ABSTRACT

Denise Kornegay Ward. SOME STUDIES ON ADENYL CYCLASE. (Under the direction of Dr. Sam N. Pennington) Department of Biology, 1973.

Adenyl cyclase from several mammalian as well as bacterial sources was studied. Highest specific activity was observed in rat cerebral cortical tissue and lowest specific activity was noted in bacteria. An increase in specific activity was noted when hormones and fluoride ion were added to the animal tissue. F^- also caused an increase in the specific activity of the bacterial cyclase but hormones elicited a decrease. The detergent Luberol-PX proved to be an effective solubilizing agent for adenyl cyclase from animal tissue. This detergent solubilized adenyl cyclase not only from heart tissue, as previously reported, but also solubilized the enzyme from brain and liver tissue. The solubilized tissue, in agreement with previous work, did not show normal hormonal response. The detergent was removed using a DEAE cellulose column. No activity could be recovered for brain adenyl cyclase from the column. Heart specific activity was higher off the column than before being placed on the column; however, the normal hormonal response was still not present and with the addition of the hormones there was a decrease in specific activity. Phospholipids did in some cases increase the specific activity over that of enzyme and hormone together. The bacterial adenyl cyclase was inhibited by the addition of hormones. Phospholipids plus hormones increased the specific activity over that where only hormones were present.

SOME STUDIES ON ADENYL CYCLASE

A Thesis

Presented to

the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts in Biology

by

Denise Kornegay Ward

August 1973

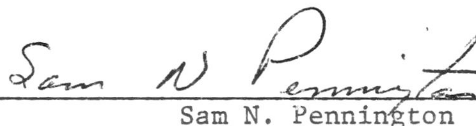
J. Y. JOYNER LIBR...
EAST CAROLINA UNIV.

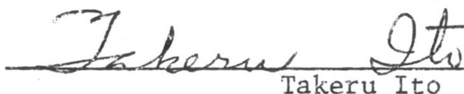
SOME STUDIES ON ADENYL CYCLASE

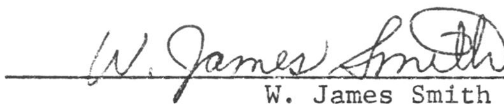
by

Denise Kornegay Ward

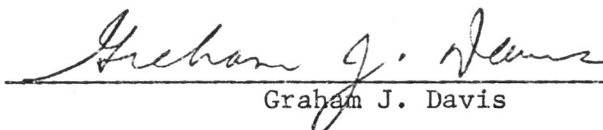
APPROVED BY:

SUPERVISOR OF THESIS 
Sam N. Pennington

COMMITTEE MEMBER 
Takeru Ito

COMMITTEE MEMBER 
W. James Smith

CHAIRMAN OF THE DEPARTMENT OF BIOLOGY


Graham J. Davis

DEAN OF THE GRADUATE SCHOOL

Joseph G. Boyette

500911

ACKNOWLEDGMENTS

I wish to acknowledge the valuable assistance and advice rendered by Dr. Sam N. Pennington, Dr. W. James Smith and Dr. Takeru Ito.

TABLE OF CONTENTS

	Page
LIST OF FIGURES	iv
LIST OF TABLES	v
I. Introduction	1
A. Review of Literature	6
B. Purpose of this Study	18
II. Materials and Methods	20
III. Results and Discussion	30
IV. Literature Cited	50
V. Appendices	52
A. Table of Abbreviations	53
B. Chemicals and Apparatus	54

LIST OF FIGURES

FIGURE Page

I. Reaction catalyzed by adenyl cyclase 3

II. Reaction catalyzed by phosphodiesterase 5

III. Sutherland's model of adenyl cyclase 9

IV. Robinson's model of adenyl cyclase 12

V. Chromatogram of cyclic-AMP separating on Dowex 50 24

VI. Standard curve for cyclic-AMP determination using anhydrous cyclic-AMP 27

VII. Standard curve for protein determination by biuret reagent 29

VIII. Effect of increasing concentration of bacterial protein on formation of cyclic-AMP 32

IX. Effect of increasing concentration of rat cerebral cortical adenyl cyclase 34

X. Effect of increasing concentration of rat cerebral cortical solubilized adenyl cyclase 36

XI. Formation of cyclic-AMP as a function of time 38

XII. Effects of hormones on heart adenyl cyclase 41

XIII. Effects of hormones on brain adenyl cyclase 44

LIST OF TABLES

TABLE		Page
I.	Effects of hormones and phospholipids on solubilized adenyl cyclase from rat heart	45
II.	Effects of hormones and phospholipids on bacterial adenyl cyclase	47

INTRODUCTION

The role of cyclic 3',5' adenosine monophosphate (cyclic-AMP) as the intercellular mediator of the glycogenolytic effect of epinephrine and glucagon in the liver was discovered by Sutherland and his co-workers. In 1957 these workers reported that cyclic-AMP could be produced from adenosine triphosphate (ATP) by tissue slices from liver, heart, brain and skeletal muscle (1). Since this report, much interest has been generated in cyclic-AMP as witnessed by the voluminous literature. This interest has been for the most part due to the biochemical role of cyclic-AMP.

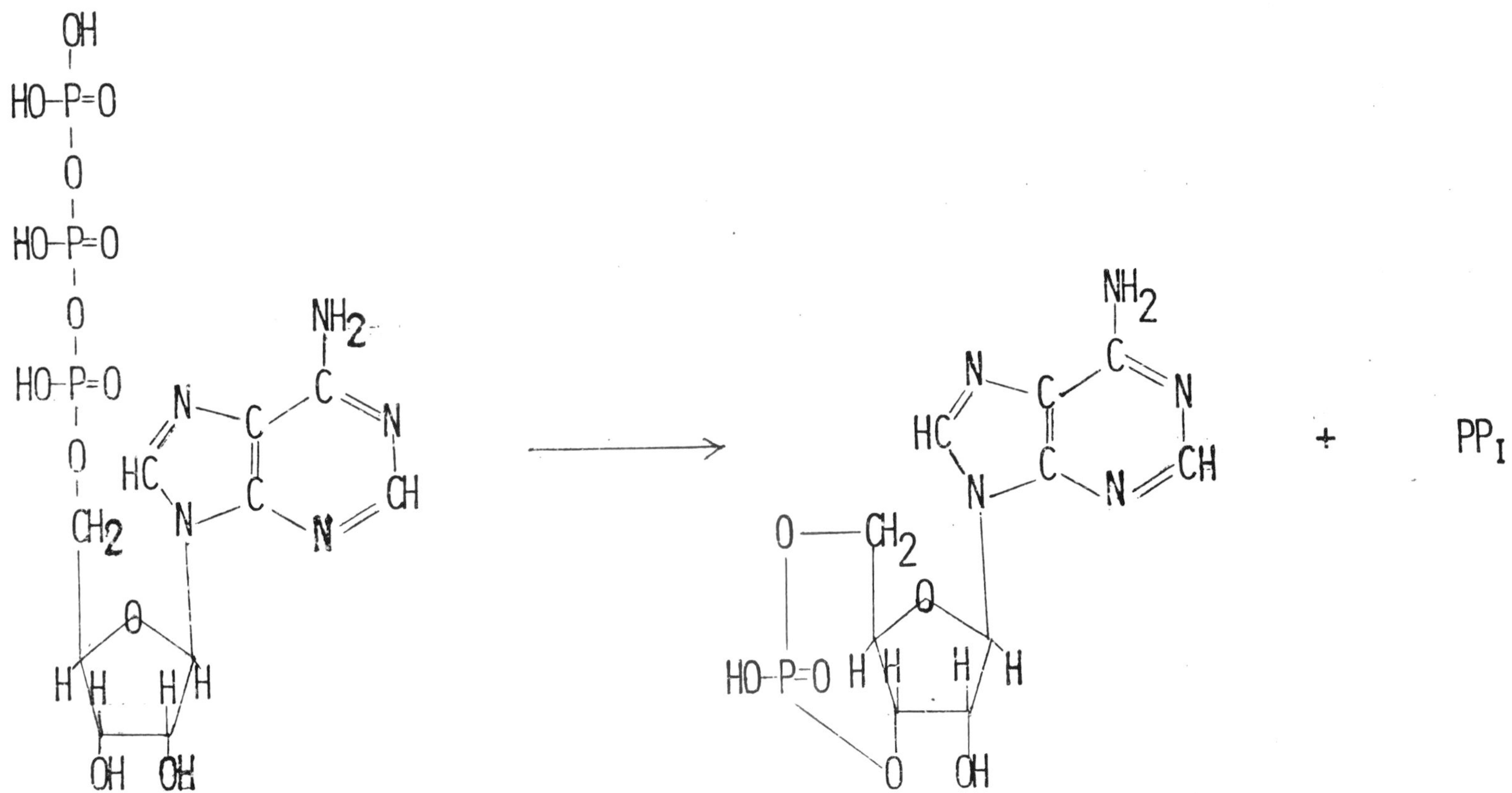
Cyclic-AMP is recognized as a regulator of cell function through its part in many biochemical pathways. This regulatory control is based on the level of cyclic-AMP in the cell. This level is directly dependent upon at least two enzyme systems. One of these, adenyl cyclase, is responsible for catalyzing the conversion of ATP to cyclic-AMP according to the reaction shown in Figure I.

The other enzyme is phosphodiesterase (PDE) which degrades cyclic-AMP to 5'-adenosine monophosphate (5'-AMP) according to the reaction shown in Figure II.

Many of the actions of hormones are thought to be related to their ability to alter the intercellular level of cyclic-AMP. Hormones or neurohumors are chemicals produced by one set of cells that effect a

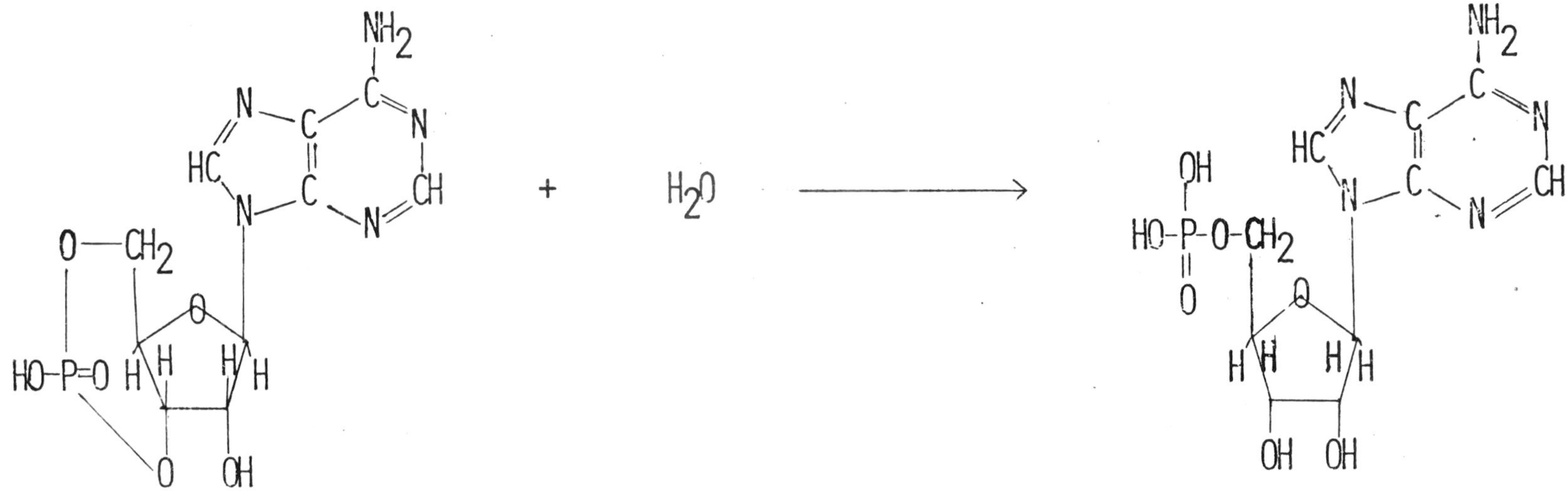
Legend for Figure I

The reaction catalyzed by adenyl cyclase. PP_i is inorganic phosphate.


