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AN ASSAY FOR ADENYL CYCLASE-

DEVELOPMENT AND APPLICATION

by

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A DISSERTATION

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INTRODUCTION

A new method for the *in vitro* measurement of adenyl cyclase activity has been evolved conceptually, has been accomplished experimentally, and constitutes the basis for this dissertation. The overall scheme for the assay involves two enzymatic reactions and is presented as follows.

Reaction 1, below,

$$1.\quad \text{ATP} \xrightarrow{\text{Adenyl Cyclase}} \text{cyclic AMP}$$

represents the enzymatic process which this assay was designed to measure -- the production of adenosine-3',5'-cyclic monophosphate (cyclic AMP) from adenosine-5'-triphosphate (ATP). This step is accomplished in an *in vitro* incubation containing: (1) a suitable tissue preparation as a source of the enzyme in question, adenyl cyclase; (2) unlabeled ATP as substrate for the reaction; (3) an ATP-regenerating system which consists of phosphoenolpyruvic acid and pyruvate kinase; (4) the co-factors necessary for enzyme activity; and (5) a means of inhibiting or removing the activity

of the enzyme, phosphodiesterase, which destroys cyclic AMP. This reaction is stopped by placing the incubation tubes in a boiling water bath for three minutes. Control samples differ only in that the substrate for the reaction, ATP, is not added until after the reaction has been stopped.

After the boiling water bath, the denatured protein is sedimented by centrifuging at 2,000 x g for 15 minutes. This results in a clear supernatant which contains the formed cyclic AMP.

The competitive reactions (2, below),

2.
$$[^3H]\text{ cyclic AMP} \xrightarrow{\text{Phosphodiesterase}} [^3H]\ 5'\text{-AMP}$$
$$\text{cyclic AMP} \xrightarrow{\text{Phosphodiesterase}} 5'\text{-AMP}$$

are designed to measure the quantity of cyclic AMP formed by reaction 1 and is accomplished in a separate in vitro incubation containing: (1) a rat brain preparation as a source of cyclic 3',5'-adenosine monophosphate phosphodiesterase (phosphodiesterase); (2) tritium labeled cyclic AMP as standard test substrate for the reaction; (3) an aliquot of the supernatant of the arrested first reaction (which contains an unknown amount of the product, unlabeled cyclic AMP); (4) co-factors for the enzyme, phosphodiesterase; and (5) adenosine-5'-monophosphate (5'-AMP) which

serves as a "sink" or "trap" for the product of the reaction that is of interest, [^{3}H] 5'-AMP. The need for this "trap" will be explained in detail later. After the incubation period, a 2,000-fold excess of unlabeled cyclic AMP (compared to the amount of [^{3}H] cyclic AMP present), is added and the reaction stopped by immediately plunging the incubation tubes into an ice water bath.

After the phosphodiesterase reaction is stopped, the labeled nucleotides are separated by a selective precipitation procedure; equimolar amounts of $Ba(OH)_2$ and $ZnSO_4$ are added to the incubate. This precipitates the product of the reaction, [^{3}H] 5'-AMP; however, the unreacted substrate of the reaction, [^{3}H] cyclic AMP, is left undisturbed in the supernatant fraction. An aliquot of the supernatant is then counted in a liquid scintillation counter to estimate the amount of tritium labeled substrate which has not reacted.

The basis of this assay rests upon the dilution of isotopic [^{3}H] cyclic AMP by the unlabeled cyclic AMP which is formed in and transferred from Reaction 1, the adenyl cyclase reaction. The higher the amount of cyclic AMP formed in the cyclase reaction, the higher the amount which is transferred in the aliquot to the phosphodiesterase reaction and therefore the

greater the displacemnt of the [^{3}H] cyclic AMP from the phosphodiesterase reaction. The quantity of displaced [^{3}H] cyclic AMP in the reaction is then monitored after the precipitation step. The quantitation of the amount of cyclic AMP transferred is accomplished by reference to a standard curve prepared by adding known amounts of authentic unlabeled cyclic AMP to the phosphodiesterase reaction. In a typical standard curve, the increasing number of counts remaining in the supernatant is plotted against increasing amounts of unlabeled cyclic AMP added to the reaction.

HISTORICAL REVIEW

This review is divided into two sections. The first section deals with discovery of the adenyl cyclase - cyclic AMP system and its place and importance in our understanding of metabolism and endocrinology. The second section reviews the major methods which have been used to measure the activity of this enzyme in *in vitro* systems.

The Adenyl Cyclase - Cyclic AMP System

In 1956, Sutherland and co-workers began a series of experiments (28, 42) which was designed to answer the question as to how two chemically dissimilar hormones, epinephrine and glucagon, similarly influenced the production of glucose by the liver. The results of these and later experiments bearing on the same question have had a dramatic and far-reaching effect on our understanding of metabolism and endocrinology for the past

fifteen years. The most important aspect of this work by Sutherland and co-workers concerns the discovery of the role of cyclic AMP in the mechanism of action of these hormones. The work leading to this discovery is summarized very briefly below but is described in detail in several review articles (3, 31, 35, 37).

The first important step in solving the above problem was accomplished when it was shown that epinephrine and glucagon exerted their action by stimulating glycogen phosphorylase. This was demonstrated by measuring glycogen phosphorylase activity in homogenates prepared from slices which had been incubated with and without the hormones. In addition, it was found (42) that the level of glycogen phosphorylase activity in liver represented a balance between activation and inactivation of this enzyme. The inactivation of glycogen phosphorylase was catalyzed by a relatively specific phosphatase. Further experiments (28) showed that the activation of glycogen phosphorylase involved a phosphorylation of the inactive enzyme. The enzyme which accomplished this activation was named dephosphophosphorylase kinase.

Hormonal stimulation of glycogen phosphorylase (by way of dephosphophosphorylase kinase) could not be achieved if the

homogenates were centrifuged and the low-speed precipitate removed. If the precipitate was added back, however, the hormonal sensitivity of the system was restored. Further experiments showed that the hormones did not activate phosphorylase in the soluble system, but instead reacted with the particulate material to form a compound which in turn was responsible for the activation of the phosphorylase. This compound was later identified as cyclic AMP (20, 36).

Cyclic AMP

In the thirteen years since the identification of cyclic AMP and the clarification of its involvement in the mechanism of action of epinephrine and glucagon in the liver, further revelations concerning its ubiquitousness in nature and its importance in metabolism and endocrinology have been truly staggering -- to the extent that monographs have been written on its role in cell function (16, 32). Cyclic AMP has been found to be widely distributed in nature and has been identified in all tissues studied (except for erythrocytes of reptiles and amphibia (37, 38). The cellular processes which cyclic AMP has been shown to influence are so numerous that a compilation will

not be attempted here; several reviews record most of its known effects (3, 6, 16, 31, 34, 35, 37, 39).

Adenyl Cyclase

Cyclic AMP is formed from ATP in an enzymatic reaction utilizing Mg^{2+} as a co-factor (38). The enzyme which catalyzes this reaction was named adenyl cyclase in 1962 by Sutherland, Rall, and Menon (38). Adenyl cyclase activity has been found to be restricted to the particulate fraction of homogenates in all cases studied thus far, and, more specifically, to the plasma membrane portion (16, 25) of that fraction.

The Adenyl Cyclase -- Cyclic AMP System in Metabolism and Endocrinology

In the years since the discovery by Sutherland and his associates of the correlations between the action of epinephrine and glucagon in the liver and cyclic AMP, there has accumulated substantial evidence which implicates the adenyl cyclase - cyclic AMP system in the mechanism of action of many other hormones. The evidence for this correlation is too extensive to catalog here (see the monograph by Greengard and Costa (16)); however, suffice it to say that those hormones which have not been shown to be

implicated in the adenyl cyclase -- cyclic AMP system appear to be the exception rather than the rule.

In this connection, Sutherland has developed the concept that many hormones may act by way of a two messenger system (31). Accordingly, the hormone itself is the first messenger which stimulates the formation of a second messenger in its target cells. The second messenger then stimulates specific changes within the cell which had been previously attributed to a direct action of the hormone or first messenger. Cyclic AMP is the only compound to date which has been firmly established to function in the second messenger role.

An Assay For Adenyl Cyclase

As a result of the overwhelming importance which a basic understanding of the adenyl cyclase-cyclic AMP system and its relation to the mechanism of action of many hormones has on our knowledge of metabolism and endocrinology, enormous effort has been expended in the past several years toward unraveling this puzzle. One of the most straightforward ways of investigating this problem in the laboratory has been to determine the effect, if any, of various hormones on the activity of adenyl cyclase in

their target tissues; however, this approach has frequently been hampered for lack of a suitable assay for this enzyme.

Methods of Measuring Adenyl Cyclase

Since the discovery of the adenyl cyclase-cyclic AMP system in 1962, many methods have been published for its measurement, an average of more than one per year. This section will not attempt to describe each assay in detail, but rather will give a synopsis of many of the methods, paying particular attention to the basis upon which each assay is founded.

In general, the assays may be divided into two groups---those which use radioisotopically labeled ATP as substrate for the reaction and those which do not. With regard to the first category, the differences among the assays depend in large measure on the methods employed to separate the labeled product of the reaction (cyclic AMP) from the unreacted labeled substrate of the reaction (ATP). Assays of the second category are more variable in nature; they may utilize either a chemical or a biochemical approach to monitor the formation of cyclic AMP.

The first widely used method to measure adenyl cyclase activity was that employed by Sutherland, Rall, and Menon (38).

ATP was used as substrate and the cyclic AMP formed was monitored by means of a bio-assay which consisted of incubating aliquots of the formed cyclic AMP with ATP, magnesium ions, and liver dephosphophosphorylase in the presence of liver homogenates containing dephosphophosphorylase kinase. After this incubation, the active phosphorylase, formed from the inactive or dephospho-enzyme, was measured. This stimulation of kinase activity was compared with that resulting from addition of known amounts of crystalline cyclic 3',5'-AMP to similar reaction mixtures. This method has been used successfully but has been criticized (18) because of its complexity and the time required to perform the assay. It has also received criticism due to its nonlinear response (5).

