sharma and Sawhey, 1978; Perchellet et al., 1978). A specific cGMP protein kinase has been suggested (Sharma, 1976). In addition to cGMP and cAMP, cyclic-3', 5'-inosine monophosphate and dibutyryl cyclic 3', 5'-adenosine monophosphate (dbcAMP) have been shown to mimick the effects of ACTH (Kitabchi and Sharma, 1971). Kitabchi and Sharma (1971), suggested that the cyclic-3',

5'-monophosphate structure was the determinate factor in steroidogenic regulation. That a cyclic nucleotide is not the exclusive intracellular mediator of ACTH action on adrenal cortical cells is suggested by several reports that calcium is a necessary requirement for steroidogenesis (Farese, 1971; Beall and Sayers, 1972; Birmingham and Bartova, 1973).

It has been determined that the rate limiting step for ACTH stimulation of steroidogenesis in the adrenal cell is between cholesterol and pregnenolone (Sharma, 1973). The pathway of corticosterone production has been summarized in Figure 1. Sharma (1973) has shown that ACTH has no effect on the transformation of pregnenolone to corticosterone. Cycloheximide, a specific inhibitor of protein translation (Shin and Sato, 1971; Koritz and Wiesner, 1975), has been used to show inhibition of ACTH stimulated steroidogenesis prior to the formation of (20 S)-20-hydroxycholesterol (Kitabchi and Sharma, 1971). Therefore, this cycloheximide sensitive step was prior to cholesterol side chain cleavage. Neither cAMP (Sharma, 1973a)nor cGMP (Sharma, 1974) had an effect on (20 S)-20-hydroxysteroid transformation to corticosterone. This suggests ACTH acts by stimulating the translation of a protein which acts between cholesterol and (20 S)-20-hydroxycholesterol. Furthermore, this protein has been

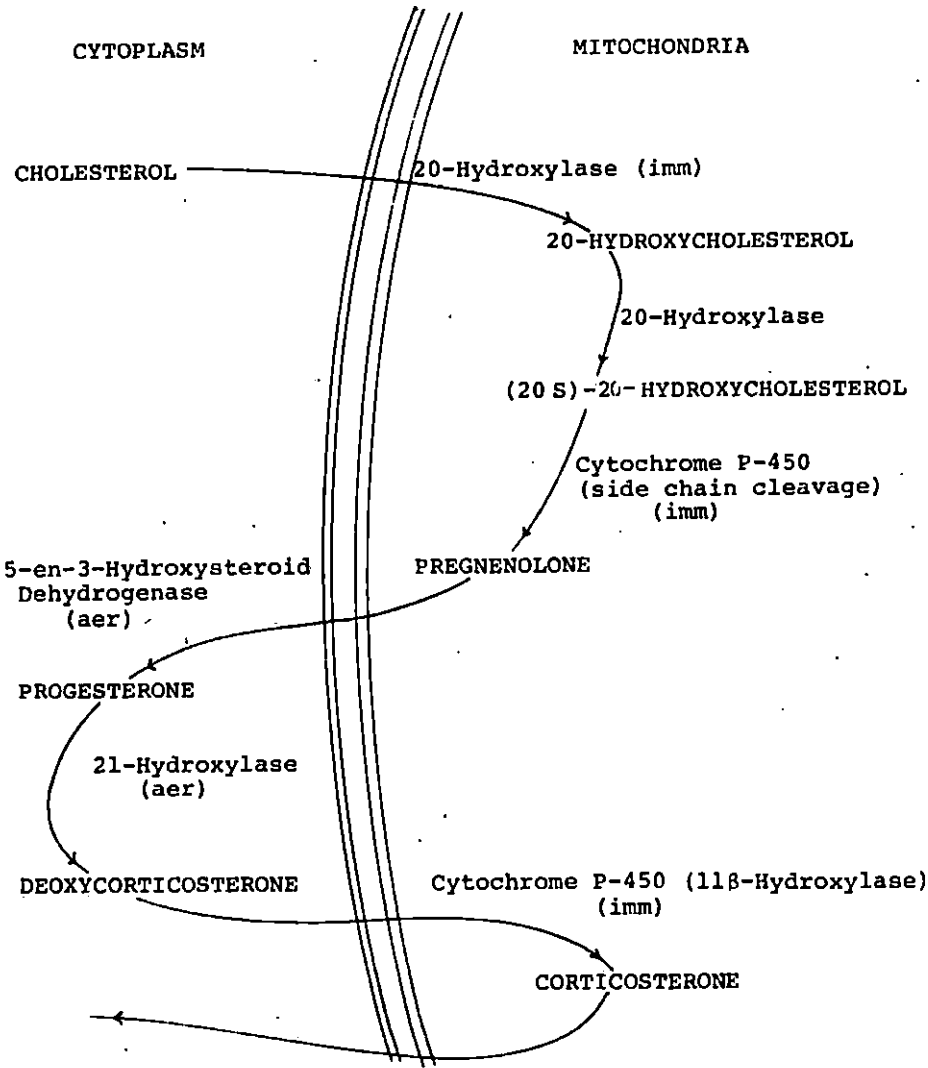

Figure 1. Biosynthesis of corticosterone from cholesterol as compiled from Simpson, Waters and Williams-Smith (1975), Gower (1974), Tamaoki (1973) and Sharma (1973).