OH
HO-P=O
O
HO-P=O
O
HO-P=O
O
CH2
NH2
N
C
C
N
HC
CH
N
C
N
O
H
H
H
H
OH
OH
NH2
C
N
N
C
HC
CH
N
C
N
O
CH2
O
HO-P=O
H
H
H
H
O
OH
+
PPI

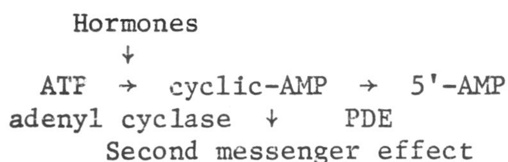
Legend for Figure II

The reaction catalyzed by phosphodiesterase.



NH2
+
H2O
OH
HO-P-O-CH2
OH OH

change in another set of cells for the good of the organism as a whole. Hormones, therefore, provide for the continued existence of an organism in an environment that, if the hormones were not present, might be decidedly unfavorable. How do these hormones act? Many appear to act by a two messenger system (2). In response to a stimulus, the hormone (first messenger) travels from the cells of their origin to the cells of their target tissue. Here they cause a change in the intercellular concentration of cyclic-AMP, the second messenger. Cyclic-AMP was the first such second messenger to be identified. Current thinking suggests that adenyl cyclase is responsive to, usually in a stimulatory manner, a variety of hormones (3). Thus the overall picture would be as follows:



The mechanism of hormonal stimulation is yet to be elucidated. The problem of identifying the cellular material with which hormones interact to produce their particular response has been investigated for years. Several papers have speculated as to the nature of an adrengeric receptor site which is the site at which catecholamine stimulation of tissue occurs (3).

Review of Literature

Sir Henry Dale first suggested the presence of an adrenergic receptor site. It has further been found that two separate sets of responses occur when stimulation by a variety of epinephrine-like

compounds was studied. The response to one or more of these compounds can be blocked by certain drugs. It was concluded by Ahlquist that there were two different adrenergic receptor sites which were designated alpha (α) and beta (β). An alpha receptor is most sensitive to norepinephrine and epinephrine and least sensitive to isoproterenol. Alpha receptors are blocked by phenoxybenzamine and phentolamine. Beta receptors are most responsive to isoproterenol and least responsive to norepinephrine except in the heart. Classical beta blocking agents include dichloroisoproterenol, proprandol and pronerthalol (4).

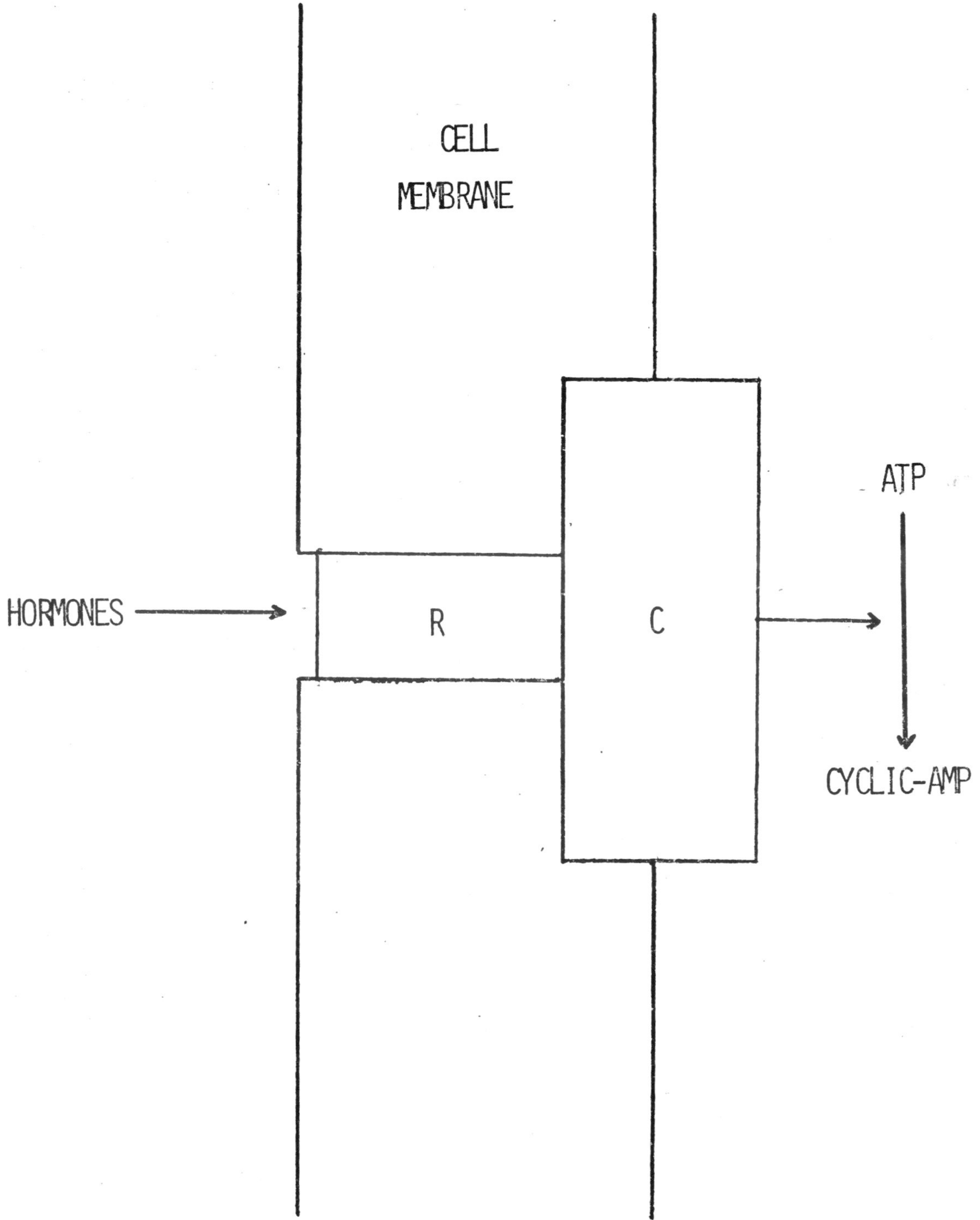
Sutherland and his co-workers have suggested that adenyl cyclase is the beta receptor and may possibly be the alpha receptor as well (3). Adenyl cyclase is membrane-bound in all animal cells and is generally localized in the plasma membranes (5). Based on this association with the plasma membrane, Sutherland has proposed several hypothetical models. One is shown in Figure III (3).

As shown, adenyl cyclase is located in the cell membrane. There are two distinct subunits of the cyclase, a regulatory subunit (R) facing the extracellular fluid and a catalytic subunit (C), the active site of which is in contact with the interior of the cell and its substrate, ATP. The model proposes that the hormone interact with the regulatory subunit which in turn influences the configuration of the catalytic subunit. In this model the regulatory subunit would be the variable component of the system, differing from tissue to tissue, the catalytic subunit being similar in all tissues.

Legend for Figure III

Hypothetical representation of the adenyl cyclase molecule.

C refers to the catalytic subunit and
R to the regulatory subunit.


CELL
MEMBRANE
HORMONES
R
C
ATP
CYCLIC-AMP

Robinson *et al.* proposed a variation on the previous model as shown in Figure IV. Here the catalytic subunit is bound to different types of regulatory subunits. One regulatory subunit has the properties associated with alpha receptors and the other has the properties associated with beta receptors (3).

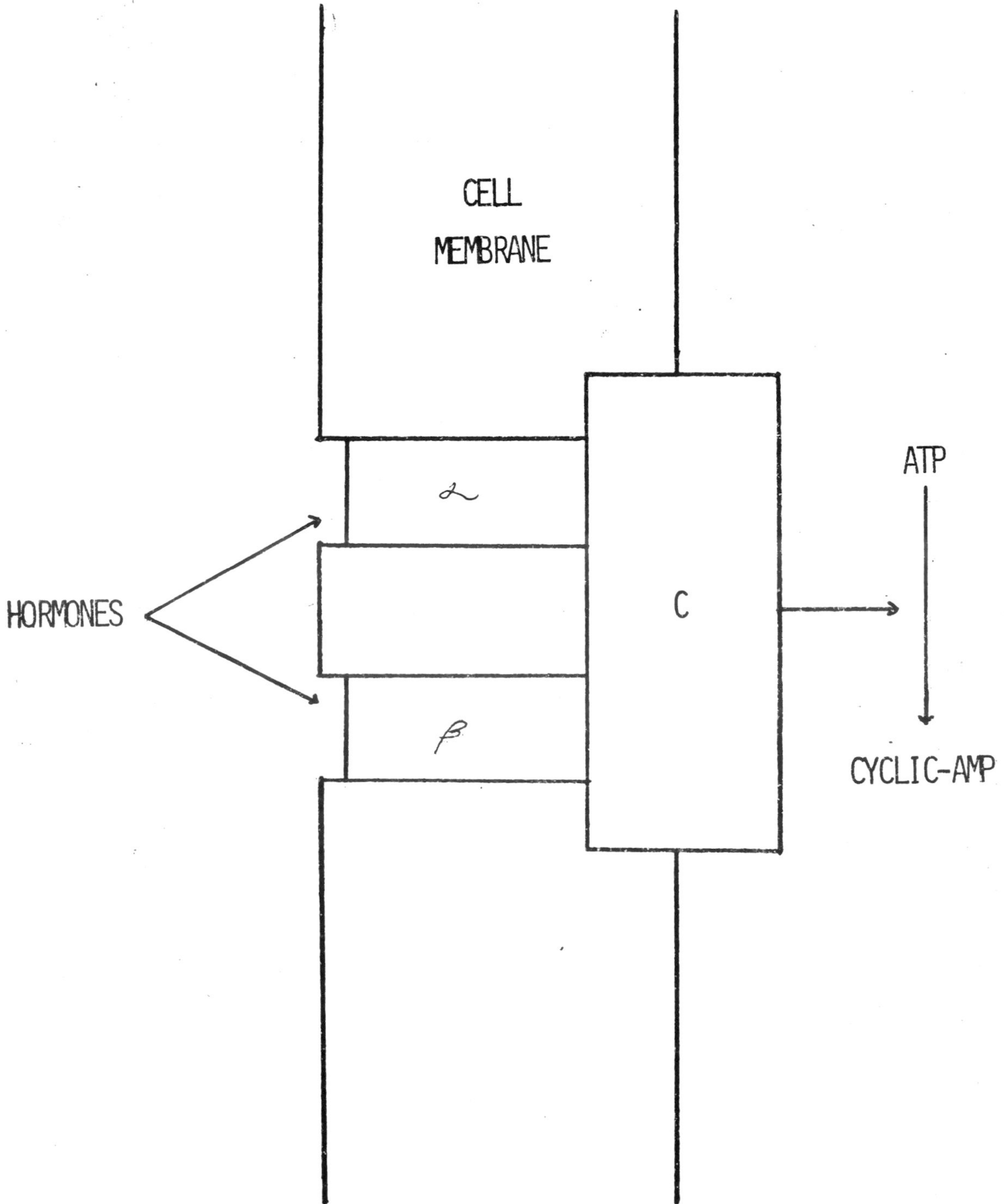
Certain phospholipids appear to be very important in hormone action of the adenyl cyclase system (6, 7). The phospholipids appear to function in coupling the regulatory and catalytic subunits within the membrane. The possibility also exists that the phospholipids may be the actual regulatory or hormone receptor unit.