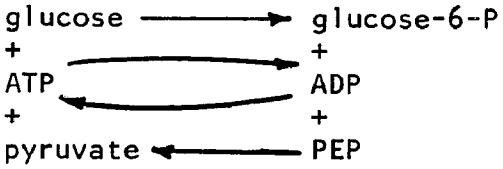
Breckenridge (5), in 1964, evolved a procedure for assaying formed cyclic AMP which may be represented in the series of steps below:

(a) Destruction of unwanted adenylates (apyrase, phosphatase)

(b) Destruction of phosphatase (pepsin)

(c) Cyclic AMP $\longrightarrow$ 5'-AMP

(d) 5'-AMP + PEP $\xrightarrow{(ATP)}$ ATP

(e)


(f) Glucose-6-P + TPN^+ $\longrightarrow$ 6-P-gluconate + TPNH

According to this procedure, cyclic AMP is converted to 5'-AMP by a specific cyclic AMP phosphodiesterase, (step (c)). 5'-AMP is then converted to ATP utilizing the enzymes adenylate kinase and pyruvate kinase, (d). Then the coupled reactions, (e), catalyzed by hexokinase and pyruvate kinase, produce glucose-6-P at a rate proportional to the total ATP present. Finally, the TPNH formed in (f), as a result of the addition of TPN^+ and glucose-6-P dehydrogenase, is measured flurometrically. The amount of TPNH is then used as an index of the amount of cyclic AMP present at the beginning of step (c).

One obvious shortcoming to the above assay is its complexity. In addition, however, it has been criticized due to the number of coupled enzymatic reactions that are employed. It has been pointed out (4) that each of these reactions is subject to interference from substances present in the enzymatic mixtures, which could drastically alter the rate of production of the measured substance, TPNH.

Also, in 1964, Bradham and Woolley (4) developed the first assay for adenyl cyclase which depended on a chemical method for the quantitative determination of cyclic AMP. Impurities present in the incubate were removed by passage through a succession of Dowex-2 and Dowex-50 columns before determining the absorbancy

at 260 mμ of the collected effluent. The quantity of cyclic AMP in the sample was calculated from the absorbancy using the molar extinction coefficient of the compound. This method suffers, first of all, because the time required for each analysis is long as a result of the use of ion-exchange columns. Secondly, the amount of cyclic AMP produced in a typical adenyl cylase reaction, and therefore available for ion-exchange chromatographic purification, is very small.

In 1965, two new methods were developed to measure adenyl cyclase activity--both utilizing radioisotope labeled ATP as the substrate for the reaction. These methods, by Rabinowitz and co-workers (27) and by Hirata and Hayaishi (17), were very similar in nature. Both used paper chromatography, developed by the descending technique, to separate the isotopically labeled nucleotides. The spot corresponding to cyclic AMP was cut out and counted for radioactivity as an index of formed product. However, one important feature was lacking in both assays; neither accounted for recovery of labeled product after chromatography.

Jungas (18), in 1966, described an assay which combined some of the procedures of previous methods. Isotopically labeled

cyclic AMP, formed in the reaction from labeled ATP, was partially separated from the other nucleotides by a Dowex-2 ion-exchange column. This fraction was lyophilized and then subjected to thin-layer chromatography on silica gel. The labeled cyclic AMP was eluted from the gel and counted for radioactivity. Cyclic AMP, labeled with a different isotope than that used for the substrate, ATP, was included in the reaction mixture in order to determine recovery of cyclic AMP. This method contains no serious hindrances other than the time and effort required for its use.

In 1967, Streeto and Reddy (33) developed a method very similar to those of Rabinowitz and co-workers (27), and Hirata and Hayaishi (17), but which corrected for the deficiencies of these two methods. Isotopically labeled ATP was used as substrate; however, a more efficient development of the paper chromatographic procedures allowed for a more precise separation of the labeled cyclic AMP from the other nucleotides present. In addition, the use of cyclic AMP labeled with an isotope other than that used for ATP allowed for the calculation of recovery. Inclusion of an ATP-regenerating system also added to the reliability of the method. Notwithstanding these benefits, however, methods which use paper chromatographic procedures for separating

nucleotides have been criticized (19) due to their time-consuming and difficult routine use. In addition, the method by Streeto and Reddy, as well as that of Rabinowitz and co-workers (27), has been critized (1) because of high counting impurities which are associated with the cyclic AMP spot on the paper chromatograms.

In 1968, Krishna, Weiss, and Brodie (19) produced a method which has been perhaps the most widely used of the methods available since its appearance. This procedure also uses isotopically labeled ATP as substrate. After the reaction is complete, ion-exchange chromatography utilizing Dowex-50 columns partially separates the labeled nucleotides. Equimolar amounts of $Ba(OH)_2$ and $ZnSO_4$ are added to the fraction containing cyclic AMP to precipitate the remaining labeled ATP; the labeled cyclic AMP is not precipitated and therefore remains in the supernatant. An aliquot of the supernatant is then counted for radioactivity. Cyclic AMP, labeled with an isotope other than that used for ATP, is added after the reaction is stopped in order to determine recovery in the column and precipitation procedures. Even though this procedure has been widely used, it has not escaped criticism. For example, Bar and Hechter (1) have shown that a significant amount of radioactivity, not due to cyclic AMP, contaminates the

cyclic AMP fraction. Also, it has been critized (8) due to a lack of precision. In addition, the methods employed for the separation of the nucleotides (the ion-exchange column and the precipitation procedure) recover only 20-30% of the cyclic AMP. This fact, when coupled to a possible contamination of the fraction, could readily lead to erroneous values for adenyl cyclase activity.

In 1970, Brooker (18) published the most recent assay for adenyl cyclase. Unlabeled ATP is used as substrate for the reaction. The formed cyclic AMP is then separated and quantitated by use of high pressure anion exchange liquid chromatography. A small volume ultraviolet flow cell is used to detect cyclic AMP in the chromatographic effluent. This method is reported to be highly reproduceable. However, it has not been available long enough for an objective evaluation to be recorded by others.

In conclusion, the purpose of this brief review has been to acquaint the reader with many of the methods employed for measuring adenyl cyclase over the past fifteen years and to enhance appreciation of the impedimenta involved in assaying this enzyme. This difficulty may be best recognized by considering the large number of methods which have evolved in the past decade for the measurement of adenyl cyclase and a further realization that none has found general acceptance. Accordingly, the development of a

quick and sensitive method for the measurement of adenyl cyclase activity appeared to be worthwhile and is the subject of the research described in this thesis.

MATERIALS AND METHODS

The materials and methods used in the majority of experiments in this study are presented below. Other materials and methodology specific for particular experiments will be noted and described at the appropriate time. Unless otherwise stated, all routine chemicals were reagent grade and purchased from J. T. Baker.

The Phosphodiesterase Reaction

Preparation of Rat Brain Phosphodiesterase

Phosphodiesterase enzyme was prepared from rat brain by the method of Brooker, Thomas, and Appleman (9). Male Sprague-Dawley rats, weighing 200-250 g, were decapitated, the brains removed, weighed, and then homogenized in five volumes of distilled water with a Potter-Elvehjem all glass homogenizer. The homogenate was centrifuged at 30,000 x g for 30 minutes using a Beckman ultracentrifuge (rotor number 50). The supernatant was removed and

adjusted to 50% saturation with $(NH_4)_2SO_4$ (Sigma Chemical Company: enzyme grade) added as a saturated solution. After centrifugation at 30,000 x g for 10 minutes and pouring off the supernatant, the pellet was resuspended (5 volumes/g original wet weight) in 60 mM Tris-HCL buffer (pH 8.0) containing 5 mM 2-mercaptoethanol (Sigma) and dialyzed for approximately fifteen hours against two changes of the buffer. An aliquot was removed for protein determination by the Lowry procedure (15); the remainder was divided into small aliquots and stored at -20° C in 2 ml glass vials. The vials, as needed for the incubation procedure, were thawed in a 4° C refrigerator and diluted with a freshly prepared solution of bovine serum albumin (BSA - 2 mg/ml, Calbiochem) containing 20 mM 2-mercaptoethanol to yield a final "phosphodiesterase protein" concentration of 200 mg/ml.

Phosphodiesterase Reaction Medium

[^{3}H] cyclic AMP (2340 mc/mmole), obtained from Amersham/Searle, was diluted to 40 µc/ml with 50% ethanol. As needed for the phosphodiesterase reaction, 7.5 µl of this solution was added to each ml of a previously prepared stock incubation preparation containing:

120 mM Tris-HCL (pH 8.0)

2.5 mM EDTA

120 mM $MgCL_2$

0.96 mM 5'-AMP

This latter solution (without [^{3}H] cyclic AMP) was prepared in bulk volumes of 100 ml and stored frozen in 10 ml portions.

To each phosphodiesterase incubation tube, 0.2 ml of the finished stock incubation preparation was added so that the final concentration of [^{3}H] cyclic AMP was 5.33×10^{-8} M. Each incubation tube (0.6 ml final volume) therefore contained 32 picomoles of [^{3}H] cyclic AMP which represented approximately 75×10^{-3} µc or 165,000 DPM.

Barium Hydroxide and Zinc Sulfate Solutions

Separate solutions of $Ba(OH)_2$ and $ZnSO_4$ were prepared at concentrations of 0.17 M for use in the selective nucleotide precipitation procedure. This concentration of $Ba(OH)_2$ resulted in a cloudy solution; however, the clear supernatant achieved upon settling was used for the precipitation.

Scintillation Fluid

The scintillation fluid used in the measurement of the radioactivity contained PPO (2,5 diphenyloxazole), POPOP (1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene) and naphthalene in p-dioxane (Sigma) and was prepared as described by Brooker (19). PPO and POPOP were purchased from Packard Instruments. The stock

scintillation solution was stored in dark glass bottles and fifteen ml were used in each counting vial.

Phosphodiesterase Incubation Procedure

The incubations were conducted in 13 x 100 mm disposable glass test tubes. All solutions containing radioactive material were delivered volumetrically with an Eppendorf pipette using disposable plastic tips (accuracy ± 0.5%). A 0.2 ml portion of the stock incubation solution containing [^{3}H] cyclic AMP was placed in each test tube and an equal volume of either H_2O, 60 mM Tris-HCl (pH 8.0), various concentrations of authentic cyclic AMP, or supernatant from an arrested adenyl cyclase reaction was added and the reaction started by adding 0.2 ml of the phosphodiesterase enzyme preparation. The incubations were carried out at 30 ± 0.05° C for various times (usually ten minutes) in an Aminco shaking constant temperature water bath. After the incubation period, 0.4 ml of 160 µM authentic cyclic AMP was added and the reaction stopped by immediately plunging the test tube into an ice water bath. After cooling, the $Ba(OH)_2$ and $ZnSO_4$ solutions (0.2 ml each) were added in that order and the tubes were shaken on a Vortex mixer. The total volume of each test tube at this point was 1.4 ml. The

tubes were then centrifuged for fifteen minutes at 2,000 x g to yield a clear supernate. A 1.0 ml aliquot of each supernate was placed in a scintillation vial, followed by the addition of scintillation fluid. The vials were counted for radioactivity with a Nuclear Chicago Unilux II liquid scintillation spectrometer. All counting data were ocrrected for quenching using a channels ratio method (24).

A series of tubes was included in each experiment to determine the exact quantity (CPM) of [^{3}H] cyclic AMP added. Enzyme was not added and these tubes were not subjected to the precipitation procedure. Instead, their volumes were made equivalent to the other tubes by the addition of distilled H_2O. These tubes will be referred to as "CPM" Check" in the experimental sections.