imm = inner mitochondrial membrane

aer = agranular endoplasmic reticulum

speculated to be derived from a preexisting messenger RNA (Garren et al., 1971). This labile protein has been proposed to have a half life of eight minutes (Koritz and Wiesner, 1975). Further speculation on the site of cycloheximide inhibition has been presented by Ungar, Kan and McCoy (1973) and summarized in Figure 2. Also presented in this scheme is the hypothesis of Koritz and Wiesner (1975) that rather than protein synthesis, there is an activation of a masked protein. Ungar, Kan and McCoy (1973) believe the activation to be a cholesterol carrier protein.

ACTH stimulation was demonstrated to increase intramitochondrial supply of cholesterol (Ungar, Kan and McCoy, 1973). Supporting this hypothesis has been the observation that the stimulation of steroidogenesis by ACTH decreases the size of the cholesterol pool contained in lipid droplets in the cytoplasm of steroidogenic cells (Gillim, Christensen and Melennan, 1969). Ungar, Kan and McCoy (1973) also proposed that ACTH caused a change in mitochondrial membrane permeability. They theorized that mitochondrial permeability changes might result from the activation of a cholesterol carrier protein. Phospholipid production is also stimulated by ACTH (Ungar, Kan and McCoy, (1973). Phospholipid micelles solubilize cholesterol and enhance