Solubilization of the membrane-bound adenyl cyclase system has been achieved only recently (8). Levey, using Luberol-PX (an ethylene oxide condensate of dodecanol) has solubilized adenyl cyclase from cat heart; however, the detergent destroyed the hormonal response normally present. Upon removal of the detergent and with the addition of phospholipids, hormonal sensitivity was restored. The phospholipids appear to be specific as to the hormone to which they restore response. Addition of phosphatidylinositol restored the norepinephrine response to adenyl cyclase, while addition of phosphatidylserine restored the response to glucagon and histamine. These experiments support the theory that hormone receptors do consist, at least in part, of phospholipids (9).

Adenyl cyclase is found to be widely distributed in nature. It has been identified in all mammalian tissues studied, in birds, reptiles, amphibians, insects, segmented worms, flatworms and in bacteria. While differences in response to hormones among tissues from various sources

Legend for Figure IV

Possible model of adenyl cyclase as related to adrenergic receptors. One type of regulatory subunit has the properties associated with alpha receptors; the other properties associated with beta receptors. C refers to the catalytic subunit, α to the alpha receptor and β to the beta receptor.


CELL
MEMBRANE
α
HORMONES
C
ATP
β
CYCLIC-AMP

have been noted, all vertebrate adenyl cyclases thus far assayed have one factor in common. This common factor is the stimulatory effect of fluoride ion (F^-) in vertebrate adenyl cyclase from broken cell preparations. The magnitude of the F^- stimulation varies from tissue to tissue, but generally it involves a three to four fold increase in activity. Even though adenyl cyclase from vertebrate broken cell preparations appears to be universally stimulated by F^-, adenyl cyclase from tissue slices or whole organs is not stimulated by F^- (10). Why tissue slices cannot be activated by F^- and tissue homogenates can be is not understood; however, it is known that F^- can pass through the cell membrane and can exist in concentrations sufficient to stimulate adenyl cyclase from broken cell preparations (11).

Fluoride ion does not activate the enzyme by the same process as do the hormones. The F^- stimulation is not reversible as is the hormonal stimulation because repeated washings do not remove the F^- stimulation. Hormonal stimulation can be dialyzed or washed away (12). Destruction of the hormonal response by detergents does not alter the F^- response in cat heart (8). However, F^- does not affect adenyl cyclase activity in rat cerebral cortex which has been solubilized (13).

The chemical mechanism of the F^- stimulation is not understood. One hypothesis is that F^- may bind to the adenyl cyclase molecule in a fluoride-magnesium complex. This step is not readily reversible and in some way enhances activity of the enzyme (12). Another possibility that has been considered is that F^- inhibits ATPase and because of this an increase in activity is noted. This mechanism

probably does not explain F^- stimulation as addition of excess ATP does not increase cyclic-AMP yield in absence of F^- (14). Another hypothesis which has proved useful in explaining both F^- and hormonal stimulation is that adenyl cyclase in higher animals is controlled by specific inhibitors. These inhibitors are proteins attached to, or near, the enzyme in the membrane and inhibit its activity. When a hormone or F^- binds to the inhibitor protein or close to it, an allosteric change occurs in the adenyl cyclase. This binding removes the inhibition and enzyme activity increases. The binding must be in some way different because the increase in activity due to F^- stimulation cannot be removed while the increase in activity due to hormonal stimulation can be removed easily by dialysis or washing (12).

Hormonal stimulation of adenyl cyclase is not consistent in all tissues as is F^- stimulation because hormones are selective as to the tissue that they stimulate. A stimulatory effect is shown only in tissues in which a hormone would normally elicit a physiological response. For example, luteinizing hormone increases cyclic-AMP levels in the corpus luteum, whereas adrenocorticotrophic hormone has no effect. In the adrenal cortex the reverse situation is true (3). This reverse effect is observed in broken cell preparations, tissue slices, and whole organs. Hormonal stimulation is far easier to show in whole organs or tissue slices than in broken cell homogenates. Many broken cell homogenates do not respond to hormonal stimulation (13).

Cations also have an effect on the activity and response of adenyl cyclase to hormones and F^-. Magnesium ion is a requirement for enzyme

activity (15). Manganese ion in liver cells causes an increase in F^- response (16), while calcium ion inhibits adenyl cyclase activity, possibly by competing with magnesium ion (17).

Mammalian adenyl cyclase systems therefore seem to be a multi-component, membrane-bound system with hormonal specificity being expressed through components that are separate from the catalytic component.

Mammalian adenyl cyclase has been extensively studied because of its role in hormone action and intercellular control. Although much less is known about the function of cyclic-AMP in bacteria, much interest has been generated since it was discovered that cyclic-AMP and a specific inducer are required for the synthesis of many inducible enzymes in E. coli. The mechanism proposed for this effect is that cyclic-AMP prevents the glucose repression of the synthesis of an inducible enzyme. Thus, glucose decreases the rate of synthesis of some inducible enzymes by lowering cyclic-AMP concentration (18).

Bacterial adenyl cyclases may be classified into two groups. The enzyme from one of these groups has an absolute requirement for pyruvate for activity while the other group of bacterial adenyl cyclases are pyruvate independent (19).

Ions affect bacterial adenyl cyclase as well as the mammalian system. A metal ion is essential for bacterial adenyl cyclase activity. Magnesium ion maximally stimulates adenyl cyclase of Escherichia coli and Brevibacterium liquefaciens, while manganese ion is the most

favorable metal ion for Nocardia erythropolis (19). F^- seems to be an inhibitor for adenyl cyclase from E. coli (20), but has no effect on adenyl cyclase from N. erythropolis (19), while F^- activates adenyl cyclase from Streptococcus salivarium (21).

E. coli phosphodiesterase also appears to be different from the mammalian enzyme. The mammalian PDE is inhibited by methylxanthines such as caffine and aminophylline which is in direct contrast to the E. coli PDE which is not inhibited by methylxanthines (18).

Bacterial adenyl cyclases have been found to be either particulate or cytoplasmic depending upon the species of bacteria. B. liquefaciens has a cytoplasmic adenyl cyclase (22), while E. coli has a particulate adenyl cyclase. Certain adenyl cyclase from bacteria does appear to be loosely membrane-bound, and where it is particulate a tris-HCl buffer will generally solubilize it (20).

The bacterial adenyl cyclases are not activated by hormones. One interesting possibility is that during the process of evolution adenyl cyclase activation has evolved according to the sequence (1) no activator, (2) an ordinary metabolite present such as pyruvate and finally (3) specific hormones. Most of the confirmed activators of adenyl cyclase may be derived from amino acids such as pyruvate by deamination, catecholamines, seratonin, and histamine by decarboxylation and peptide hormones by polymerization (19).

One of the drawbacks to the study of adenyl cyclase has been the lack of a simple, sensitive method for assaying adenyl cyclase. There are several problems involved with the development of a simple assay procedure. One of these problems is the maintenance of substrate

concentration. It is difficult to maintain enough ATP for maximal rates of activity due to the ATPase enzymes associated with the membrane-bound cyclase. To overcome this problem most assays add large concentrations of ATP. This however can alter the free Mg^{++} concentration sufficiently to alter the hormone response. ATP regenerating systems have also been employed. However, these require an ATP concentration of at least 0.5mM to be efficient. A recent method for solving this problem proposes the use of the diphosphoimide analogue of ATP which serves as a substrate for cyclase and is not affected by ATPases (6).

The second problem that must be overcome if the assay is to work is the prevention of the hydrolysis of the newly formed cyclic-AMP by PDE. This is usually done by addition of methylxanthines. If the assay is measuring the conversions of radioactive ATP to radioactive cyclic-AMP, large quantities of unlabeled cyclic-AMP may be added during incubation to slow down destruction of the labeled cyclic-AMP. The high concentration of cyclic-AMP apparently does not affect the adenyl cyclase (6).

The most difficult problem is to separate and quantitate the cyclic-AMP formed in the assay mixture. Many methods are available. The most widely used is that of Krishna _et al._ whichuses radioactive ATP (23). In this method the incubation mixture is passed through a Dowex 50 (hydrogen form) column which separates the cyclic-AMP from the ATP, ADP and the 5'-AMP. The cyclic-AMP fraction obtained is precipitated by addition of $ZnSO_4$ and $Ba(OH)_2$ which precipitates all

nucleotides and inorganic phosphates with the exception of cyclic-AMP. A portion of the supernatant fluid can then be added to a counting vial and counted. This method has the advantages of being sensitive, simple and relatively rapid.

Another method which has gained acceptance is the radioimmunoassay of cyclic-AMP. Antibodies which are specific for cyclic-AMP are used. A known amount of radioactive cyclic-AMP is added to the unlabeled sample. The endogenous and labeled antigen compete for binding sites on the antibodies. The amount of the labeled complex is inversely related to the unlabeled antigen concentration (24).

Another method utilizes the high speed liquid chromatograph which separates and detects the nucleotides present in the incubation mixture. This method has been shown to be rapid, reproducible and sensitive in brain tissue; however, preliminary purification is necessary before assaying other tissue due to interfering substances present (25).

Purpose of this Study

We proposed to study adenyl cyclase from several mammalian tissue sources as well as bacterial adenyl cyclase. Norepinephrine, epinephrine, glucagon and histamine were used to test for hormonal activation of adenyl cyclase. The effect of F^- was tested. The adenyl cyclase from the mammalian tissue sources was solubilized with Luberol-PX. The detergent was removed and phospholipids were added to the solubilized adenyl cyclase in an attempt to restore hormonal sensitivity. Phospholipids were added to bacterial adenyl cyclase to determine if a hormonal response could be elicited.