Another series of tubes was generally included to determine if [^{3}H] cyclic AMP was being lost by the precipitation procedure. These tubes contained either no enzyme or an enzyme which had been inactivated in a boiling water bath. These samples were subjected to the procedure and will be referred to as "Blanks" in the experimental section.

THE SEPARATION OF THE LABELED NUCLEOTIDES - CYCLIC AMP AND 5'AMP

The first step in the experimental development of this assay for adenyl cyclase concerned the second reaction in the assay sequence -- i.e., the conversion of [^{3}H] cyclic AMP to [^{3}H] 5'AMP by the enzyme phosphodiesterase. This section will deal specifically with separation of the substrate and product of this reaction by the $Ba(OH)_2$ - $ZnSO_4$ precipitation procedure.

Introduction

Preliminary experiments indicated that a substantial amount of 5'-nucleotidase-like activity was present in the rat brain phosphodiesterase preparation and would, if allowed to proceed, interfere with the proposed cyclic AMP determination by converting the reaction product, [^{3}H] 5'-AMP, into [^{3}H] adenosine. The latter compound has been reported (19) not to be precipitated by the $Ba(OH)_2$ - $ZnSO_4$ procedure and therefore would not be distinguishable from the reaction substrate, [^{3}H] cyclic AMP, during counting of the supernatant. Hypothetically, this problem

could be solved by including unlabeled 5'-AMP in the phosphodiesterase reaction mixture. This would serve as a "trap" or "sink" for the [^{3}H] 5'-AMP formed by the reaction; the unlabeled 5'-AMP present in great excess would, in effect, spare the [^{3}H] 5'AMP from further conversion to [^{3}H] adenosine and thus reduce the amount of radioactive reaction product which fails to be precipitated.

Methods, Results and Discussion

An experiment was therefore performed to test the effect of unlabeled 5'-AMP on the phosphodiesterase reaction. The procedure was the same as that given in the Materials and Methods section except that 0.2 ml of 60 mM Tris-HCl (pH 8.0) occupied the volume usually reserved for either unlabeled cyclic AMP (for a standard curve determination) or the supernatant from a previously arrested adenyl cyclase reaction (for cyclase activity determination). Also, water rather than 160 µM unlabeled cyclic AMP was added at the conclusion of the incubation procedure.

The results are shown in Table 1. The presence of 5'-AMP in the series incubated with enzyme increased the precipitation of the label, as shown by the smaller percentage of counts remaining in the supernatant. This was considered a result of the diluting effect of unlabeled 5'-AMP on the [^{3}H] 5'-AMP pool, thus retarding its conversion to [^{3}H] adenosine. The "Blank" results indicate

Table 1

The effect of 5'-AMP (0.04 mM) on the phosphodiesterase reaction and on the precipitation of [^{3}H] cyclic AMP.

	Percent of original counts remaining in the supernatant*	
	5'-AMP Absent in Incubation Medium	5'-AMP Present in Incubation Medium
CPM Check (No Incubation)	100.0 ± 2.7†	100.0 ± 2.5
Blank (No Enzyme)	69.9 ± 2.0	24.7 ± 1.3
Incubation (Enzyme Present)	79.1 ± 0.7	30.2 ± 0.3

*Incubation condition: 10 minutes at 30° C.

†Mean ± 1 S.D. (n = 3).

that 5'-AMP in the incubation medium also decreased the counts in the series which did not contain enzyme -- possibly by co-precipitation of cyclic AMP with 5'-AMP. However, there was also significant precipitation of [^{3}H] cyclic AMP in the "Blanks" without 5'-AMP and the amount of substrate lost by this route was intolerable. The remaining experiments in this section were designed to correct this undesired precipitation of [^{3}H] cyclic AMP.

One possible way to decrease the precipitation of [^{3}H] cyclic AMP is by adding an abundance of unlabeled cyclic AMP to the preparation just prior to precipitation. This should work especially well if only a small constant amount of cyclic AMP is precipitated rather than a fixed percentage of the total amount present. An experiment was designed to test this hypothesis. [^{3}H] cyclic AMP, contained in the "Incubation Preparation," was subjected to the precipitation procedure in the presence and absence of a 1,000 fold excess of unlabeled cyclic AMP. The effect of 5'-AMP on the precipitation of [^{3}H] cyclic AMP was also tested because of the data in Table 1 which suggested loss of [^{3}H] cyclic AMP by co-precipitation. As shown in Table 2, the addition of 5'-AMP did not increase the precipitation of [^{3}H] cyclic AMP as was expected. In fact, a smaller quantity of the label compound was lost in the presence of 5'-AMP than when it was absent. However,

Table 2

The effect of adding unlabeled cyclic AMP (50 nM) on the precipitation of [^{3}H] cyclic AMP.

5'-AMP*	Unlabeled Cyclic AMP	% CPM Before Precipitation	% CPM After Precipitation
-	-	100.0 ± 1.0†	44.9 ± 2.0
-	+	100.0 ± 2.6	100.0 ± 1.5
+	-	100.0 ± 0.2	58.4 ± 0.7
+	+	100.0 ± 1.6	96.7 ± 0.8

*0.04 mM Final Concentration.

†Mean ± 1 S.D. (n = 3).

the addition of a 1,000 fold excess of unlabeled cyclic AMP did decrease greatly the loss of [^{3}H] cyclic AMP counts with precipitation. In the series with and without added 5'-AMP, the addition of unlabeled cyclic AMP resulted in count recoveries of 96.7% and 100.0% respectively.

With the problem of [^{3}H] cyclic AMP loss apparently solved, the experiment reported in Table 1 was repeated in order to determine correctly the effect of 0.04 mM 5'-AMP on the phosphodiesterase reaction. This experiment differed from that of Table 1 by the addition of 0.2 ml of 160 µM unlabeled cyclic AMP to each incubation tube immediately after the reaction was stopped and therefore before the precipitation procedure. The counting rates of the "Blank" tubes, Table 3, indicated that a substantial amount of radioactivity was still being lost on precipitation even in the presence of cold cyclic AMP. The loss was reduced but still excessive as the "Blank" tubes must be kept as close as possible to 100% of the CPM Check in order to have a good control value for the precipitation procedure. The only difference between this experiment and that of Table 2 was that additional ingredients, necessary for the phosphodiesterase incubation, were present. In this experiment, the effect of 5'-AMP during the incubation was very similar to that shown in Table 1.

Table 3

The effect of 5'-AMP (0.04 mM) on the phosphodiesterase reaction and on the precipitation of [^{3}H] cyclic AMP in the presence of 50 nM unlabeled cyclic AMP.

	Percent of original counts* remaining in the supernatant	
	5'-AMP Absent in Incubation Medium	5'-AMP Present in Incubation Medium
CPM Check	100.0 ± 0.2†	100.0 ± 1.2
Blank (No Enzyme)	70.0 ± 2.2	66.4 ± 5.2
Incubation (Enzyme Present)	73.8 ± 0.5	45.1 ± 1.9

*Incubation conditions: 10 minutes at 30° C.

†Mean ± 1 S.D. (n = 3).

Table 4 shows the results of an experiment designed to test the effect of the individual constituents of the phosphodiesterase reaction medium on the precipitation of [^{3}H] cyclic AMP. There was no incubation in this experiment. The $Ba(OH)_2$ - $ZnSO_4$ precipitation procedure was carried out after the addition of each ingredient shown and after the addition of 0.2 ml of either 60 mM Tris-HCl (pH 8.0) or H_2O. From the results, one point was readily obvious -- in those tubes to which the Tris-HCL buffer was added, consistently more [^{3}H] cyclic AMP was lost by precipitation. This is seen when one compares series numbers one with four, two with five, three with six, and two with seven.

An experiment was therefore designed to determine the correlation between the volume of 60 mM Tris-HCl (pH 8.0) added and the amount of [^{3}H] cyclic AMP precipitated. Varying amounts of the buffer were added to tubes containing 0.2 ml of the [^{3}H] cyclic AMP incubation preparation (containing 24 micromoles Tris) and 0.2 ml of 160 µM unlabeled cyclic AMP. A series of samples also contained an appropriate amount of the phosphodiesterase enzyme which had been inactivated by heating in a boiling water bath for three minutes. The samples were precipitated with $Ba(OH)_2$ and $ZnSO_4$ and the supernatant counted. As noted in Figure 1 both series resulted in an essentially linear correlation between the volume of Tris solution added (up to 0.4 ml) and the number of CPM remaining in the supernatant.