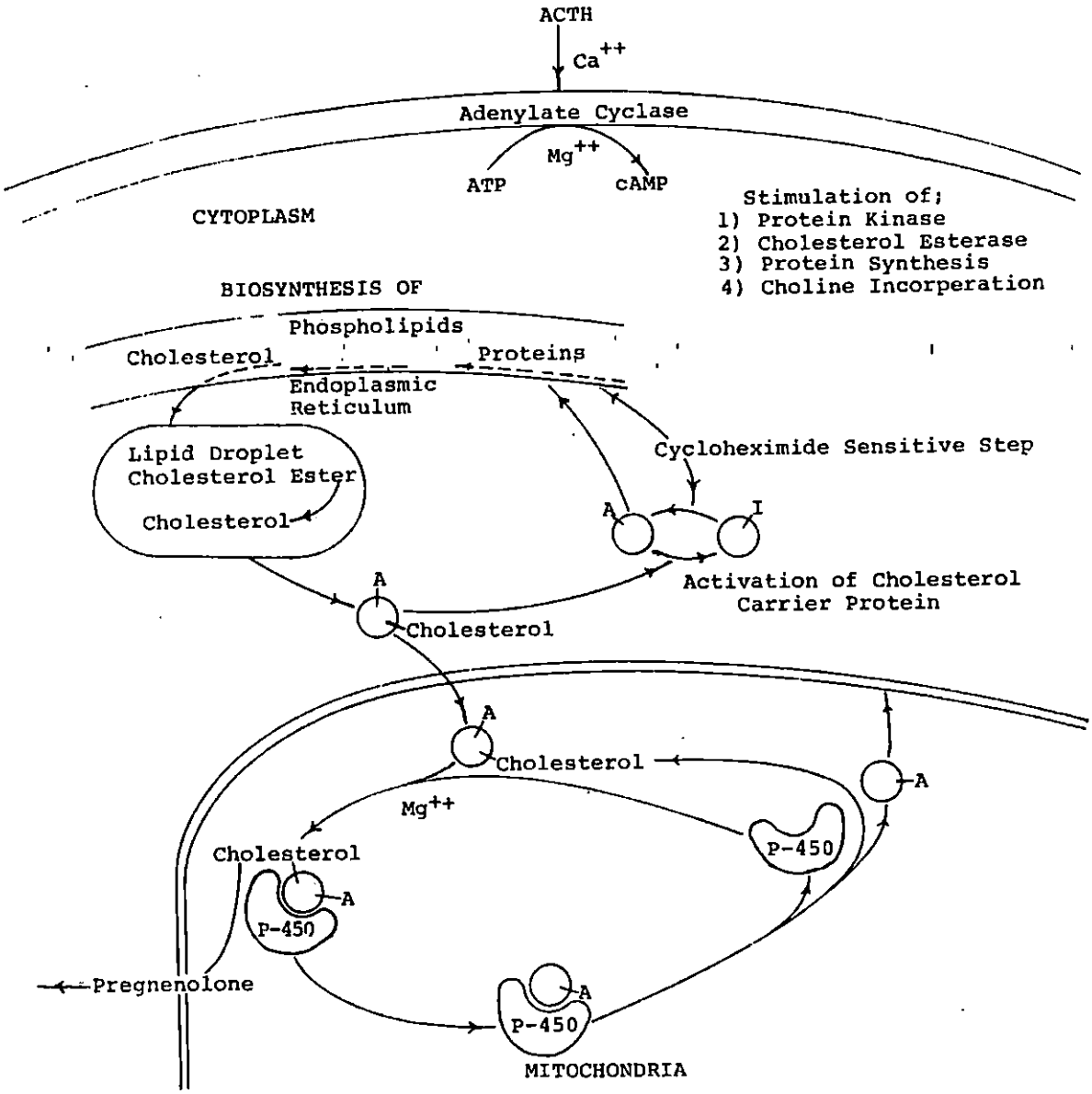

Figure 2. Site of cycloheximide inhibition in the conversion of cholesterol to pregnenolone as described by Ungar, Kan and McCoy (1973).

its intracellular transport (Guraya, 1971).

In addition to the activation of protein synthesis by the activation a preformed messenger RNA (mRNA) (Garren et al., 1971; Pearlmutter, Rapino and Saffran, 1973), an additional factor which resembles an RNA species and characterized with a 6-7 hour half life and several hour production rate has been proposed (Mostafapour and Tchen, 1971).

Experiments employing the transcription inhibitor actinomycin D (Act D) suggest another factor involved in the control of steroidogenesis (Reich et al, 1962; Shin and Sato, 1971). Administration of actinomycin D potentiates the effect of ACTH. It has been postulated that Act D inhibits the transcription of a mRNA that codes for an inhibitory protein. The result would be a decline in the amount of inhibitor present in the cell. The build up of this inhibitor would explain the presence of a delayed refractory period (Mostafapour and Tchen, 1972).

Although cycloheximide inhibits both protein synthesis and corticosterone production, some researchers do not believe that ACTH acts by increasing the rate of synthesis of a labile protein (Koritz and Wiesner, 1975). Instead they contend that ACTH affects the activation of a rapidly turning over protein.

Following the introduction of ACTH, there is a lag period of disputed duration before corticosterone production can be detected. According to Schulster and Jenner (1975) the lag period was 24 seconds. Pearlmutter, Rapino and Saffran (1973) claim that the lag period after ACTH, cAMP and dbcAMP administration were all 3 minutes. This indicates that the lag period is due to processes following the activation of cAMP. Beall and Sayers (1972) showed a rise in cAMP within one minute after the introduction of 1000 μU of ACTH. Corticosterone production could not be measured until after a two minute lag period (Beall and Sayers, 1972). If a protein is synthesized after ACTH stimulation, synthesis could account for this lag period in corticosterone production. In vivo studies in mammals show that protein synthesis proceeds at a rate varying from 40 amino acids to 600 amino acids per minute (Pearlmutter, Rapino and Saffran, 1973). Therefore a lag period of 3 minutes would enable the production of a protein 120 to 1800 amino acids in length. If the lag period is 24 seconds, a protein of 16 to 240 amino acids long may be synthesized. Schulster and Jenner (1975) feel that a 24 second lag period is not sufficient for the translation of a protein. They proposed a preexisting labile protein that facilitates the conversion of cholesterol to pregnenolone. Inhibition of protein

synthesis would cause a decrease in steroidogenesis due to a decrease in the cells content of the labile precursor protein (Schulster and Jenner, 1975).

It is generally accepted that ACTH acts via a second messenger. But the support that cAMP is the exclusive mediator has been questioned. It has been shown that cAMP may not have an obiligatory role in stimulating corticosterone secretion (Ramachandran, Long, and Liles, 1976). Evidence that weakens the possible role of cAMP as the sole intracellular mediator of ACTH was presented by Beall and Sayers (1972), which showed that low doses of ACTH (below 25uU) stimulated corticosterone production but without a detectable increase in cAMP. Therefore, it is postulated that cAMP mediates only some of the actions of ACTH.

The following study proposes to clarify the intracellular molecular mechanisms through which ACTH acts on the isolated rat adrenal cell, and to present supportive evidence of these mechanisms of action. Specifically, which cyclic nucleotide mediates ACTH action? Is protein synthesis involved? At what point are the steroidogenic pathways activated?

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. This solution was 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 eighths. Pooled adrenals were rinsed with 10 ml of cold Krebs Ringer Bicarbonate buffer (Umbreit, Burris, and Stauffer, 1959), to which 200 mg% glucose was added (KRBG).