The purpose of this study may be summarized as follows:

I. Solubilization of adenyl cyclase from several tissue sources using Luberol-PX

II. Restoration of hormonal sensitivity with phospholipids

III. Addition of phospholipids to bacterial adenyl cyclase to see if a hormonal response can be elicited

IV. Comparison, if any, between bacterial and mammalian response

MATERIALS AND METHODS

The bacterial strain used was Escherichia coli ATCC 8739. They were grown in soy trypticase broth for 18 to 24 hours at 37° and sedimented by centrifugation at 4,000 xg for 20 minutes at 0-4°C. The entire harvesting and enzyme isolation procedure was carried out at 0-4°C until incubation. After centrifugation, the cells were washed twice in a cold 0.25M sucrose solution. The cells were then placed in a precooled mortar with an equal weight of alumina, type 305, and ground for one minute. Ten volumes of tris buffer, 0.01M pH 9.0, containing 0.01M $MgSO_4$, $0.25X10^{-3}$M EDTA, 1mM dithiothreitol and 10% sucrose were then added. This mixture was centrifuged at 10,000 xg for ten minutes. The supernatant was used as the source of the bacterial enzyme.

Animal tissues were from rats of the Holtzman strain. The rats were sacrificed by a sharp blow to the head followed by decapitation. Tissues were removed immediately and placed in a tris buffer solution that was 0.01M in tris pH 7.7, 0.25M in sucrose, and 10^{-3}M in EDTA. If the adenyl cyclase was to be solubilized the solution was also 0.02M in Luberol-PX.

Cerebral cortical gray matter was dissected free in the cold room and homogenized in ten volumes of tris sucrose solution by hand with 8 to 10 strokes in a Pyrex ground glass hand homogenator. The heart was minced with scissors and then homogenized using a Potter Elvejhan homogenator. The solution used for homogenation was tris buffer with

sucrose and EDTA with or without added Luberol-PX. The Luberol-PX tubes were spun at 30,000 xg and the supernate was used as the source of the solubilized adenyl cyclase.

Bacterial adenyl cyclase was assayed by a method similar to that of Krishna, et al. The incubation medium contained 0.91 mg Tris pH 9.0, 2.4 mg $MgCl_2$, 0.1 mg ATP, 0.5×10^{-3} mCi of adenosine-2-H^3-5'-triphosphate and 0.1 ml of enzyme in a final volume of 0.6 ml. Protein concentration of the enzyme was 3 to 4 mg/ml. Hormones, when added, were at a concentration of 0.1 mg per assay tube. Phospholipids, when added, were at a concentration of 50 micrograms per assay tube. Incubation was conducted at 37°C for either 5 or 20 minutes. The incubations were terminated by immersion of the incubation tube in a boiling water bath for three minutes. One-tenth ml of water containing 0.5 mg of carrier cyclic-AMP was then added to each tube. The tubes were centrifuged at 3,000 rpm for three minutes, and four-tenths ml of the supernatant was placed on top of a small column (0.4 x 3.3 cm) of Dowex 50 hydrogen form which had been fined (50W-X4, 200-400 mesh) and eluted with water. The cyclic-AMP was eluted in the 1.5 to 4.0 ml fractions (see Figure V). These fractions were combined and treated with 0.2 ml of 5% $ZnSO_4$ and 0.2 ml of 0.3 N $Ba(OH)_2$ with vigorous mixing. This treatment precipitated all nucleotides and inorganic phosphates and left the cyclic-AMP in the supernate. After centrifugation and without disturbing the packed precipitate, the precipitation treatment was repeated. After recentrifuging, the supernate was transferred to another tube by decanting. This mixture was made

acidic by adding a few drops of dilute HCl. (If the solution is not made acidic the scintillation fluid decomposes and reliable results will not be obtained.) A one ml aliquot of the supernate was added to ten ml of scintillation fluid. Scintillation fluid was prepared by mixing one part Triton-X 100 to two parts of a cocktail containing four grams PPO(2,5-Diphenyloxazole) and 0.2 grams POPOP(1,4-bis-[2-(4-Methyl-5-Phenyloxazoyl)] benzene) in one liter of scintanalyzed toluene. Radioactivity was counted in a Hewlett Packard liquid scintillation detector. Heat denatured enzyme was run as a blank. The absorption at 260 nm of an aliquot was determined in order to calculate the recovery of cyclic-AMP.

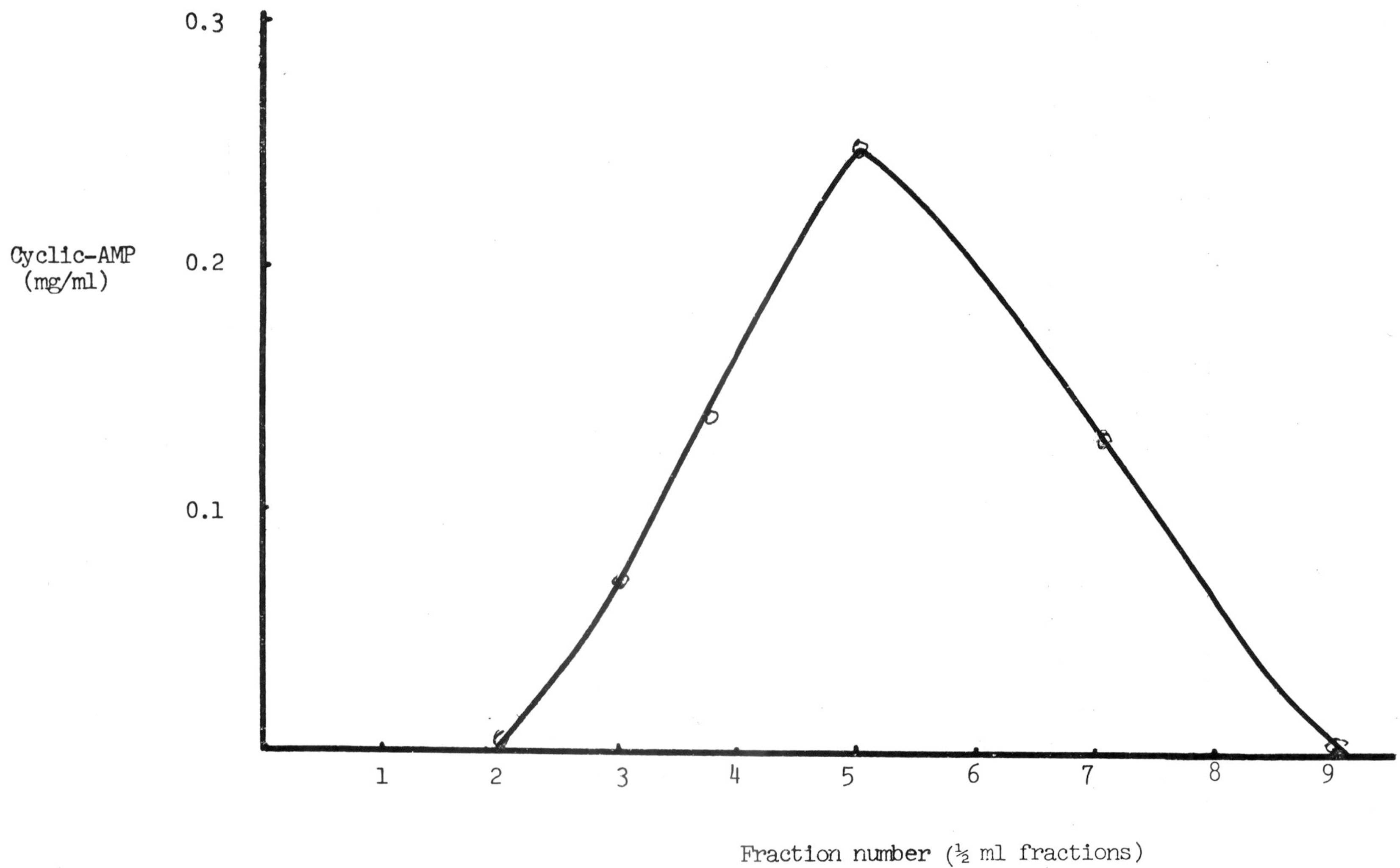
The assay of animal tissue was similar to that used for the bacteria. The final assay volume was 0.6 ml and contained 0.1 ml of enzyme (10 mg/ml), 6.6 mg tris (pH 7.7), 0.45 mg $MgCl_2$, 0.1 mg ATP, 10.5 mg aminophylline and $0.5x10^{-3}$mCi of adenosine-2-H^3-5'-triphosphate. Hormones when added were at a concentration of 0.1 mg per assay tube. Phospholipids when added were at a concentration of 50 micrograms per assay tube. The remainder of the procedure for assay was the same as for the bacteria.

Phospholipids were obtained as a solution in either $CHCl_3$ or benzene. The solvent was evaporated with a stream of nitrogen. Tris-HCl, (10mM, pH 7.7) was added to the residue and the lipid was dispersed with sonication at 0-4°C. This procedure took about one minute to disperse the lipid.

Legend for Figure V

Fractions were collected in 1/2 ml portions.

Fraction 3 through fraction 8 were combined and used for assaying.


0.3
0.2
0.1
Cyclic-AMP
(mg/ml)
1
2
3
4
5
6
7
8
9
Fraction number (½ ml fractions)

To separate the Luberol-PX from the adenyl cyclase the following procedure was used. Approximately 1.3 ml of the Luberol-PX supernate obtained for the 30,000 xg spin was applied to a DEAE-cellulose column (1.0 x 12 cm) equilibrated at $4^{o}C$ with 10mM tris pH 7.7. The DEAE used was extensively fined before use. The flow rate was approximately 0.2 ml per minute. The column was washed with 15 to 20 volumes of 10mM tris pH 7.7 to remove the detergent. Adenyl cyclase was eluted with 1M tris pH 7.7.