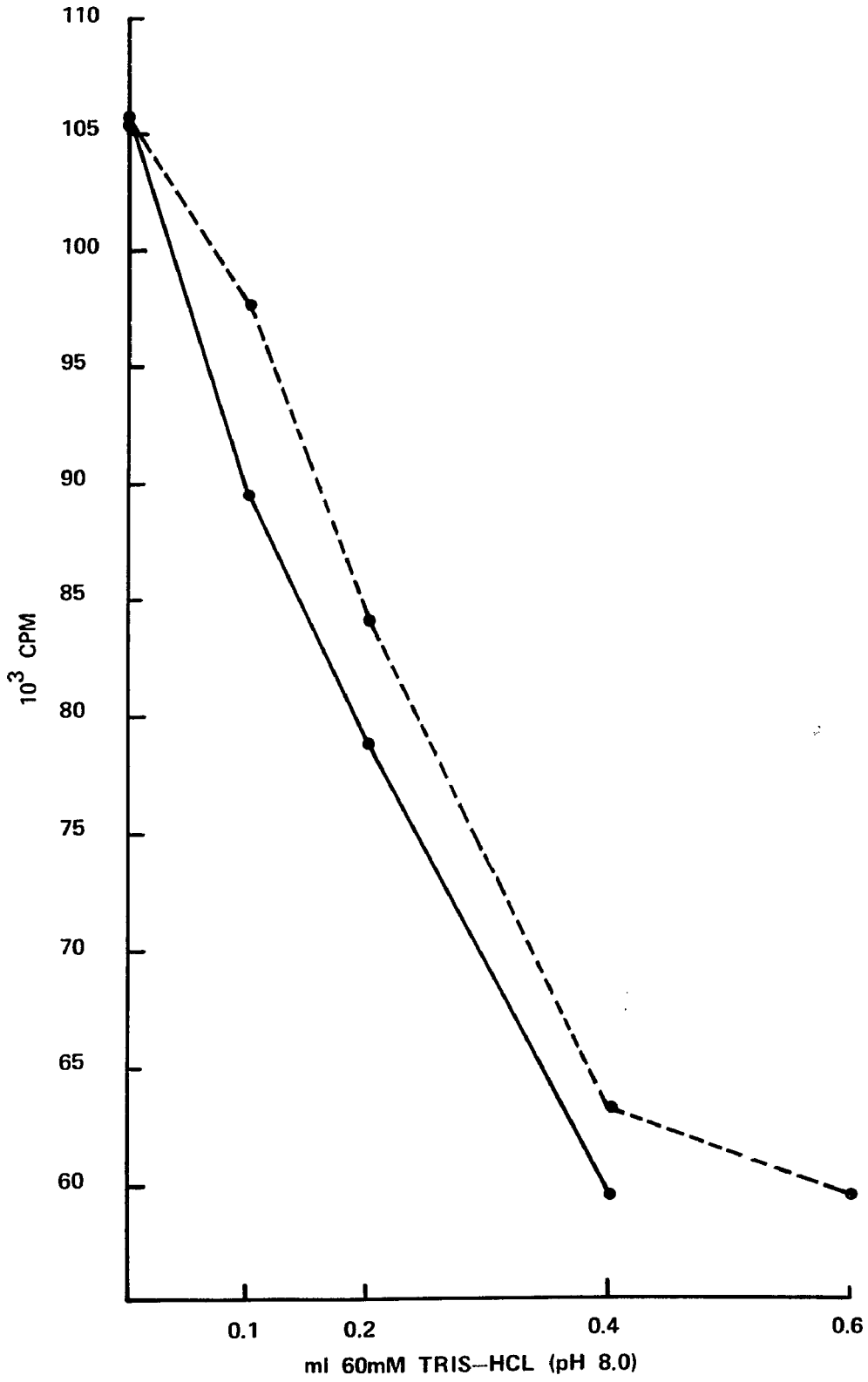
Table 4

The effect of constituents of the phosphodiesterase incubate on the precipitation of [^{3}H] cyclic AMP.

Series Number	60 mM Tris-HCl (pH 8.0) Added Plus	% Initial CPM Remaining After Precipitation
1	Blank (60 mM Tris-HCl (pH 8.0))	78.2 ± 2.4*
2	BSA and Mercaptoethanol in 60 mM Tris-HCl (pH 8.0)	85.8 ± 0.5
3	Phosphodiesterase in BSA and Mercaptoethanol in 60 mM Tris-HCl (pH 8.0)	62.7 ± 0.6
	H_2O Added Plus	
4	Blank (H_2O)	101.8 ± 2.8
5	BSA and Mercaptoethanol in H_2O	100.4 ± 3.1
6	Phosphodiesterase in BSA and Mercaptoethanol in H_2O	88.7 ± 0.3
7	BSA and Mercaptoethanol in 60 mM Tris-HCl (pH 8.0)	97.0 ± 0.1

*Mean ± 1 S.D. (n = 3).

Figure 1. The effect of Tris on the precipitation of [^{3}H] cyclic AMP by $Ba(OH)_2$ and $ZnSO_4$. Each point refers to the number of counts (average of three determinations) remaining in the supernate after the precipitation procedure. The solid line indicates the series of tubes which contained a killed enzyme preparation. The broken line refers to the series which did not contain enzyme.


110
105
100
95
90
85
80
75
70
65
60
10^3 CPM
0.1
0.2
0.4
0.6
ml 60mM TRIS–HCL (pH 8.0)

In order to confirm that the above was an effect of the Tris molecule or ion and not due to a pH change as a result of increased buffer capacity as more Tris was added, the previous experiment was repeated, checking the pH of the solution before and after the precipitation procedure. The results are presented in the upper half of Table 5. It should be noted that the change in the corrected CPM was very similar to the previous experiment (Figure 1) and reflects a direct correlation as before. The change in the pH of the solution before precipitation was very slight -- a range of only 0.11 pH units.

An additional series was run to test the effect of an intentional change in pH on the precipitation procedure; aliquots of 0.1 N NaOH were added to affect the pH change. As shown in the lower half of Table 5, elevations in pH were associated with small decreases in counts. Since a pH change of 1.5 units (7.68 to 9.20) resulted in a change of only 7,000 counts, the change expected from the observed pH change (0.11 units) in the previous series would be insignificant.

Summary

The problem which suggested this series of experiments -- i.e., development of a method of precipitating the [^{3}H] 5'-AMP in the reaction mixture without disturbing the [^{3}H] cyclic AMP -- has

Table 5

The effect of Tris-HCl (pH 8.0) concentration and of pH on the precipitation of [^{3}H] cyclic AMP.

ml of 60 mM Tris-HCl (pH 8.0) Added	CPM Remaining After Precipitation	pH Before Precipitation	pH After Precipitation
0.0	111,675 ± 681*	7.76 ± 0.01	7.42 ± 0.08
0.1	102,103 ± 2,122	7.80 ± 0.00	7.52 ± 0.00
0.2	94,166 ± 2,863	7.82 ± 0.01	7.57 ± 0.05
0.4	81,452 ± 4,285	7.83 ± 0.00	7.59 ± 0.03
0.6	73,643 ± 3,540	7.87 ± 0.02	7.62 ± 0.02

ml of 0.1 N NaOH Added	CPM Remaining After Precipitation	pH Before Precipitation	pH After Precipitation
0.0	109,322 ± 1,366*	7.68 ± 0.01	7.40 ± 0.02
0.1	106,464 ± 541	8.14 ± 0.01	7.55 ± 0.00
0.2	103,313 ± 1,046	8.55 ± 0.00	7.73 ± 0.02
0.3	101,576 ± 1,082	9.20 ± 0.00	7.85 ± 0.01

*Mean ± 1 S.D. (n = 3).

been solved. The changes instituted in the assay procedure to correct this difficulty are summarized below:

(1) Limitation of the amount of Tris in the reaction mixtures to 24 micromoles, an amount insuring an adequately buffered preparation but one which has only a slight effect on the precipitation of cyclic AMP.

(2) The addition of a 2,000-fold excess of unlabeled cyclic AMP just prior to precipitation.

THE MASKING OF 5'-NUCLEOTIDASE-LIKE ACTIVITY

Introduction

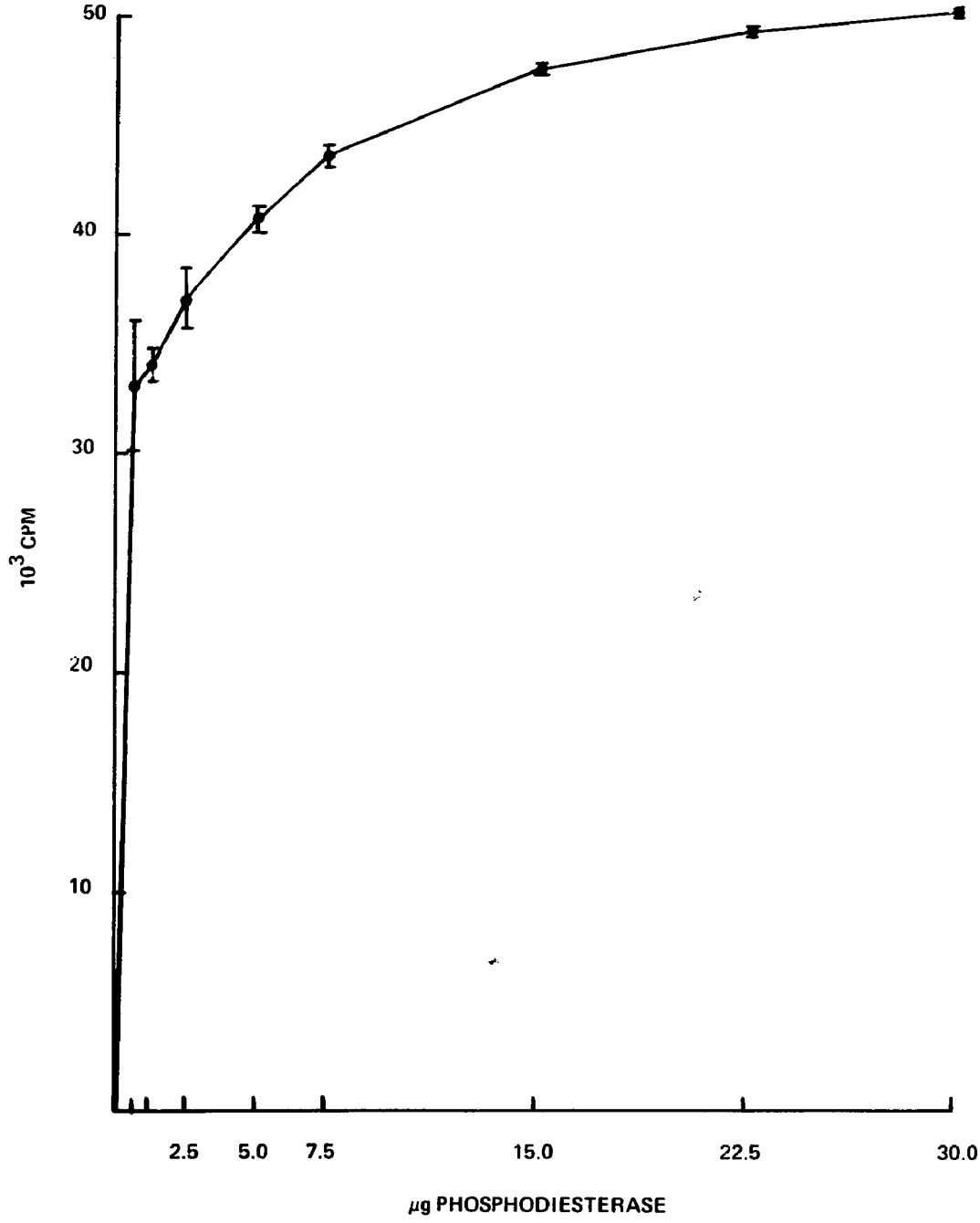
As mentioned in the previous section, experiments indicated there might be substantial amounts of 5'-nucleotidase-like activity (hereafter referred to as 5'-nucleotidase) in the rat brain phosphodiesterase preparation. As was pointed out, this enzyme would interfere with the cyclic AMP assay because the [^{3}H] 5'-AMP formed would be converted to a substance, [^{3}H] adenosine, which could not be separated from the unreacted [^{3}H] cyclic AMP by the precipitation procedure. This is not a problem in the most frequently used assay for phosphodiesterase because that method (2) actually employed the formation of adenosine catalyzed by adding an excess of alkaline phosphatase. The parameter measured to estimate phosphodiesterase activity was the inorganic phosphate release from 5'-AMP under those conditions.

This chapter will consider both the evidence for 5'-nucleotidase in the rat brain phosphodiesterase preparation and methods for inhibiting or making inconsequential (masking) its activity.