Isolation of Adrenal Cells

All glassware used in the isolation procedure was siliconized with Siliclad (Clay-Adams), rinsed in distilled water and dried in an oven at 60°C. 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 eighths were transfered to a 25 ml erlenmeyer flask containing a digesting medium consisting of the following constituents per pair of adrenals: 1 ml 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 35 minutes in a Dubnoff Metabolic Shaking Incubator (Precision Instruments). Cells were dispersed with approximately 30 repeated excursions with a Pasteur pipet. The digestant was incubated for another 10 minutes and redispersed with an additional 20 excursions. 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 0.5ml 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 (Precision Scientific Co.). The supernatant was aspirated with a Pasteur pipet and discarded. 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 extra 0.3 ml was added for final cell counts.

Cell counts were taken after each cell isolation to determine the number of cells per adrenal equivalent. Two drops of a methylene blue solution were added to the cell suspension and the stained cells were counted with a hemocytometer. An average of two counts was recorded.

Incubations

The cell suspension was mildly vortexed (Vortex-genie, Scientific Industries, Inc.) between each addition of 1 ml aliquots with an automatic pipet (Oxford Sampler, Multirange Micropipetting System, Oxford Laboratories) 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^{o}C under an atmosphere of oxygen-95% and carbon

dioxide - 5%, with 40 shaking oscillations per minute. Incubations were for 2 hours unless short term 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/mmole) 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 Biochemical, Inc.), 1 mM and 98.26 ng; cAMP (Sigma Biochemical, 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 (Fisher) with the aid of a repipetor (Scientific Products). The mixture was swirled and poured into a 12 ml glass conical centrifuge tube. Each incubation 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 aspirated and discarded or saved for radioactive 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 $4^{o}C$ 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) 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 vials along with 10 ml of Insta Gel (Packard Inc.) scintillation fluid or a 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 positioned into a liquid scintillation counter (Packard Model 2009) and left for 5 hours before counting to allow for settling and a decrease of chemoillumination. Counts were taken 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 Silica 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 counting vial was brought up to 10 ml.

Statistical Analysis

Data were evaluated by the two-tailed paired-sample t test at 95% confidence level with a degree of freedom of four (tabulated $t = 2.776$).

RESULTS

The isolation of adrenal cells after collagenase digestion produced cell counts varying from 334,000 to 550,000 cells per adrenal equivalent. The mean cell count with standard deviation was $434,000 \pm 61,000$. Using the uptake of methylene blue stain as an indicator for cell viability, it was determined that 96% of the cells were viable. Only those cells stained with methylene blue and with large lipid droplets were counted.

A dose related release of corticosterone from isolated adrenal cells is shown in Figure 3. Corticosterone production is expressed in percent free steroid with the calculation as follows: Percent free steroid = $(x/0.8y) \times 100$, where x = cpm of free ^{3}H-corticosterone and y = total cpm added to each tube. Figure 3 shows the log dose for half maximal stimulation (LD_{50}) is 35 μU ACTH. A dose of 500 μU of ACTH was used for the succeeding experiments unless stated otherwise.

The effect of the transcriptional inhibitor Actinomycin D on ACTH stimulated steroidogenesis in adrenal cells is depicted in Figure 4. At a concentration of 4.0 μg/ml, Act D inhibits greater than 98% of transcription (Shin and Sato, 1971). A dosage of 12 μg/ml Act D produced no significant change in corticosterone