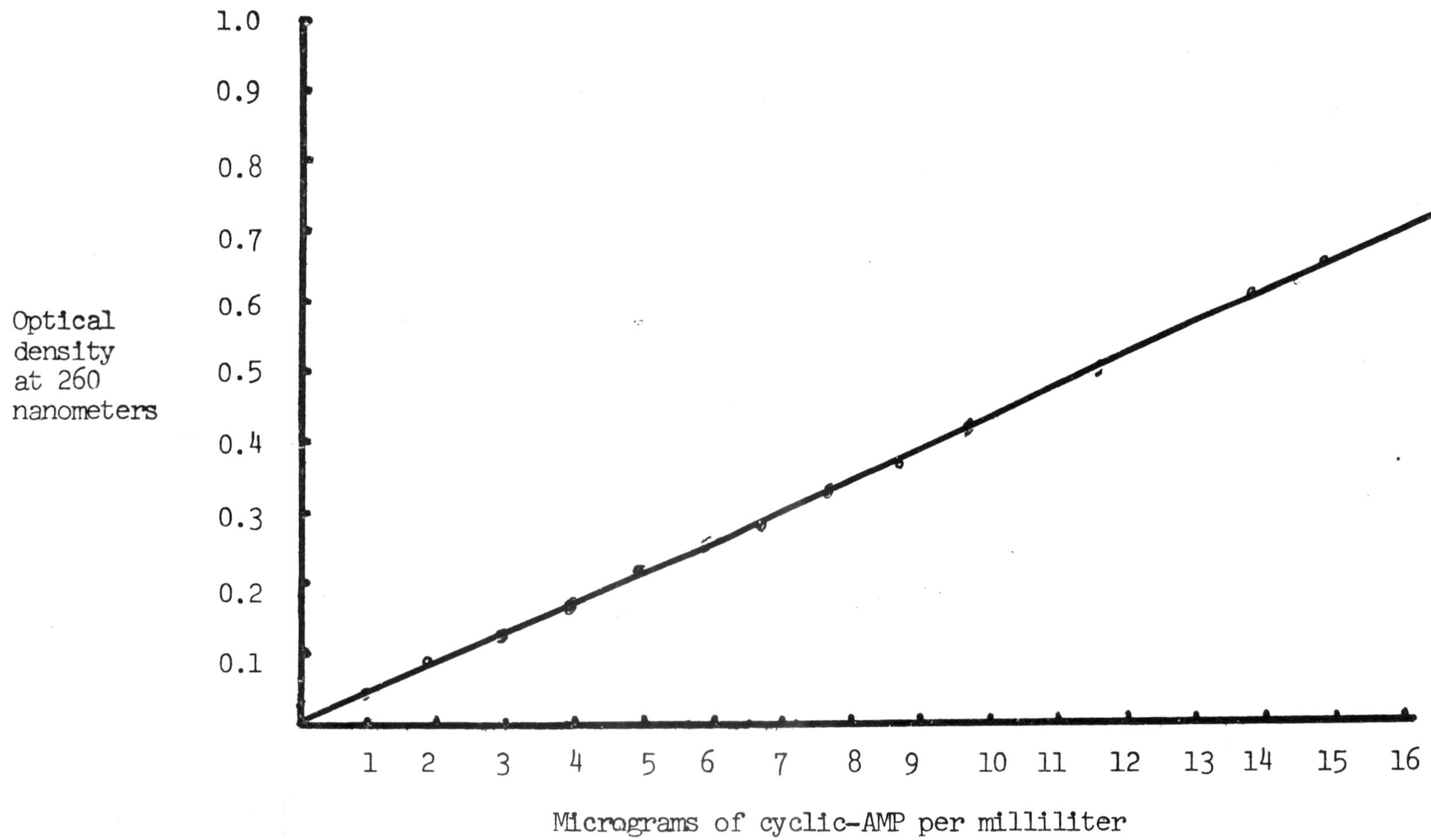
A standard curve for cyclic-AMP was obtained using anhydrous cyclic-AMP. The optical density was read at 260 nm. This curve was used to calculate the recovery of the cold carrier cyclic-AMP (see Figure VI).

Protein determinations were by the biuret method using bovine serum albumin as the protein standard (see Figure VII) (26).

All chemicals and instruments used are listed in the appendix.

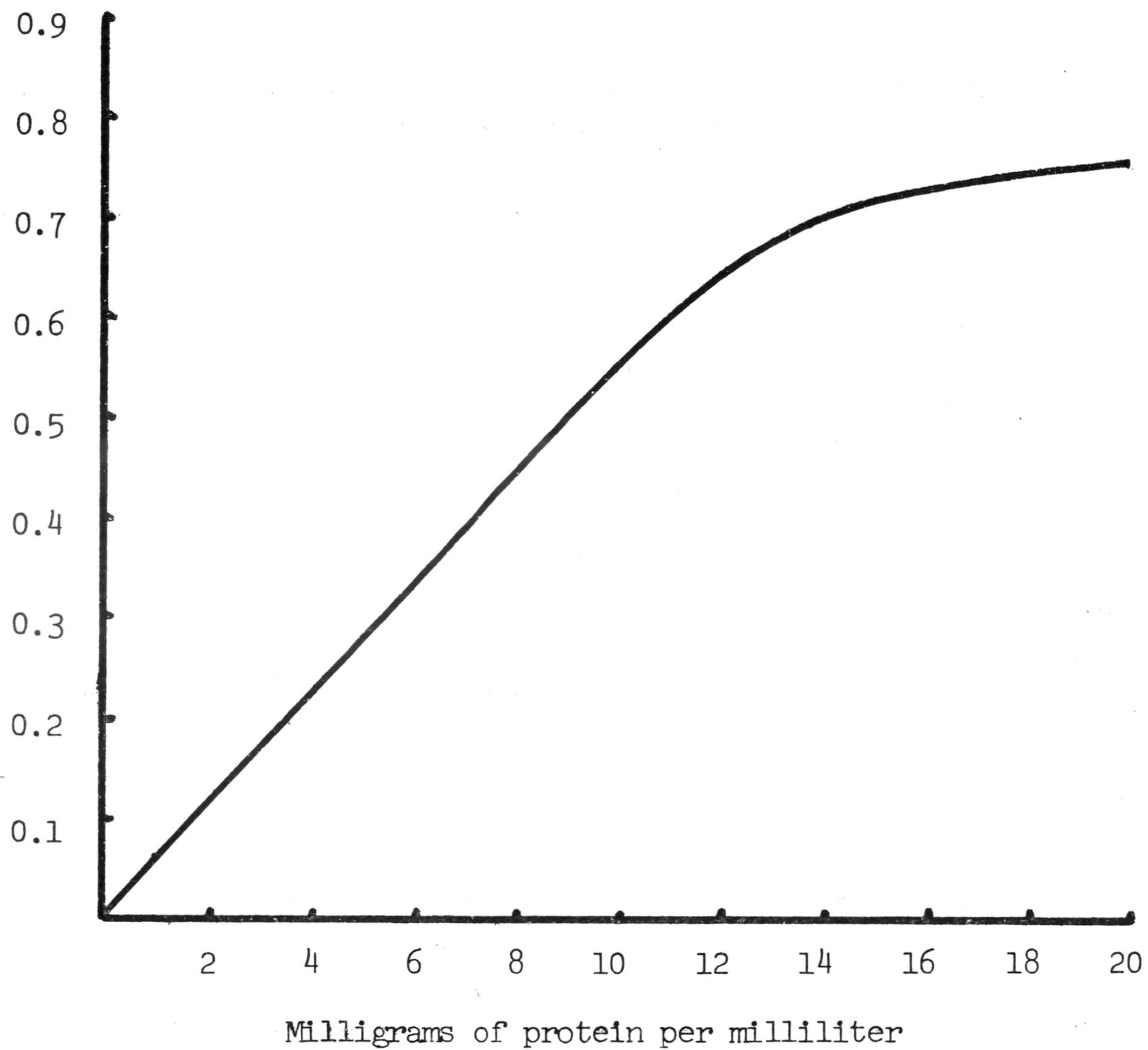
Legend for Figure VI

Standard curve for cyclic-AMP recovery study.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Optical density at 260 nanometers
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Micrograms of cyclic-AMP per milliliter

Legend for Figure VII

Protein standard curve obtained using bovine serum albumin by the biuret method.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
2
4
6
8
10
12
14
16
18
20
Milligrams of protein per milliliter

RESULTS AND DISCUSSION

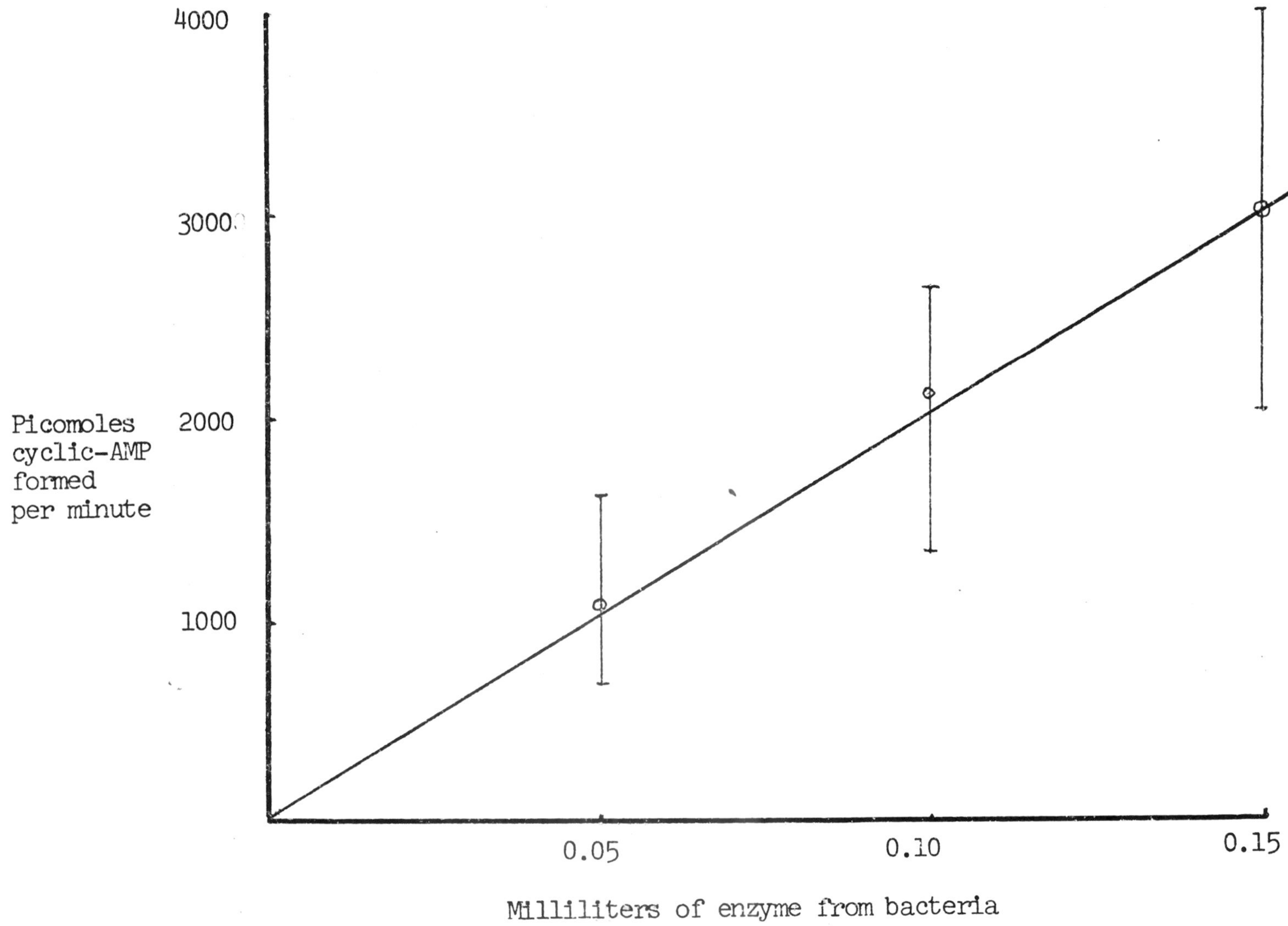
Before working with the solubilized preparations and the phospholipids, basic techniques for assaying and monitoring the adenyl cyclase were developed. Lack of a sensitive assay procedure remained a problem through much of the experimentation. Liquid chromatography was tried as a means of separating and quantitating the cyclic-AMP produced. Only brain adenyl cyclase had sufficient activity such that the quantity of cyclic-AMP produced could be detected by the liquid chromatograph. The quantity produced was still so slight that accurate results could not be obtained using the liquid chromatograph. Krishna's radioisotopic method was thus selected to assay the adenyl cyclase.