Methods and Materials

In this series of experiments, the methods used in the phosphodiesterase incubations were as recorded previously except that the labeled compounds present after incubation (in the experiment of Figure 2) were separated by anion-exchange resins as described by Brooker, Thomas, and Appleman (9). Their method involves quantitative conversion of the labeled product of the phosphodiesterase reaction, [^{3}H] 5'-AMP, into [^{3}H] adenosine by using a second reaction catalyzed by adding an excess of 5'-nucleotidase from King Cobra Venom (Sigma Chemical Company). After the incubation is complete, the labeled compounds, [^{3}H] cyclic AMP and [^{3}H] adenosine, are separated (for radioisotope counting purposes) by adding a suitably prepared anion-exchange resin, Bio Rad AG-IX-2, Cl^- 200-400 mesh. Scintillation fluid is then added and the radioactivity measured in a scintillation counter. Radiographic separation of the labeled compounds is accomplished because the unreacted substrate, [^{3}H] cyclic AMP, is absorbed by the resin so that the compound is completely isolated from the scintillation fluid -- resulting in a 100% quenching of its tritium. However, the nucleoside, [^{3}H] adenosine is not bound by the resin and is therefore measured essentially unquenched. The rate of [^{3}H] adenosine formation is used to calculate the activity of phosphodiesterase.

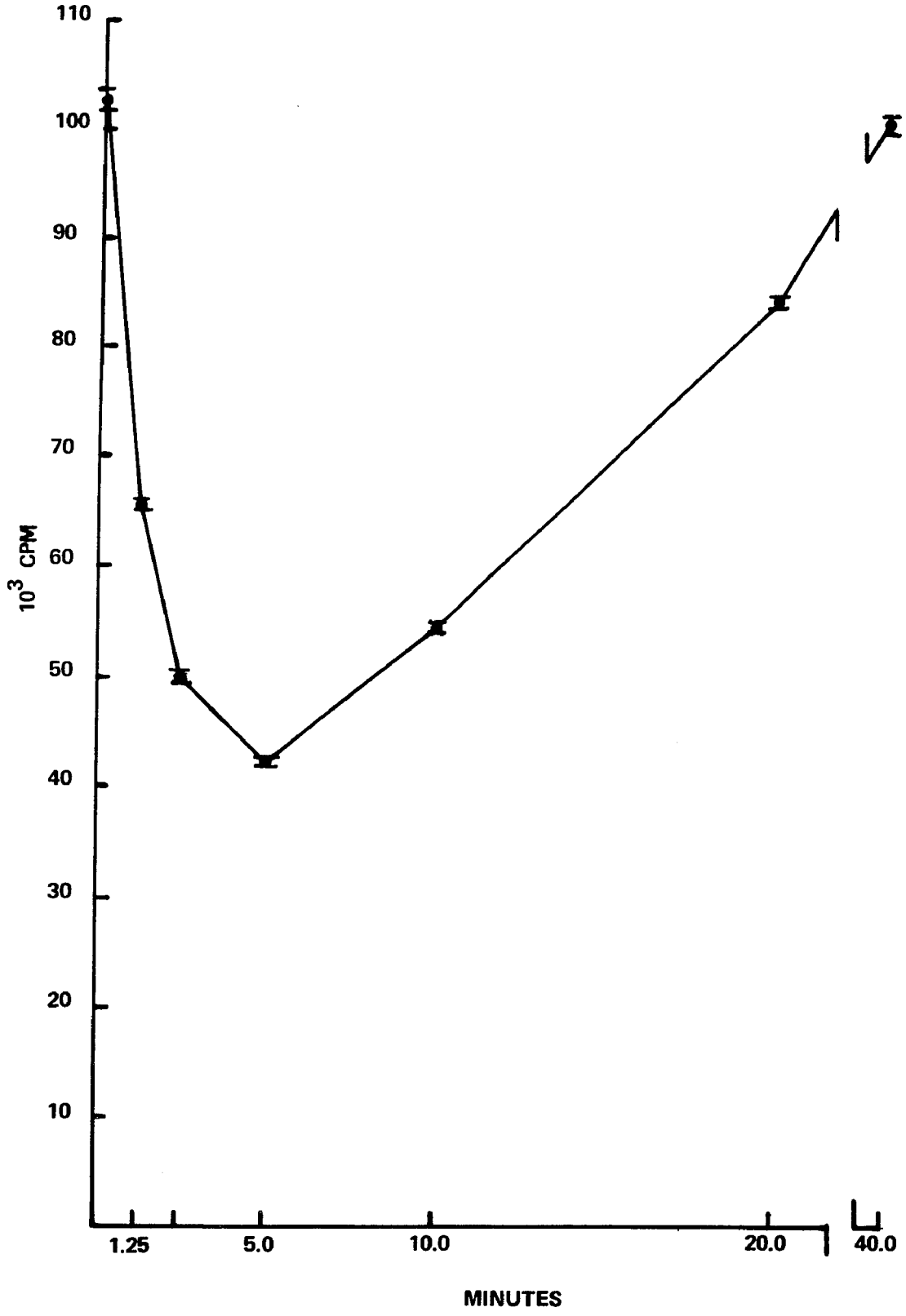
Figure 2. Conversion of cyclic AMP to adenosine as a function of the increase in the phosphodiesterase preparation concentration. Each point represents the mean of three determinations $\pm$ S.D. of [^{3}H] adenosine radioactivity after completion of the incubation. µg phosphodiesterase refers to the amount of phosphodiesterase preparation protein included in each experimental tube. Incubation conditions: 10 minutes at 30° C.


50
40
30
20
10
10^3 CPM
2.5
5.0
7.5
15.0
22.5
30.0
μg PHOSPHODIESTERASE

Results and Discussion

As shown in Figure 2, increased concentrations of rat brain phosphodiesterase were associated with the increased conversion of its reaction product, [^{3}H] 5'-AMP, into [^{3}H] adenosine. Since no exogenous 5'-nucleotidase was contained in the reaction medium of this experiment, it appeared highly probable that there was sufficient 5'-nucleotidase present in the rat brain phosphodiesterase preparation to convert major fractions of the formed [^{3}H] 5'-AMP into [^{3}H] adenosine. This is verified by the experiment of Figure 3 which was designed to measure conversion with time of [^{3}H] cyclic AMP to [^{3}H] 5'-AMP catalyzed by the rat brain phosphodiesterase preparation. The methods utilized in this experiment were those given in the Materials and Methods section for the measurement of phosphodiesterase except that 0.12 mM (rather than 0.96 mM) 5'-AMP was included in the [^{3}H] cyclic AMP Incubation Preparation. As can be seen, the activity of phosphodiesterase during the initial five-minute incubation interval was apparent, indicated by the decrease in CPM remaining as [^{3}H] cyclic AMP. However, during the incubation interval between five minutes and forty minutes, there was an increase in CPM remaining in the supernatant after precipitation. This was interpreted as 5'-nucleotidase activity in the phosphodiesterase

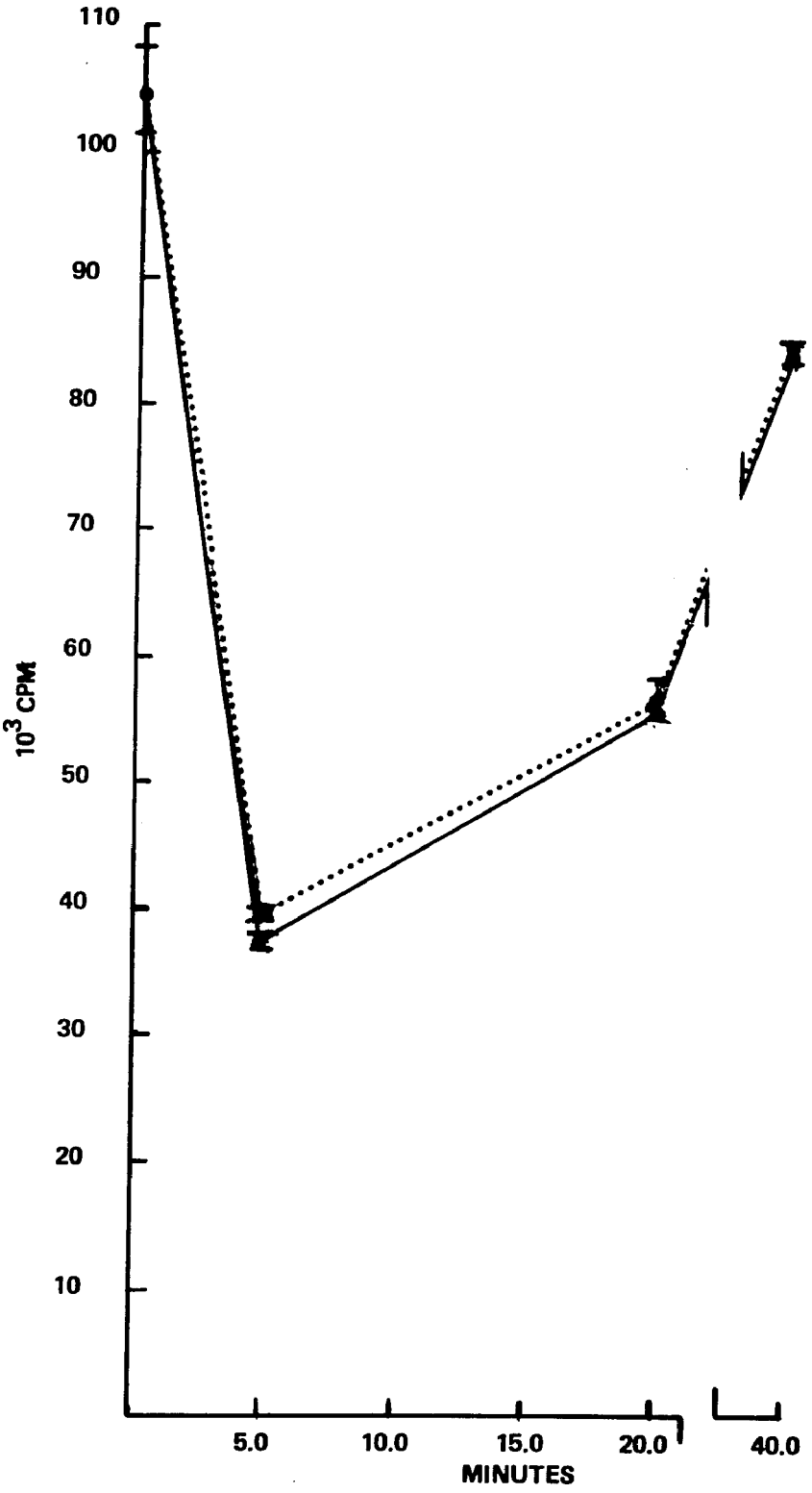
Figure 3. The reaction profile of whole rat brain phosphodiesterase with time. Each point is the mean CPM of three determinations ± S.D. and represents the radioactivity of the labeled compounds not subject to precipitation. Minutes refer to the length of incubation at 30° C.