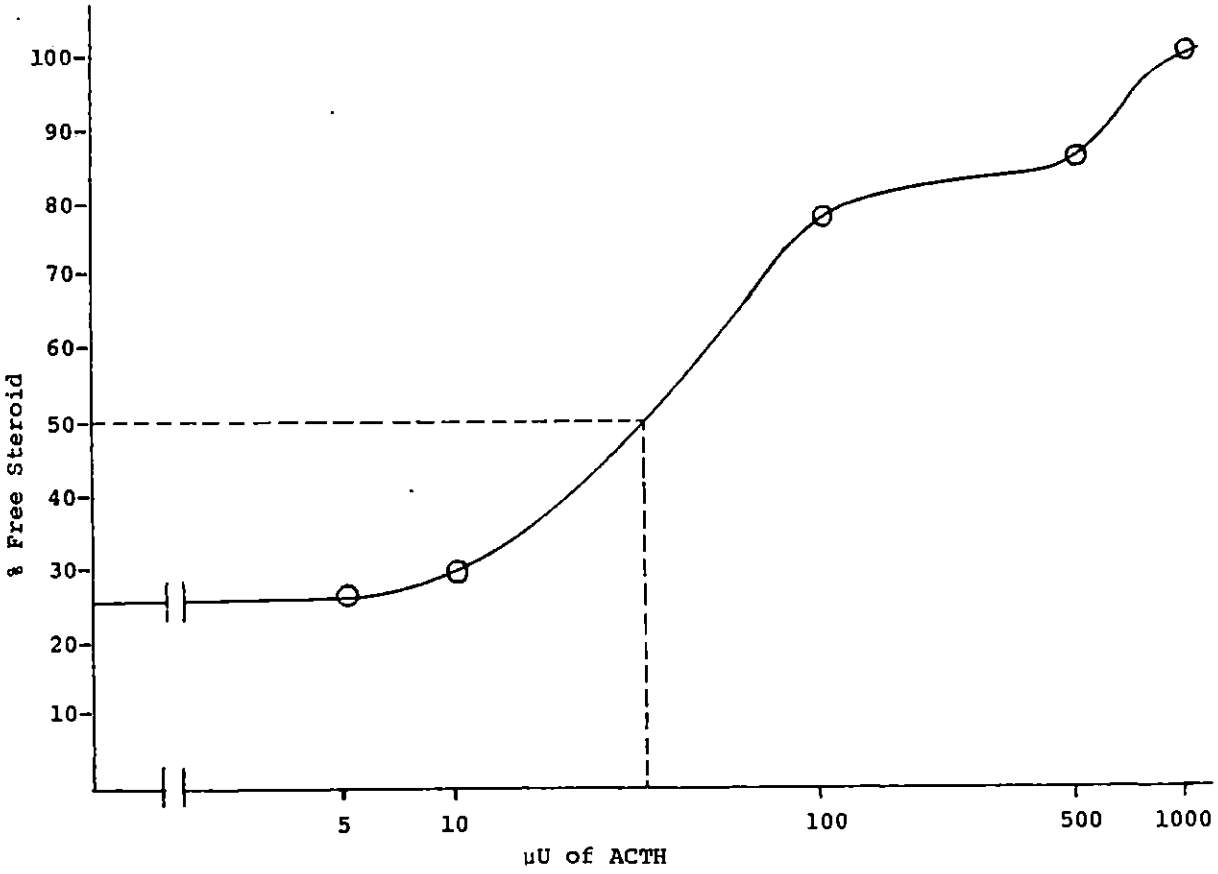

Figure 3. Dose-Response curve of corticosterone production to ACTH stimulation

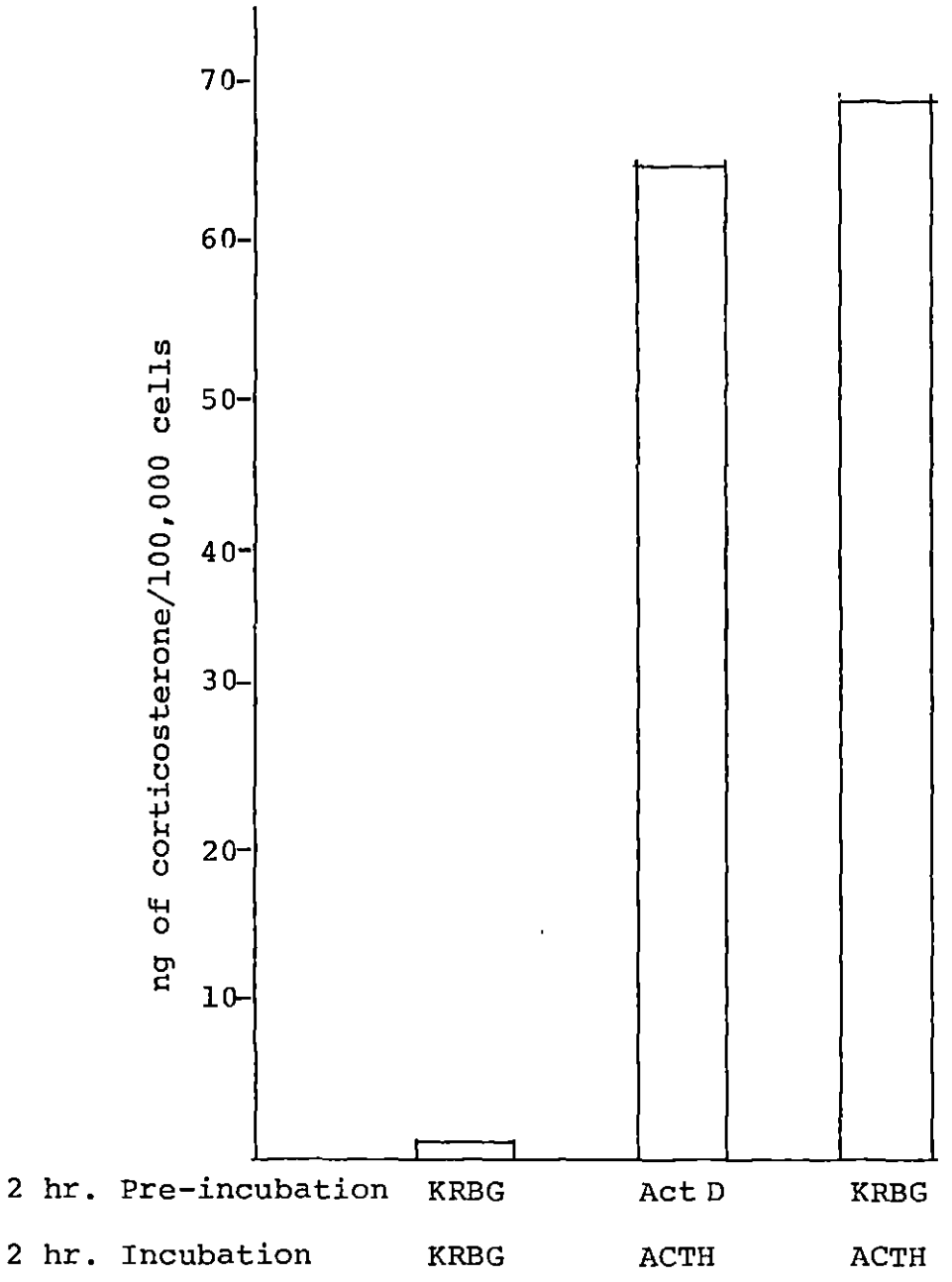

Figure 4. Long term effects of Actinomycin D on corticosterone production. Dosages used were 500μU of ACTH and 12μg/ml of Act D.

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 studied. 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 ($t = 0.709$).

Cycloheximide inhibition of ACTH stimulated steroidogenesis is shown in Figure 5. It can be seen that concentrations of 0.1 μM and 1.0 μM cycloheximide produced a 65% inhibition of 500 μU dose of ACTH. A 10.0 μM concentration of cycloheximide inhibited ACTH stimulation by 84%.

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 6 and tabulated in Table 1, neither ACTH alone nor ACTH plus cycloheximide inhibit the conversion of pregnenolone to corticosterone. However, an apparent inhibitory effect of Act D on ACTH stimulated steroidogenesis is observed. This inhibition proved to be insignificant.