The enzyme was assayed to determine if its activity would have a linear response over a range of concentrations (see Figures VIII, IX, and X). Linear curves were obtained with bacterial adenyl cyclase and rat cerebral cortical enzyme. The solubilized rat cerebral cortical enzyme appears to have a plateau as shown in Figure X. However, the number of counts per minute used for this data were quite low and thus variations between repetitive counting periods would often be a change of at least 50%. This fact is reflected in the wide range of the data obtained.

Figure XI shows the effect of the length of the assay incubation on the quantity of cyclic-AMP produced. This curve was expected to be linear and probably does not have the break as the graph indicated. This may be due to the low counts obtained.

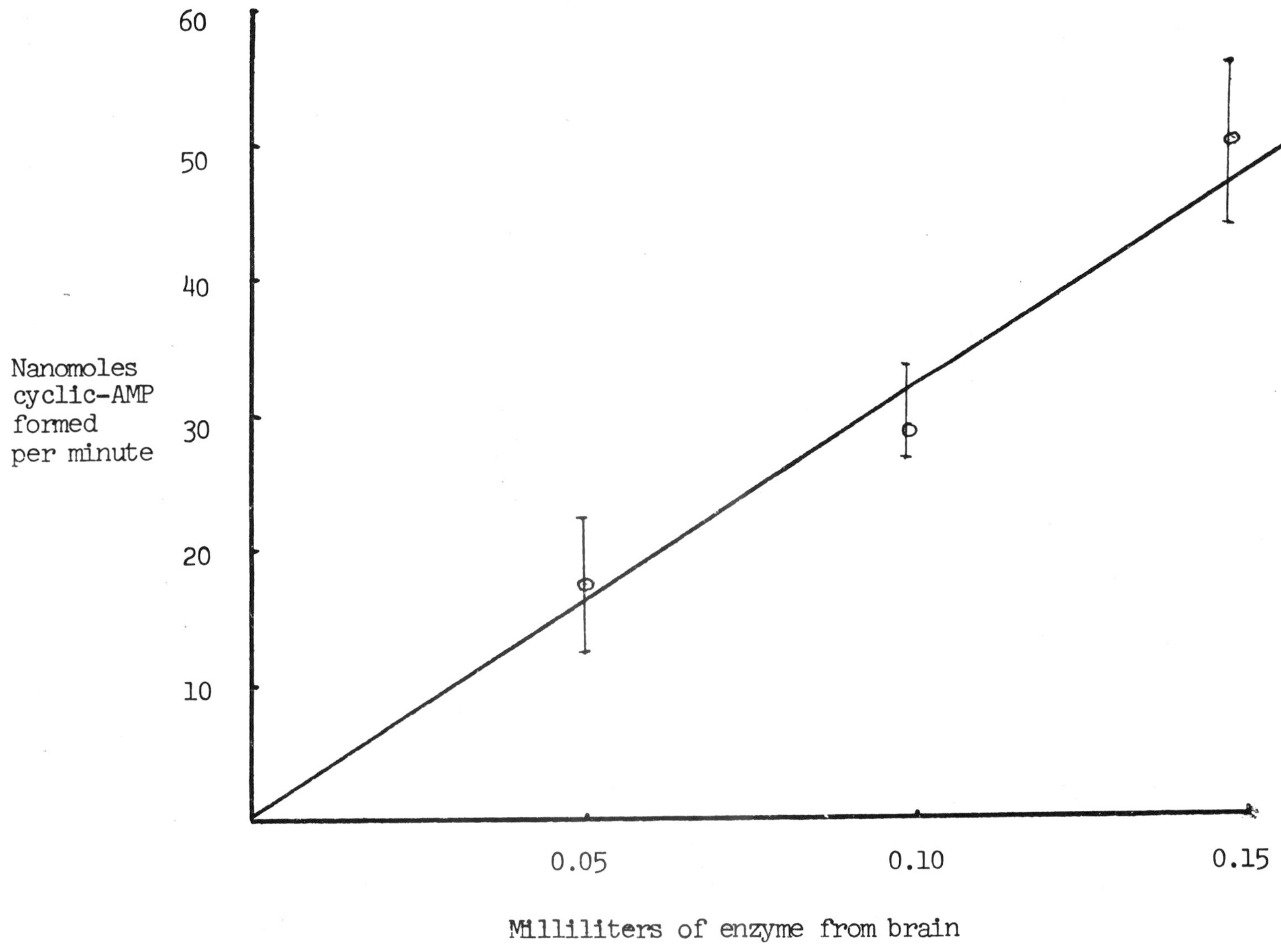
Legend for Figure VIII

Effect of increasing concentrations of bacterial adenyl cyclase on the production of cyclic-AMP


4000
3000
2000
1000
Picomoles
cyclic-AMP
formed
per minute
0.05
0.10
0.15
Milliliters of enzyme from bacteria

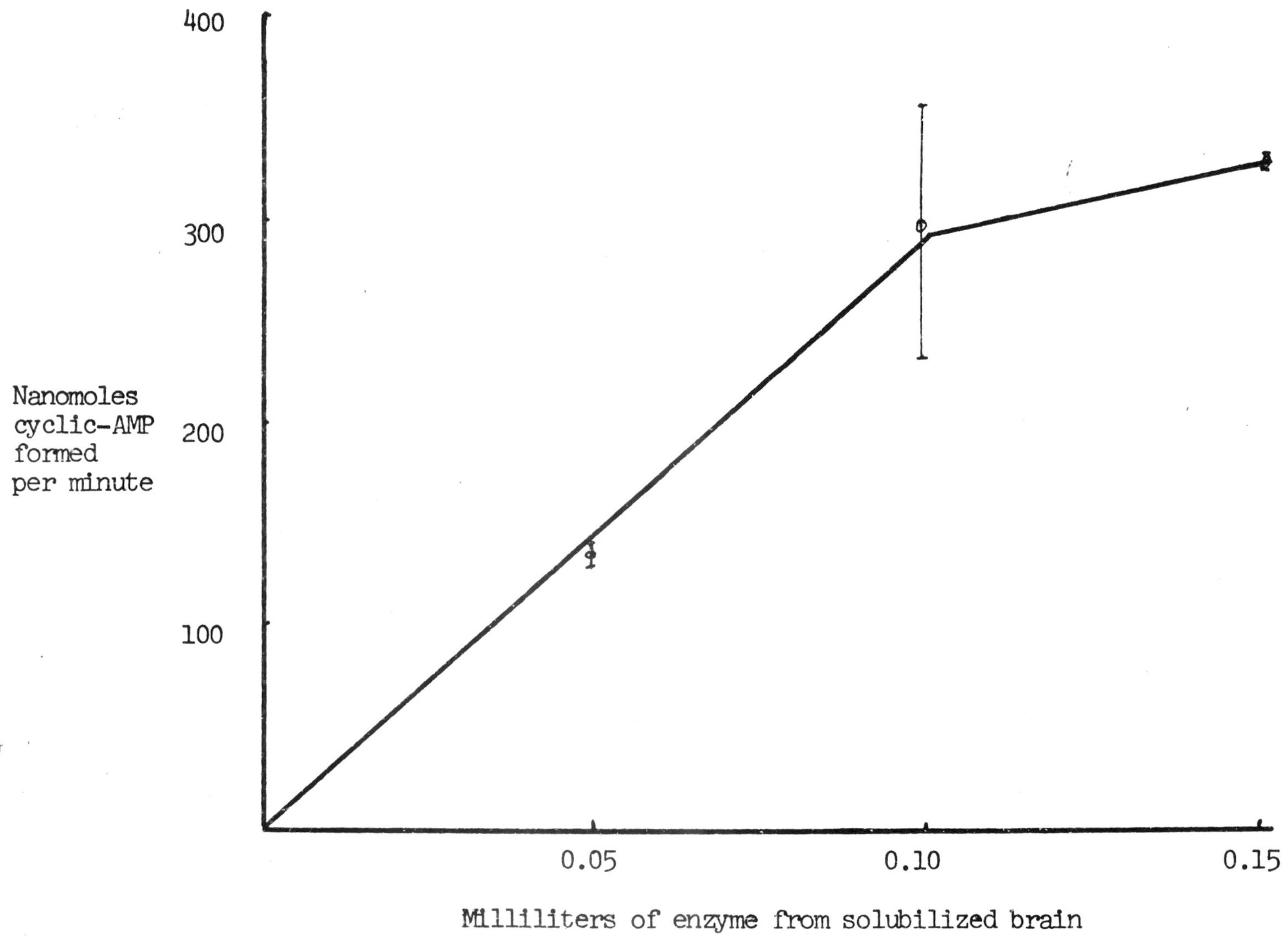
Legend for Figure IX

Effect of increasing concentrations of rat cerebral cortical adenyl cyclase on the production of cyclic-AMP


60
50
40
30
20
10
Nanomoles
cyclic-AMP
formed
per minute
0.05
0.10
0.15
Milliliters of enzyme from brain

Legend for Figure X

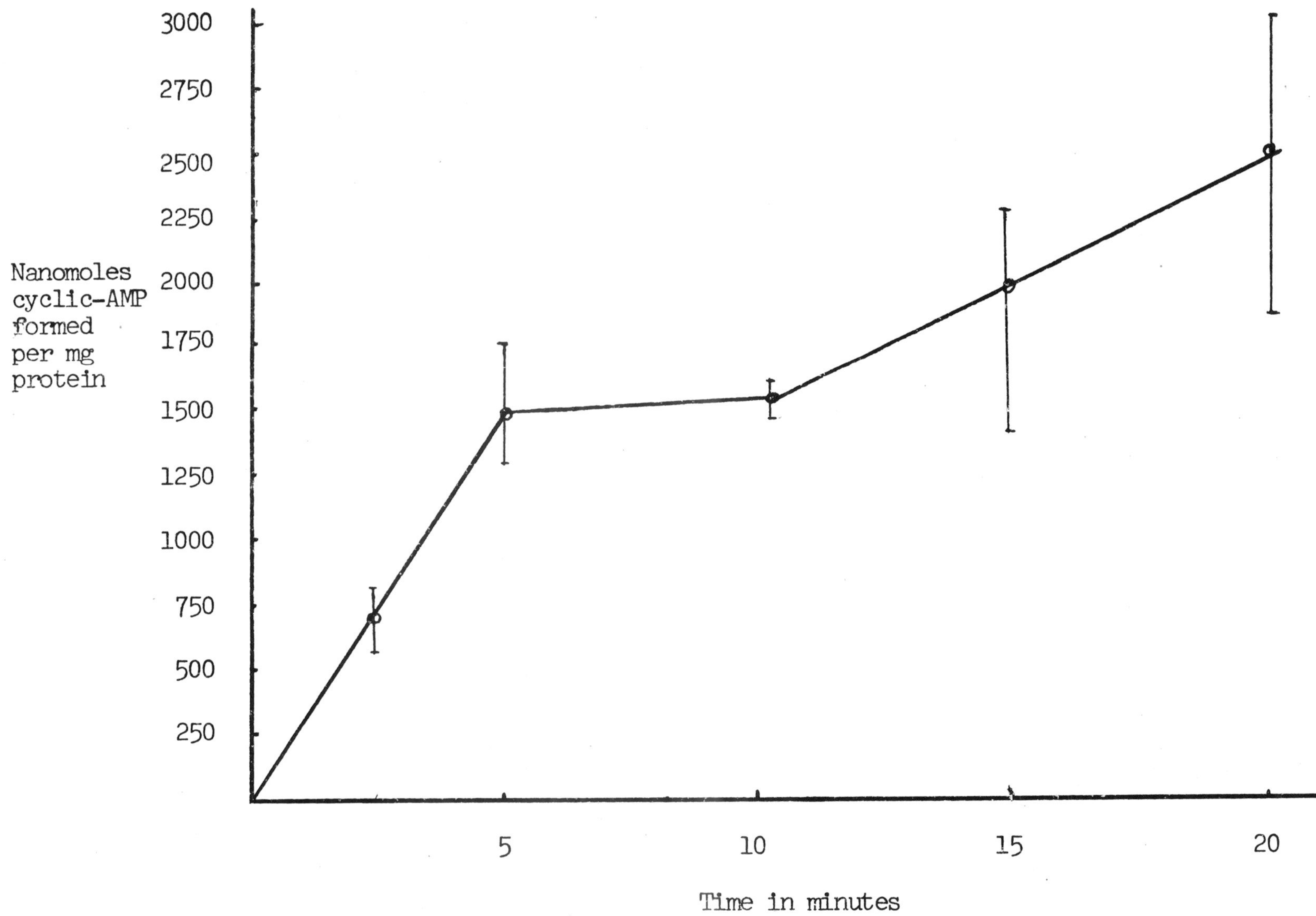
Effect of increasing concentrations of solubilized rat cerebral cortex adenyl cyclase on the production of cyclic-AMP