110
100
90
80
70
60
50
40
30
20
10
10^3 CPM
1.25
5.0
10.0
20.0
40.0
MINUTES

preparation which was sufficient to degrade major fractions of the [^{3}H] 5'-AMP as well as the unlabeled 5'-AMP added to mask the activity of the nucleotidase.

The rat brain phosphodiesterase used in the experiment of Figure 3 had been prepared using tissue from whole rat brain. Since Weiss and Costa (41) had reported that different regions of the rat brain contained different activities of phosphodiesterase, two additional phosphodiesterase preparations were made -- one utilizing tissue from the cerebral hemispheres, the other using the medulla oblongata. These regions of the rat brain were chosen only because they represented a large tissue sample. The purpose of this experiment was to find, if possible, a region of the brain with a high ratio of phosphodiesterase to 5'-nucleotidase activity. As can be seen in Figure 4, this approach to the problem was unrewarding. In both preparations, the activity of phosphodiesterase was essentially the same as the whole brain preparation; compare the decline in CPM after five minutes incubation with that shown in Figure 3. There appeared to be less 5'-nucleotidase activity; compare the increases in CPM at forty minutes in Figures 3 and 4. However, the decrease was not sufficient to warrant pursuit of the problem by this approach.

Figure 4. The reaction profile of phosphodiesterase prepared from different regions of rat brain tissue. The solid line indicates the activity of a preparation of the cerebral hemispheres. The broken line refers to a preparation that utilized the medulla oblongata. Each point is the mean CPM of three determinations $\pm$ S.D. plotted against incubation time at 30^{o} C.


110
100
90
80
70
60
50
40
30
20
10
10^3 CPM
5.0
10.0
15.0
20.0
40.0
MINUTES

Cheung (10) reported that preparations of rat brain phosphodiesterase could be stored at -20° C for weeks without significant loss of activity. Since many enzymes are labile under these conditions, it was considered worthwhile to test the stability of 5'-nucleotidase activity during freezing. Accordingly, rat brain preparations were frozen for varying periods of time at -20° C, after which the phosphodiesterase and 5'-nucleotidase activities were monitored. As before, an increase in CPM during the latter periods of incubation was used as an index of 5'-nucleotidase activity at levels too high for the assay procedure under consideration. Incubation periods of five, twenty, and forty minutes were considered sufficient to see any change in 5'-nucleotidase activity due to the freezing process. The change in % CPM during the initial five minute incubation periods (Table 6) indicated that only a slight loss of phosphodiesterase activity occurred in preparations frozen up to 60 days, thus confirming the report of Cheung (10). However, the 180 day value of 67.0% indicates that a substantial amount of activity had been lost by that time. The decrease, as the freezing time increased, in % CPM at both the 20 and 40 minute incubation periods did indicate some loss of 5'-nucleotidase activity; however, even after 6 months the decrease was not sufficient for the proposed

Table 6

The effect of freezing on rat brain phosphodiesterase and 5'-nucleotidase activities.

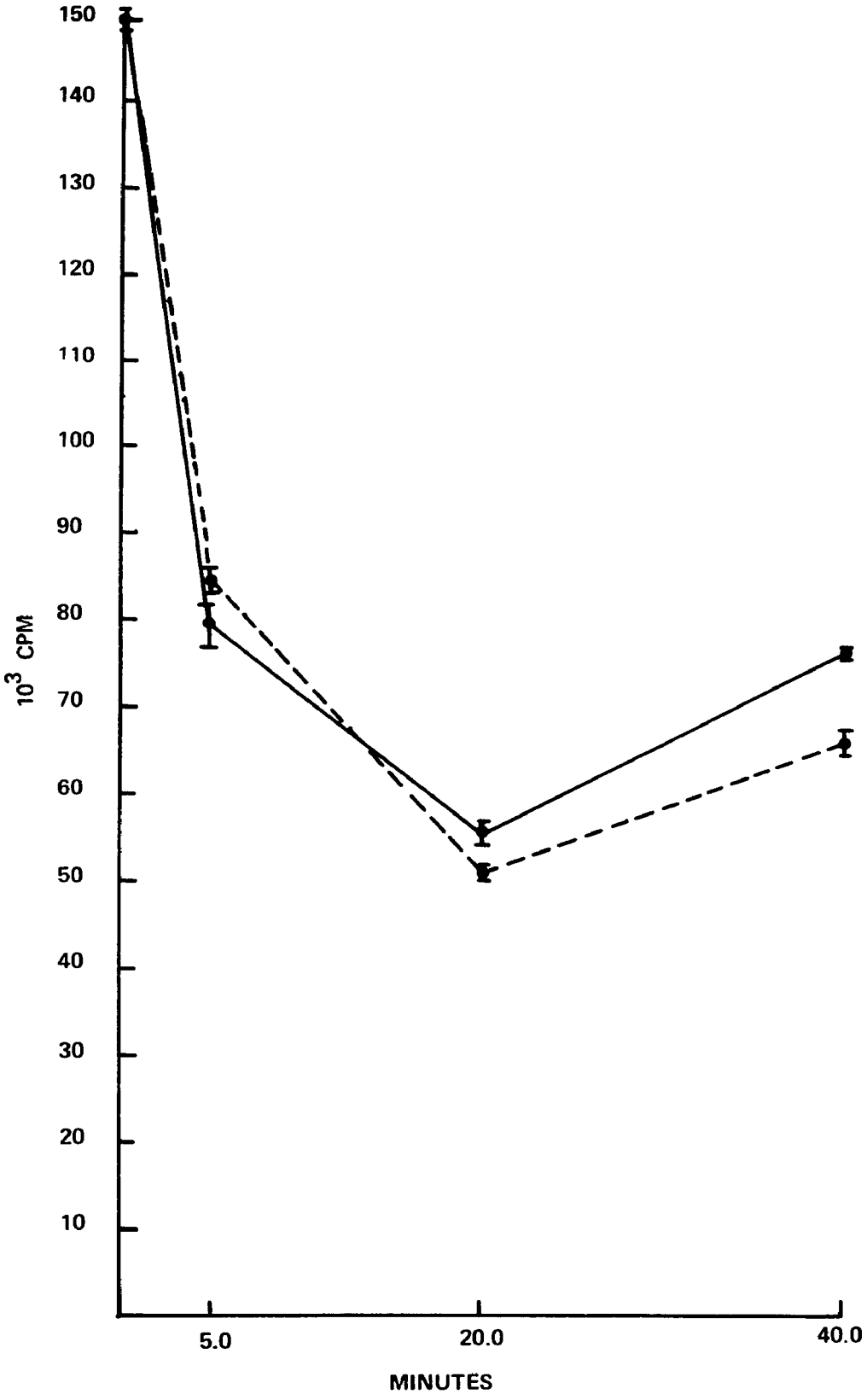
Number of Days Frozen	% Initial CPM Remaining in Supernate 5 Minutes	20 Minutes	40 Minutes
Control (not frozen)	41.1 ± 0.1*	82.7 ± 0.9	98.6 ± 1.1
5	43.7 ± 0.5	83.5 ± 0.8	94.7 ± 1.2
13	43.6 ± 1.4	45.9 ± 1.4	70.0 ± 0.6
60	47.0 ± 0.3	32.9 ± 0.1	50.4 ± 0.5
180 (6 months)	67.0 ± 1.5	26.3 ± 1.9	29.4 ± 2.0

*Mean ± 1 S.D. (n = 3).

assay. This is shown by the increase in % CPM in the interval between the 20 and 40 minute incubation periods for the 180 day preparation. Other preparations, specifically those prepared from rat brain cerebral hemispheres and medulla oblongata, were also subjected to the freezing process (results not shown). The decay of 5'-nucleotidase in these preparations followed the same rate as that noted above for the whole brain preparation. It was therefore decided that means other than freezing, would have to be instituted in the procedure in order to remove or mask the activity of 5'-nucleotidase.

Chemical inhibitors were tested next as a means of reducing 5'-nucleotidase activity. It had been reported (14) that adenosine and inorganic phosphate exerted an inhibitory effect on this enzyme. Accordingly, 0.1 mM adenosine and 10 mM KH_2PO_4 (the recommended concentrations) were included in the phosphodiesterase incubation. The inclusion of adenosine in the incubation did not significantly alter 5'-nucleotidase activity (results not shown); however, incubation with phosphate, Figure 5, did indicate a substantial degree of inhibition. Unfortunately, the inhibition was not sufficient for the needs in this procedure as there was still an increase in corrected CPM noted at the 40 minute incubation period. The use of higher concentrations of

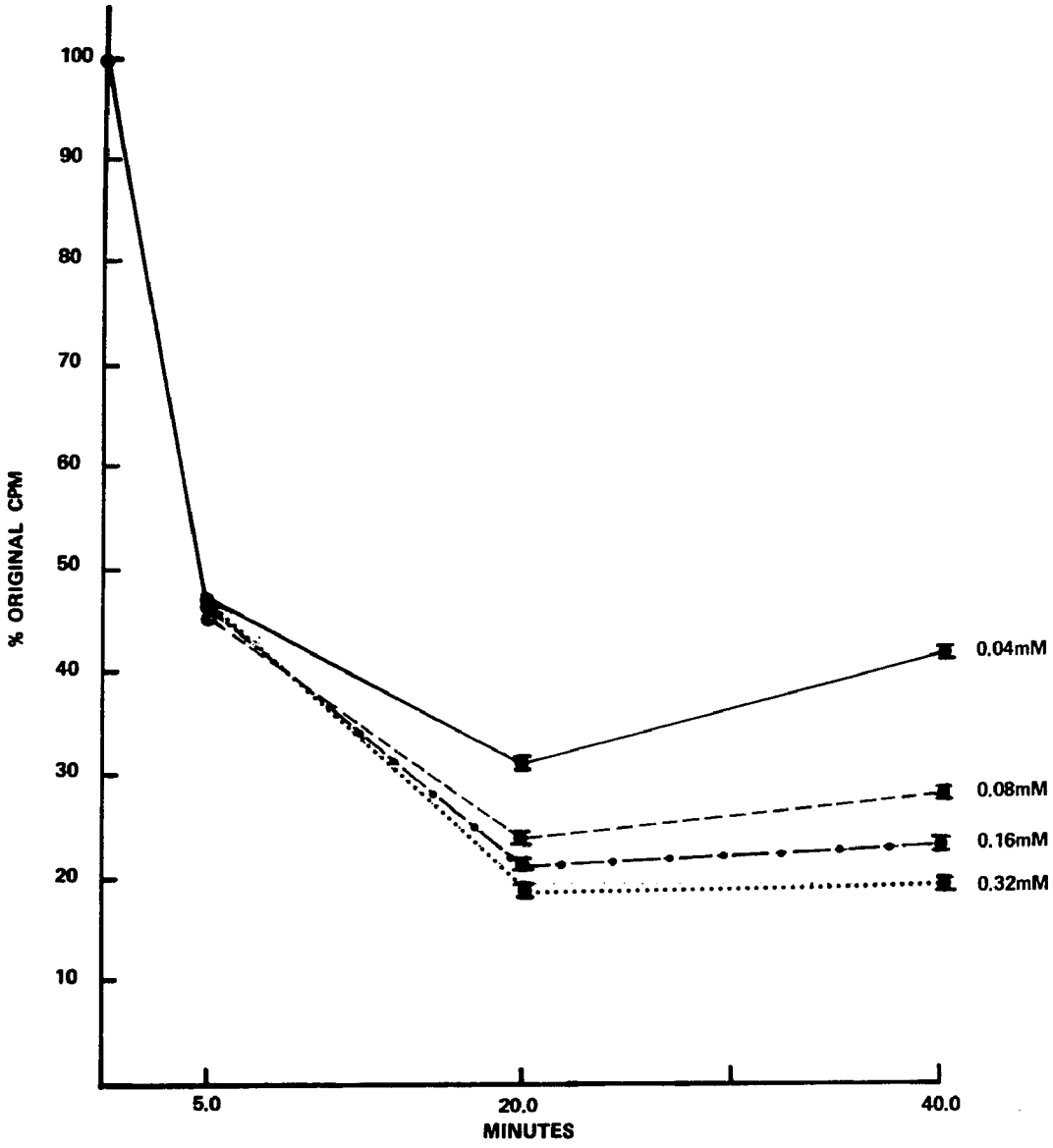
Figure 5. The effect of KH_2PO_4 on 5'-nucleotidase and phosphodiesterase activities. The solid line indicates the control tubes while the broken line refers to the experimental tubes. Each point is the mean CPM of three determinations $\pm$ S.D. Minutes refer to the length of incubation at 30° C.