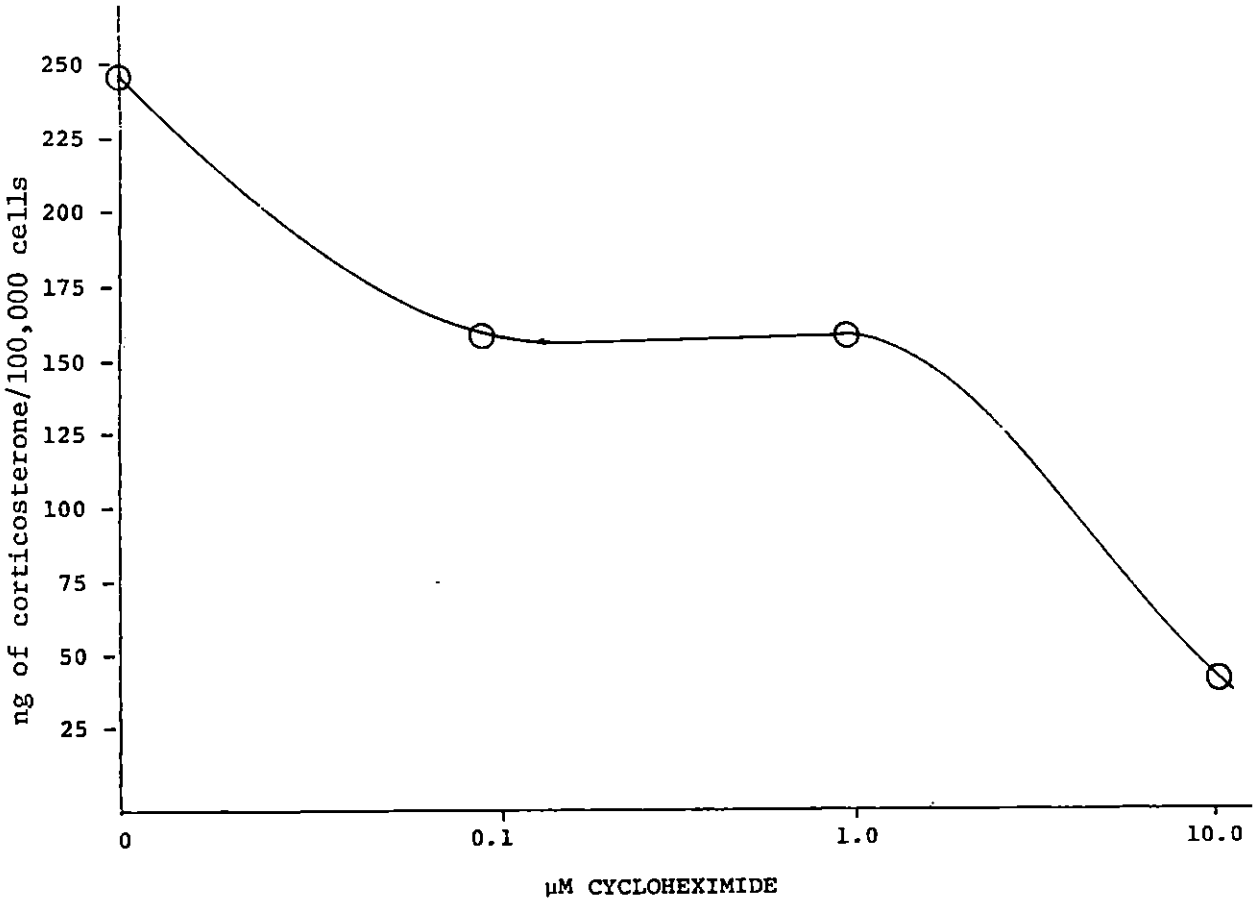

Figure 5. Effects of varying concentrations of cycloheximide on corticosterone production in response to 500 μU ACTH.

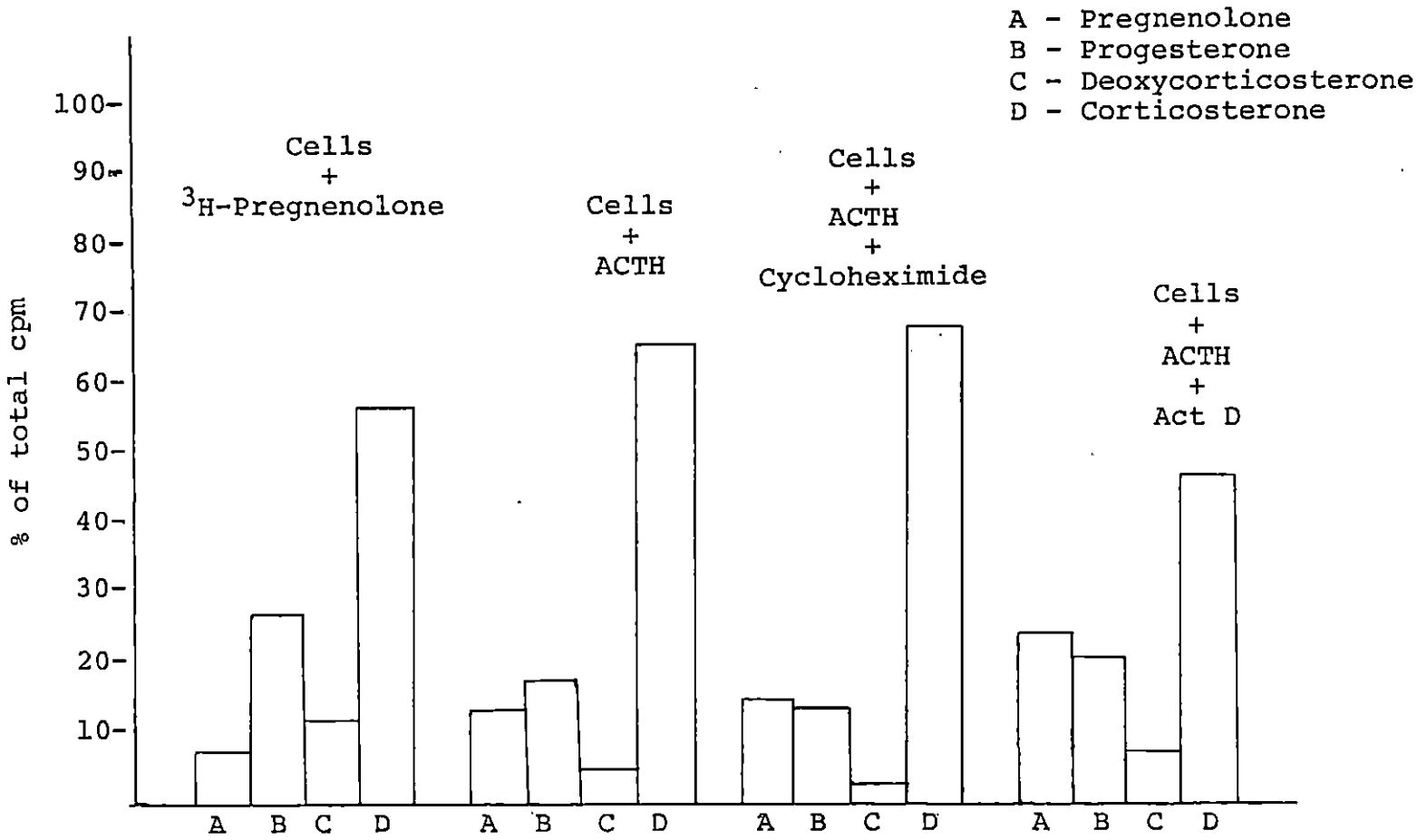

Figure 6. Long term effects of inhibitors on the conversion of ^{3}H-pregnenolone to corticosterone. ACTH was added in the amount of 500μU, and cycloheximide to a final concentration of 10μM.