400
300
200
100
Nanomoles
cyclic-AMP
formed
per minute
0.05
0.10
0.15
Milliliters of enzyme from solubilized brain

Legend for Figure XI

Formation of cyclic-AMP as a function of time.

Solubilized rat cerebral cortex is the source of the enzyme.


3000
2750
2500
2250
2000
1750
1500
1250
1000
750
500
250
Nanomoles cyclic-AMP formed per mg protein
5
10
15
20
Time in minutes

Following these preliminary experiments, whole homogenates from heart, liver, and cerebral cortex were tested for response to various hormones and to F^-. Liver adenyl cyclase was stimulated by epinephrine and both the heart and brain cyclases were stimulated significantly by histamine and norepinephrine. Brain cyclase was stimulated several fold by F^- (see Figures XII and XIII). These effects were expected and have been reported many times in the literature.

Solubilization of the cyclase and retention of activity in the presence of the detergent has been reported by only one lab and only in cat heart (8). Using the detergent that those authors used (Luberol-PX) both rat brain and rat heart were solubilized. The activity of identical supernatant that had not been treated with Luberol-PX was checked and compared with the activity of the supernatant that had been treated with Luberol-PX. The supernatant of heart and brain homogenates without Luberol-PX had no adenyl cyclase activity. The Luberol-PX proved to be a simple and effective means of solubilizing the adenyl cyclase.

The solubilized adenyl cyclase was tested for response to hormones and F^-. The solubilized adenyl cyclase was not expected to be responsive to hormones because the phospholipids that are necessary for the adenyl cyclase to respond to the hormones have been removed by the detergent (9). The heart cyclase was not stimulated by norepinephrine or histamine in the solubilized state; however, there was still a significant activation in response to F^- (see Figure XII). This was in agreement with Levey's work with cat

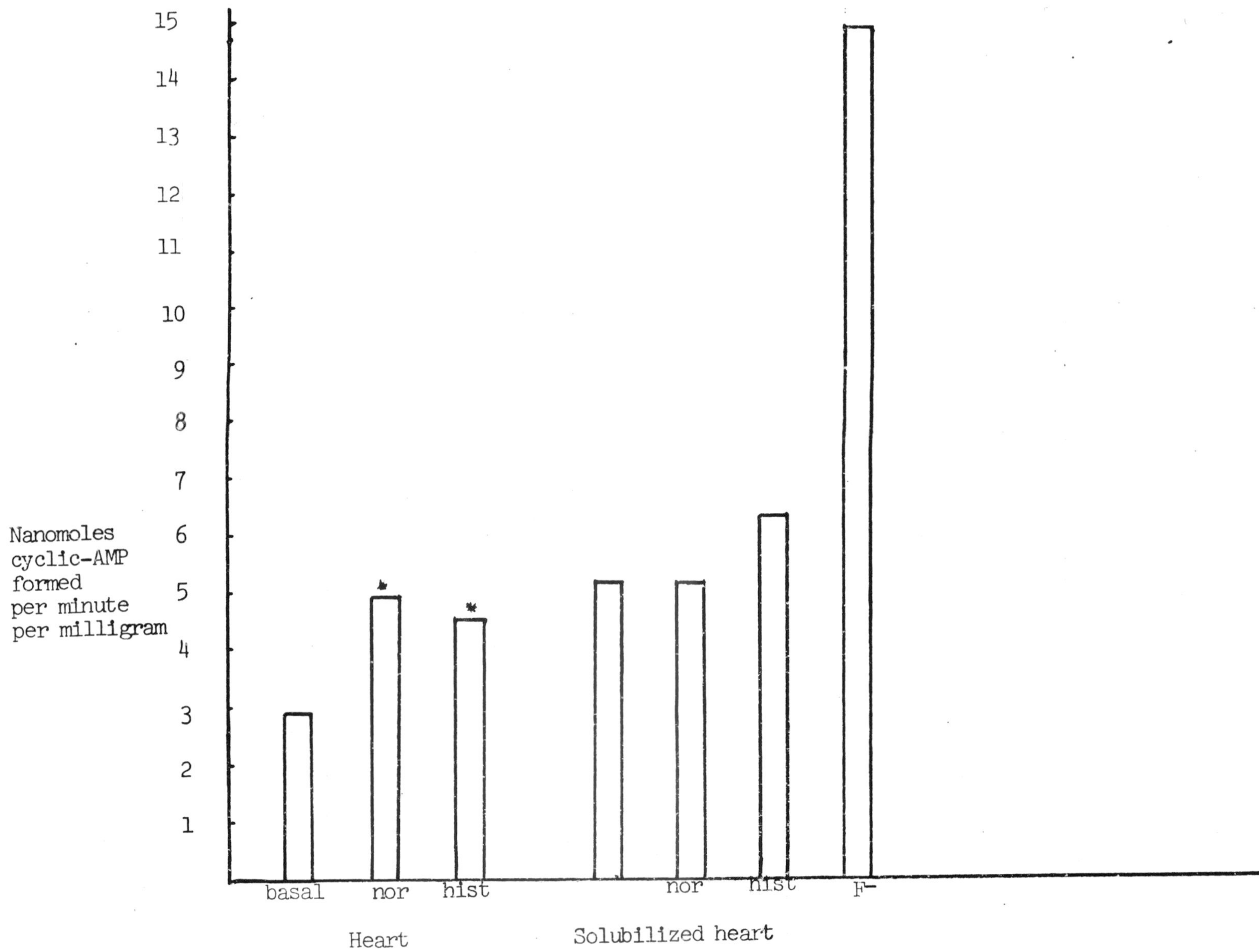
Legend for Figure XII

The effect of norepinephrine, histamine and F^- on heart adenyl cyclase and solubilized heart adenyl cyclase.

Basal = Basal level of cyclase activity

Nor = norepinephrine

Hist = histamine


Nanomoles
cyclic-AMP
formed
per minute
per milligram
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
basal
nor
hist
nor
hist
F-
Heart
Solubilized heart

heart (8). The solubilized brain cyclase was also not further stimulated by the addition of histamine and norepinephrine (see Figure XIII). In direct contrast to the heart cyclase, the brain cyclase was not further stimulated by F^-. This indicates that the solubilization by Luberol-PX maximally stimulated the brain adenyl cyclase. Perkins and Moore have found that rat cerebral cortex adenyl cyclase treated with Triton X-100 was not further activated by F^-. However they did find that the activity of F^- activated enzyme could be increased by Triton X-100 (13).

Following characterization of the solubilized adenyl cyclase, attempts were made to separate the enzyme from the detergent using a DEAE-cellulose column. It was necessary to remove the detergent to find out if the detergent was the inhibitor of the hormonal response. Using brain cyclase no activity could be recovered from the column even though significant amounts of solubilized adenyl cyclase were added to the column. Heart adenyl cyclase activity however could be recovered from the column. Levey reported that specific activity of cat heart adenyl cyclase decreased when the cyclase was recovered from the column. The rat heart used here showed an increase in specific activity, from 5.5 nanomoles cyclic-AMP per minute per milligram, indicating that a purification or activation step had occurred (see Table I).

When histamine and glucagon were added to the cyclase from which the Luberol-PX had been removed the specific activity decreased to