150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
10^3 CPM
5.0
20.0
40.0
MINUTES

phosphate did not seem warranted as the amount used in the present experiment was sufficient to also produce inhibition of the phosphodiesterase enzyme. This can be noted by comparing the CPM values at the 5 minute incubation interval. This effect has been previously reported by Goldberg and co-workers (15).

Finally, an experiment was designed to test the effect of an increase in the 5'-AMP trap on masking the activity of 5'-nucleotidase. This approach was attempted with the realization that the increased 5'-AMP might significantly compromise the conversion efficiency in the phosphodiesterase reaction or that the precipitation procedure might not be sufficiently complete to remove the increased amount of 5'-AMP present. Figure 6 shows the effect of increasing the concentrations of 5'-AMP in the incubate from 0.04 mM to 0.08 mM, 0.16 mM and 0.32 mM (6,000 times the maximum amount of [^{3}H] 5'-AMP which could be formed). The CPM remaining in the supernatant at 20 and 40 minutes decreased with each doubling of 5'-AMP concentration in the incubation medium. The general shape of the curve obtained with the highest concentration of 5'-AMP employed indicated (and this will be proven in the following section) a complete masking of 5'-nucleotidase activity in the rat brain phosphodiesterase preparation. In addition, the data at 5 minutes indicated that

Figure 6. The effect of different concentrations of 5'-AMP on masking the activity of 5'-nucleotidase. mM values refer to the final concentration of 5'-AMP in the incubation medium of each series. % CPM refers to the percentage of original radioactivity present. Each point is the mean of three determinations $\pm$ S.D. Minutes refer to the length of incubation at 30° C.


100
90
80
70
60
50
40
30
20
10
% ORIGINAL CPM
5.0
20.0
40.0
MINUTES
0.04mM
0.08mM
0.16mM
0.32mM

the increase in 5'-AMP did not interfere with initial velocity of the phosphodiesterase reaction.

THE PHOSPHODIESTERASE REACTION PROFILE

Introduction

In Figure 6, it may be noted that the number of CPM in the series of tubes containing 0.32 mM 5'-AMP was reduced only to about 19% of the initial amount of radioactivity present. From the general profile of the curve, it was surmized that this resulted from either a failure of the reaction to go to completion or a failure of the means instituted to monitor the progress of the reaction. Because of the important function rendered by the phosphodiesterase reaction in the proposed adenyl cyclase assay, both of the above possibilities had to be eliminated. Accordingly, this section will record the experiments used to solve this problem.

Specifically, experiments will be presented which investigate the following hypotheses as reasons for the observed reaction profile:

(1) The increased amount of added unlabeled 5'-AMP (0.32 mM) exceeded the capacity of the precipitation reaction. In this case, the reaction could be proceeding to completion but the analytical proce-

dure would be monitoring only a fraction of the actual activity.

(2) The reaction kinetics were on the first order (with respect to substrate) part of a Michaelis-Menten type curve. This would mean that the velocity of the reaction decreased as the substrate concentration decreased so that the reaction velocities during the latter incubation periods would be approximately zero.

(3) The reaction rate was decreasing with time due to an inactivation of the phosphodiesterase enzyme.

(4) The substrate of the reaction, [^{3}H] cyclic AMP, was contaminated with some material such as [^{3}H] adenosine which was not precipitable. If this were the case, the reaction could have been proceeding to completion but this would not have been detected.

Results and Discussion

Hypothesis number 1 was tested in the following manner. Varying concentrations of 5'-AMP (up to 0.32 mM) were subjected to the $Ba(OH)_2$ - $ZnSO_4$ precipitation procedure. The completeness of precipitation was checked by monitoring the absorbancy of

the solution at 260 mμ before and after the precipitation procedure. As can be seen in Figure 7, complete removal of the 5'-AMP was effected at all concentrations tested. Therefore, the reaction profile of Figure 6 could not have been due to the inefficient removal of the product of the reaction, [^{3}H] 5'-AMP.

Hypothesis number 2, i.e., that reaction velocity decreases because of declining substrate concentration, was tested in the following manner. It was reasoned that if the hypothesis were correct, then a decreased initial concentration of [^{3}H] cyclic AMP should be reduced to approximately the same level reached by an initially higher concentration of substrate. Accordingly, an experiment was designed employing 10 picomoles and 30 picomoles of [^{3}H] cyclic AMP as the initial amounts of substrate present. As may be seen in Figure 8, the observed CPM was reduced after 20 minutes incubation from 105,000 to about 24,000 (representing 23.4% of the initial number present) in the tubes containing 30 picomoles and from 33,000 to about 8,000 (representing 24.7% of the initial number present) in the tubes containing 10 picomoles. These data appear to exclude hypothesis (2) as an explanation for the reaction profile of phosphodiesterase recorded in Figure 6.

However, this experiment was very informative, indicating that regardless of initial substrate concentration, reduction in CPM was essentially complete by 10 minutes. This would lend

Figure 7. The effectiveness of the $Ba(OH)_2$ - $ZnSO_4$ precipitation procedure on the removal of 5'-AMP. The absorbance was monitored at a wave length of 260 mμ using a Beckman DU spectrophotometer. The solid line refers to the absorbance before the precipitation procedure, the broken line after the procedure.