Table 1. Effect of inhibitors on ^{3}H-pregnenolone conversion to corticosterone in isolated adrenal cells.

Treatment	% of total cpm				
	Pregnenolone	Progesterone	Deoxycorticosterone	Corticosterone	t value comparison to ACTH stimulation
^{3}H-Pregnenolone	5.92	26.00	11.95	56.07	1.28
+ ACTH [a]	12.71	17.14	4.48	65.68	—
+ ACTH + + Cycloheximide [b]	14.8	14.28	2.95	67.97	0.235
+ ACTH + + Act D [c]	24.02	20.73	7.78	49.16	1.72

a. 500 µU ACTH b. 10.0 µM Cycloheximide c. 12 µg Act D

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 2). These experiments showed significant incorporation of amino acids into protein using 500 μU ACTH ($t = 4.310$) and dbcAMP ($t = 3.104$). Protein production was not significant from controls using cAMP ($t = 0.040$) or cGMP ($t = 0.990$). At physiological levels of 10 μU ACTH and 2 μM cyclic nucleotides, no amino acid incorporation was seen.

Table 2. 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	8,6440	85	0.990

DISCUSSION

The mechanism of action of ACTH is not completely understood. It is well substantiated that ACTH binds to the adrenocortical cells and induces the formation of a cyclic nucleotide. Whether this nucleotide is cAMP (Haynes and Berthet, 1957; Lefkowitz et al., 1970; Shin and Sato, 1971) or cGMP (Sharma and Sawney; 1978; Perchellet et al., 1978) is still to be determined. The action of this cyclic nucleotide is to activate a specific protein kinase which in turn phosphorylates a regulatory protein. It has been suggested by Garren et al. (1971) that this protein induces the translation of a pretranscribed mRNA. The resultant protein is necessary for the stimulated conversion of cholesterol to (20 S)-20-hydroxycholesterol (Kitabchi and Sharma, 1971). 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 6).

Ungar et al. (1973) along with Sharma and Sawney (1978) have focused the action of ACTH on stimulating the transport of cholesterol from the cytoplasmic pools into the mitochondria. Supporting experimentation by Sharma and Sawhney (1978) showed that there was no

stimulatory effect by ACTH, cAMP or cGMP observed on the conversion of mitochondrial cholesterol to corticosterone. This proposed stimulated transport mechanism of cholesterol by ACTH, cGMP and cAMP (Sharma and Sawhney, 1978) has been shown to be sensitive to the translation inhibitor cycloheximide (Figure 5). Unger et al. (1973) suggest that cycloheximide inhibited the production of a cholesterol carrier protein or a protein that changed mitochondrial membrane permeability. Further investigation using Act D, a transcription inhibitor, showed insignificant depression of ACTH stimulated steroidogenesis (Figure 4). Thus adding support to Garren et al. (1973) hypothesis that there is a preformed mRNA which is translated into a protein in response to ACTH stimulation.

Results reported in this paper of Act D action on isolated adrenal cells are in contradiction to those reported by Mostafapour and Tchen (1972). They observed a stimulation of steroidogenesis when cells were incubated with Act D. Their explanation was that Act D prevented the transcription of a mRNA that codes for an inhibitory protein. Results reported in Figure 4 show in contrast, a slight inhibitory effect induced by Act D.

Previous experimentation by other researchers have suggested that cAMP (Haynes and Berthet, 1957; Lefkowitz

et al., 1970; Shin and Sato, 1971) or cGMP (Perchellet et al., 1978; Sharma and Sawhney, 1978) acts as the intracellular mediator for ACTH. A study was performed to observe the effects of these cyclic nucleotides and ACTH on protein synthesis. Effects were observed by determining the amount of ^{3}H-leucine incorporation into proteins. It has been shown that ACTH and dbcAMP caused a significant increase in protein synthesis over that of a control incubate (Table 2). However when isolated adrenal cells were stimulated with cGMP and cAMP, they failed to produce a significant increase in ^{3}H-leucine incorporation into proteins (Table 2). The difference in dbcAMP and cAMP action can be explained by results obtained by Kitabchi and Sharma (1971). They observed that dbcAMP is more permeable to cell membranes than cAMP, and is less likely to be degraded by phosphodiesterase. Therefore dbcAMP is a more potent stimulator than cAMP.

In conclusion, it may be said that inhibition of mRNA synthesis did not affect ACTH or cyclic nucleotide action. At pharmacological doses, dbcAMP mediates ACTH action on adrenocortical cells with respect to the induction of protein synthesis from a preformed mRNA. Pharmacological levels of both cAMP and cGMP failed to induce amino acid incorporation. The action of cyclic

nucleotides is by activation of protein synthesis. Inhibition of protein synthesis proved that this site of action is between cholesterol and pregnenolone.

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