Legend for Figure XIII

The effect of norepinephrine, histamine and F^-

on brain adenyl cyclase and solubilized brain adenyl cyclase

Basal - basal level of cyclase activity

Nor = norepinephrine

Hist = histamine

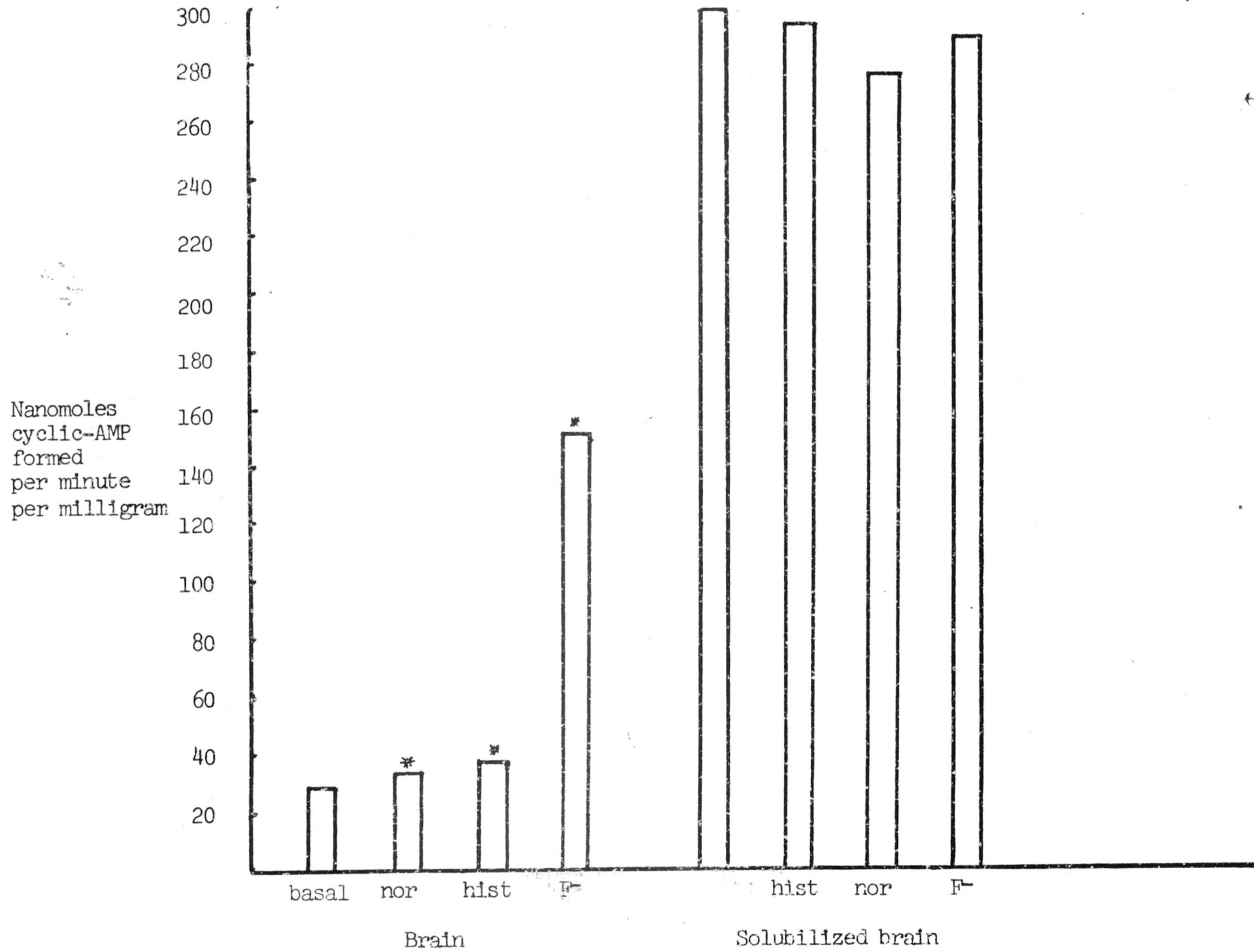

300
280
260
240
220
200
180
Nanomoles
cyclic-AMP
formed
per minute
per milligram
160
140
120
100
80
60
40
20
basal
nor
hist
F⁻
hist
nor
F⁻
Brain
Solubilized brain

Table I

The adenyl cyclase used in these experiments had been solubilized with Luberox-PX from cat heart. The detergent had been removed by passing the solubilized cyclase through a DEAE-cellulose column before being tested for hormone response. Values given are in nanomoles cyclic-AMP produced per milligram per minute.

	No hormone	histamine	glucagon
Enzyme	15.0±4.7	5.9±2.7	6.0±0.0
+ phosphatidylserine		5.4±0.4	6.3±2.5
+ phosphatidylethanolamine		7.5±2.1	12.1±0.0
+ lecithin		7.6±5.5	12.0±1.4
+ phosphatidylinositol		16.1±3.9	11.2±5.2

5.9±2.7 nanomoles cyclic-AMP per minute per milligram for samples with histamine and 6.0±0.0 nanomoles cyclic-AMP per minute per milligram for samples with glucagon. This activity is very similar to the activity of the cyclase before the Luberol-PX was removed. Phospholipids were then added in an attempt to restore hormonal response. In some cases the phospholipids did increase the specific activity over that of cyclase and hormone together. However the specific activity was lower in all cases than the specific activity for heart cyclase in the absence of phospholipid and hormone (see Table I). Phosphatidylinositol added to samples containing either histamine or glucagon increased the specific activity. Phosphatidylethanolamine and lecithin also increased the specific activity of samples containing glucagon.

To determine if there were any similarities between the bacterial adenyl cyclase and the mammalian adenyl cyclase, the bacterial cyclase was tested for response to hormone, F^- and phospholipids. During a five minute incubation, glucagon, histamine and norepinephrine were found to inhibit the adenyl cyclase activity (see Table II). Ide has reported that both norepinephrine and epinephrine had no effect on the bacterial adenyl cyclase (19). At the pH of his assays however both norepinephrine and epinephrine are oxidized to adrenochrome. We have not found an effective antioxidant for either norepinephrine or epinephrine. The oxidation of norepinephrine is slowed by the addition of vitamin E; however, vitamin E does not slow the oxidation of epinephrine. Therefore we were unable to determine the effect of epinephrine on bacterial adenyl cyclase.

Table II

The adenyl cyclase used in these experiments was bacterial obtained as described under Materials and Methods. Values given are nanomoles cyclic-AMP per minute per milligram.

	No hormone	histamine	glucagon
Enzyme	34.0±9.9	7.5±2.0	7.3±2.9
+ phosphatidylserine	22.1±0.7	10.4±5.7	5.3±3.4
+ phosphatidylethanolamine	10.6±3.1	12.2±1.6	14.1±1.5
+ lecithin	19.1±7.9	12.6±1.1	7.9±1.2
+ phosphatidylinositol	16.4±1.7	10.4±0.5	12.2±6.9
+ F^-	67.4±11.6		
+ F^- and Luberol-PX	55.4±12.5		

F^- was found to have a definite stimulatory effect on the bacterial adenyl cyclase (see Table II). This is in direct contrast to what Tao has reported (20). Quite possibly the strain of bacteria used for this experiment was a mutant strain and thus a different response to F^- was noted.

The results of the addition of phospholipids to bacterial adenyl cyclase paralleled those of the mammalian system. Highest activity was observed when neither phospholipids nor hormones were present. When the phospholipids were added they decreased the specific activity but not as much as the histamine or glucagon did. Addition of phospholipids to samples containing either glucagon or histamine caused an increase in the specific activity of these samples; however, the full activity of the enzyme was not restored. In these systems the hormone may be acting as an inhibitor and the phospholipid may be removing the hormone. Hormones also possibly activate by removing an inhibitor protein. Apparently they must also react in some way with a phospholipid to have a hormonal response.

As previously stated (see Introduction) activators of adenyl cyclase may have developed from (1) an enzyme that has no activators, (2) to an ordinary metabolite as an activator, (3) to a specific hormone as an activator. Bacterial adenyl cyclases are not activated by hormones, but some bacterial adenyl cyclases are activated by pyruvate. Mammalian adenyl cyclase appears to be more highly specialized than do the bacterial cyclases. It appears in both

mammalian and bacterial cyclase that there is a common site possibly on the catalytic subunit at which F^- acts. F^- may complex with an inhibitor protein at this site, causing an allosteric change in the cyclase and thereby activating it.

LITERATURE CITED

1. Rall, T. W. and Sutherland, Earl W. The Journal of Biological Chemistry, 232: 1065 (1958).

2. Sutherland, Earl W., Robison, G. Alan and Butcher, Reginald W. Circulation, 37: 279 (1968).

3. Robison, G. A., Butcher, R. W. and Sutherland, E. W. Anals New York Academy of Science, 139: 703 (1967).

4. Turner, C. Donnell and Bagnara, Joseph T. General Endocrinology. W. B. Saunders Company, Philadelphia, Pa. (1971) p. 345.

5. Birnbaumer, Lutz and Martin, Rodbell. The Journal of Biological Chemistry, 244: 3477 (1969).

6. Rodbell, Martin. Acta Endocrinol., 1971: 337 (1971).

7. Levey, Gerald S. Biochem. Biophys. Res. Comm., 43: 108 (1971).

8. Levey, Gerald S. Biochem. Biophys. Res. Comm., 38: 86 (1970).

9. Levey, Gerald S. The Journal of Biological Chemistry, 246: 7405 (1971).

10. Øye, I. and Sutherland, E. W. Biochim. Biophys. Acta, 127: 347 (1966).

11. Kornegay, Denise A. and Pennington, Sam. Fluoride, 6: 19 (1973).

12. Schramm, Michael and Naim, Esther. The Journal of Biological Chemistry, 245: 3225 (1970).

13. Perkins, John P. and Moore, Marilyn M. The Journal of Biological Chemistry, 246: 62 (1971).

14. Weiss, Benjamin. Journal of Pharm. Exp. Therp., 166: 330 (1969).

15. Sutherland, Earl W., Rall, T. W. and Menon, Tara. The Journal of Biological Chemistry, 237: 1220 (1962).

16. Pohl, Stephen L., Birnbaumer, Lutz and Rodbell, Martin. The Journal of Biological Chemistry, 246: 1849 (1971).

17. Durmmond, G. I. and Duncan, L. The Journal of Biological Chemistry, 245: 976 (1970).

18. Pastan, Ira and Pulman, Robert. Science, 169: 339 (1970).

19. Ide, Misao. Arch. Biochem. Biophys., 144: 262 (1971).

20. Tao, Mariano and Lipmann, Fritz. Biochemistry, 63: 86 (1969).

21. Khandelwal, Ramji L. and Hamilton, Ian R. The Journal of Biological Chemistry, 246: 3297 (1971).

22. Hirata, Saharu and Hayaishi, Osamu. Biochim. Biophys. Acta, 149: 1 (1967).

23. Krishna, Gopal, Weiss, Benjamin and Brodie, Bernard B. Journal of Pharm. Exp. Therp., 163: 379 (1968).

24. Steiner, A. L., Kipnis, D. M., Utigen, R. and Parker, C. Proc. Nat. Acad. Sci., 64: 367 (1969).

25. Brooker, Gary. Analytical Chemistry, 42: 1108 (1970).

26. Clark, John M. (ed.). Experimental Biochemistry. W. H. Freeman and Company. San Francisco (1964) p. 75.

APPENDIX

ABBREVIATIONS

Aminophylline	$(\text{Theophylline})_2 \cdot$Ethylenediamine
ATP	Adenosine triphosphate
Tris	Tris(Hydroxymethyl)Amino methane
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
cyclic-AMP	Cyclic adenosine monophosphate
PPO	2,5-Diphenyloxazole
POPOP	1,4-bis-[2-(4-methyl-5-phenyloxazoyl)]-Benzene
EDTA	(Ethylenedinitrilo)tetra acetic acid
DEAE	Diethyl amino ethyl cellulose
PP_i	Inorganic phosphate

INSTRUMENTS

Perkin-Elmer Coleman 124 spectrophotometer (Hitachi Tokyo, Japan)

with a Perkin-Elmer Coleman 56 recorder (Hitachi Tokyo, Japan)

Fischer accumet model 320 pH meter

Packard liquid scintillation spectrometer

Liquid chromatograph - Varian ultraviolet detector and power supply

Chemicals	Company	Lot No.
Soy trypticase broth	BBL	203684
Sucrose	Dixie Crystal	
Alumina	Sigma	42C-2870
Tris	Fisher	703519
$MgSO_4$	Fisher	751594
EDTA	Eastman	671A
Dithiothreitol	Sigma	92C-6140
$MgCl_2$	Fisher	716243
ATP	Sigma	81C-7540
Adenosine-2-H3-5' triphosphate	Amersham Searle	Batch 9
Dowex-50W (50X4-400)	Sigma	51C-2300
Cyclic-AMP	Sigma	81C-0220
$ZnSO_4$	Fisher	702464
$Ba(OH)_2$	Fisher	712594
Triton X-100	Packard	020
PPO	Packard	5220
POPOP	Packard	4329
Toluene	Fisher	795817
Luberol-PX	Savco	
Aminophylline	Sigma	31C-2200
DEAE cellulose	Sigma	30C-2400
Bovine serum albumin	Sigma	30C-2070
Histamine	Sigma	62C-5330
Norepinephrine	Sigma	92C-2190
Na F^-		
Phosphotidylinositol	Applied Science Lab.	1128
Phosphotidylethanolamine	Applied Science Lab.	1286
Letichen	Applied Science Lab.	1246
Phosphotidylserine	Applied Science Lab.	1235
Glucagon	Sigma	62C-2450