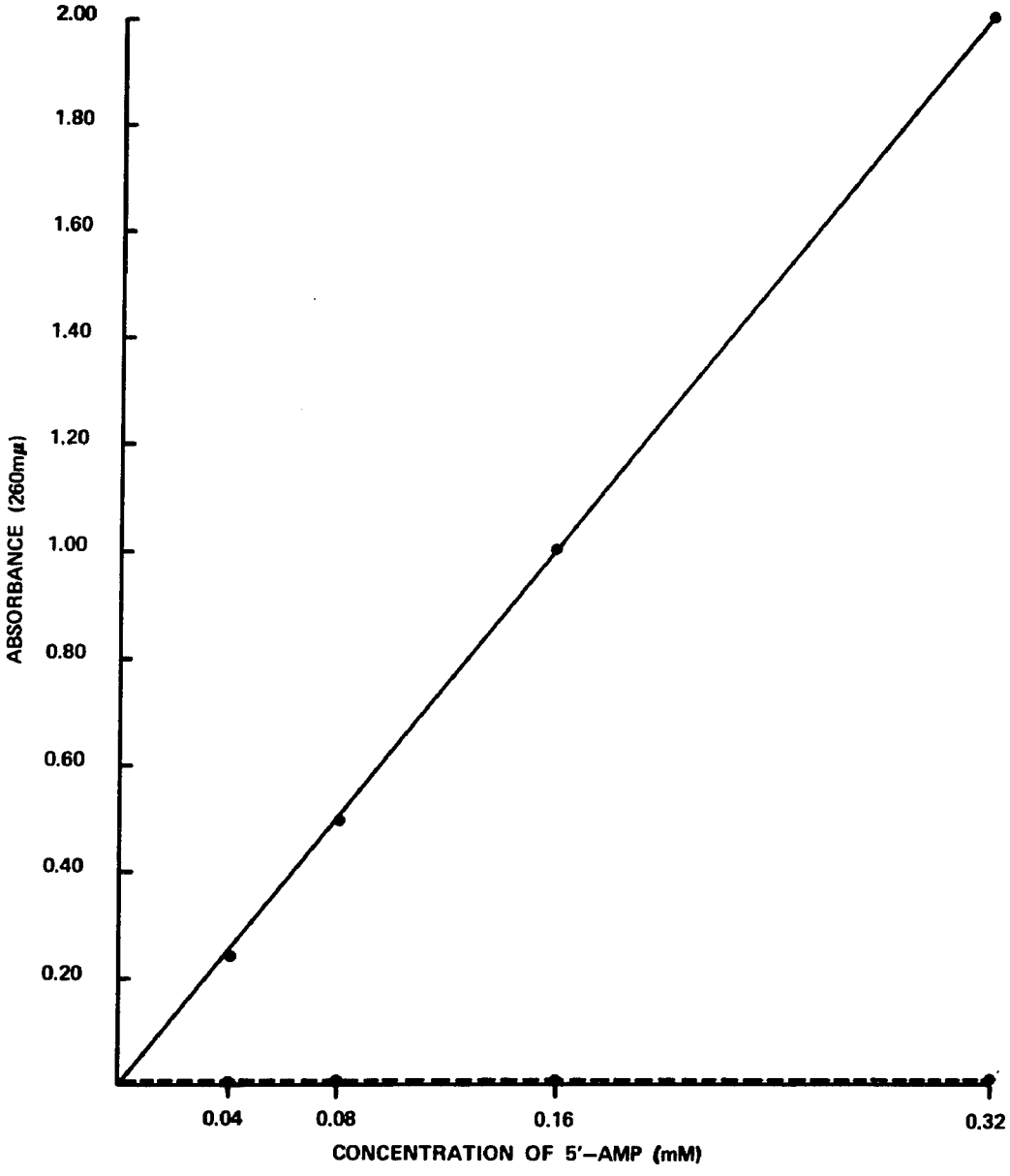
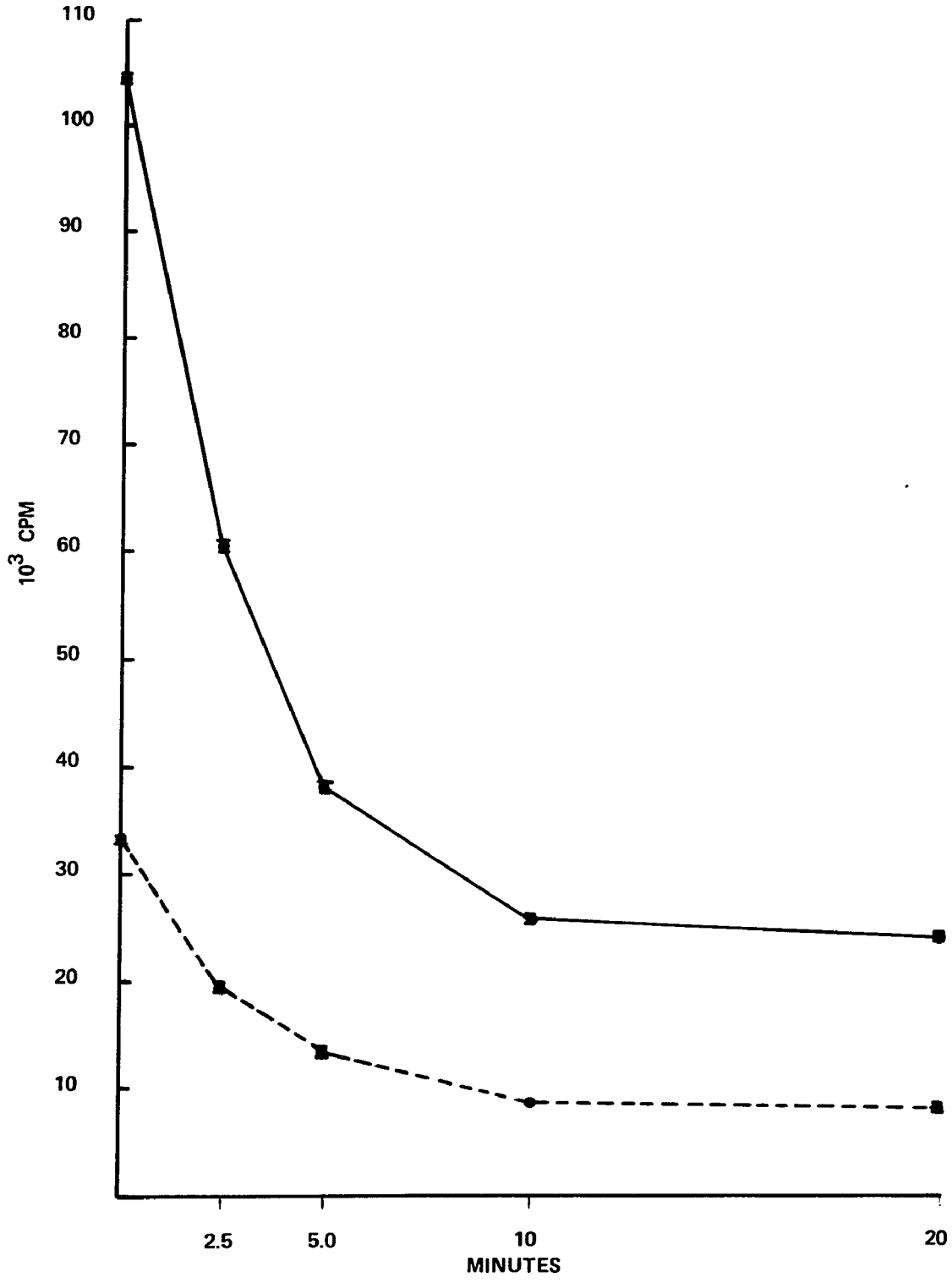

2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
ABSORBANCE (260mμ)
0.04
0.08
0.16
0.32
CONCENTRATION OF 5'–AMP (mM)

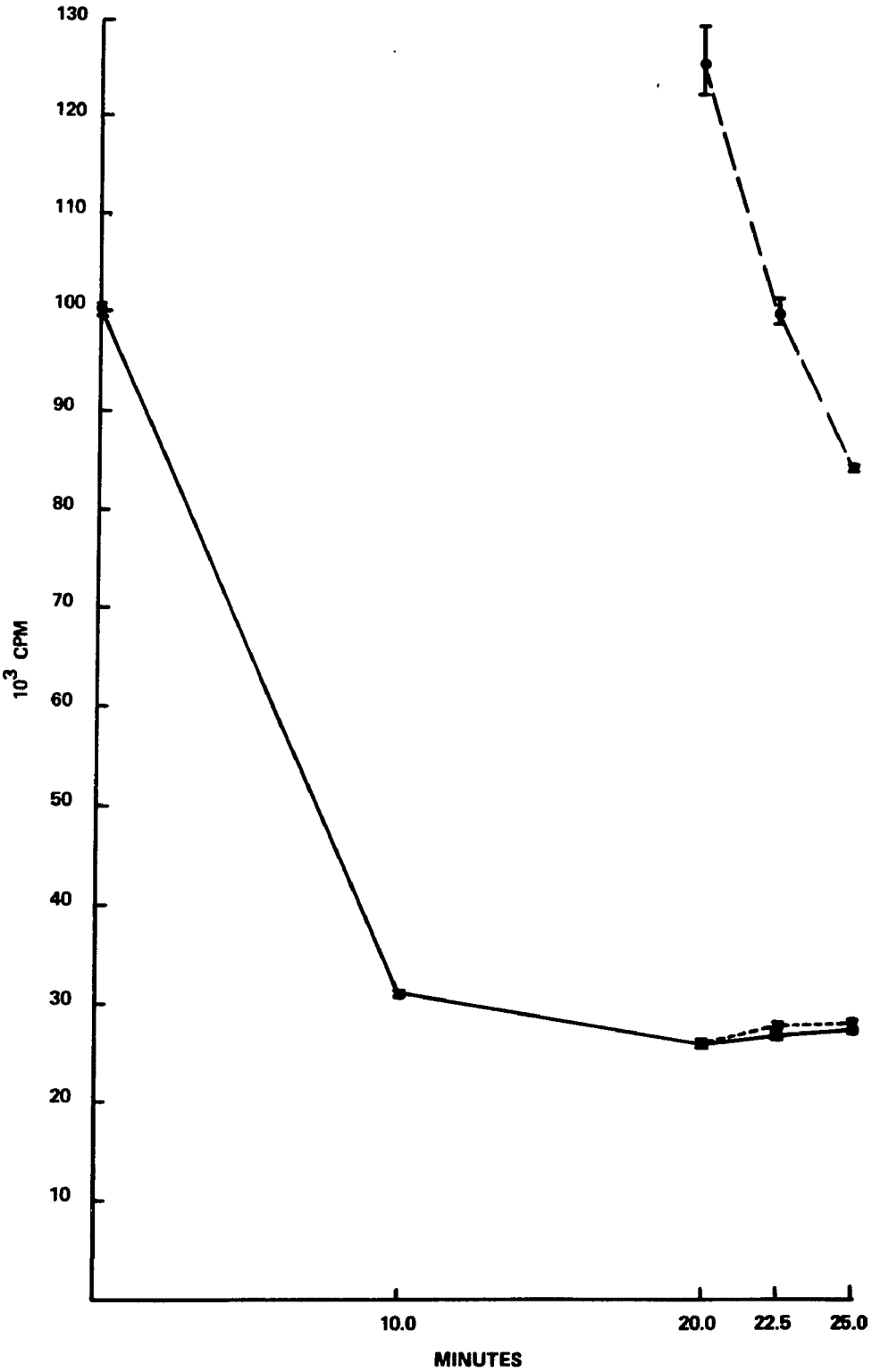
Figure 8. The reaction profile of phosphodiesterase with time, employing 10 picomoles (broken line) and 30 picomoles (solid line) as the initial amount of [^{3}H] cyclic AMP present as substrate. Each point is the mean of three determinations $\pm$ S.D. and refers to the CPM present in the supernate after the precipitation procedure. Minutes refer to the length of incubation at 30^{o} C.


110
100
90
80
70
60
50
40
30
20
10
10^3 CPM
2.5
5.0
10
20
MINUTES

credence to the possibility that the reaction profile of Figure 6 was due to a loss of activity in the phosphodiesterase preparation. It may also be observed that the final percentage of radioactivity was similar (23.4 vs 24.7) at both initial substrate concentrations, lending credence to the interpretation that the reaction profile was due to a contamination of the substrate with a substance which was not precipitable -- e.g., [^{3}H] adenosine.

Hypothesis (3), concerning the loss of activity of the enzyme, was checked in the following manner. An incubation was performed which included a fresh aliquot of phosphodiesterase added after 20 minutes incubation. If the reaction profile were due to enzyme inactivation, the addition of fresh enzyme should stimulate an immediate decrease in CPM. In some of the tubes in this experiment, additional [^{3}H] cyclic AMP was added at the 20 minute interval. If the enzyme were still active, the elevated CPM should immediately begin to decrease; if the enzyme were inactive, the elevated CPM would not begin to fall. The results are shown in Figure 9. As can be seen, the radioactivity added at 20 minutes as fresh substrate ([^{3}H] cyclic AMP) began immediately to be converted into a form removed from the supernatant by the precipitation reaction---indicating that

Figure 9. The effect of adding fresh substrate or enzyme during the latter periods of the phosphodiesterase incubation. The solid line represents a control series of tubes to which BSA and mercaptoethanol-control for the phosphodiesterase addition (dotted line)-were added at the 20 minute incubation interval. The broken line from 20 - 25 minutes indicates the effect of adding [^{3}H] cyclic AMP equivalent to the initial amount present at zero time. CPM refers to the radioactivity remaining in the supernate after the precipitation procedure. Minutes refer to the length of incubation at 30^{o} C. Each point represents the mean of three determinations and the vertical bars $\pm$ 1 S.D..


130
120
110
100
90
80
70
60
50
40
30
20
10
10^3 CPM
10.0
20.0
22.5
25.0
MINUTES

the enzyme was still active. Moreover, the addition of fresh enzyme to tubes which had been incubating for 20 minutes did not further reduce the number of CPM remaining in the supernatant. Both phases of this experiment, therefore, ruled out the hypothesis that the phosphodiesterase reaction profile was due to inactivation of the enzyme.

This experiment provided, however, an additional piece of evidence in support of the last hypothesis--the contamination of the substrate with a non-precipitable substance. The inability of the fresh enzyme, added at 20 minutes, to reduce the radioactivity indicated that there was little if any substrate left in the incubation medium or in other words that the reaction had already gone to completion.

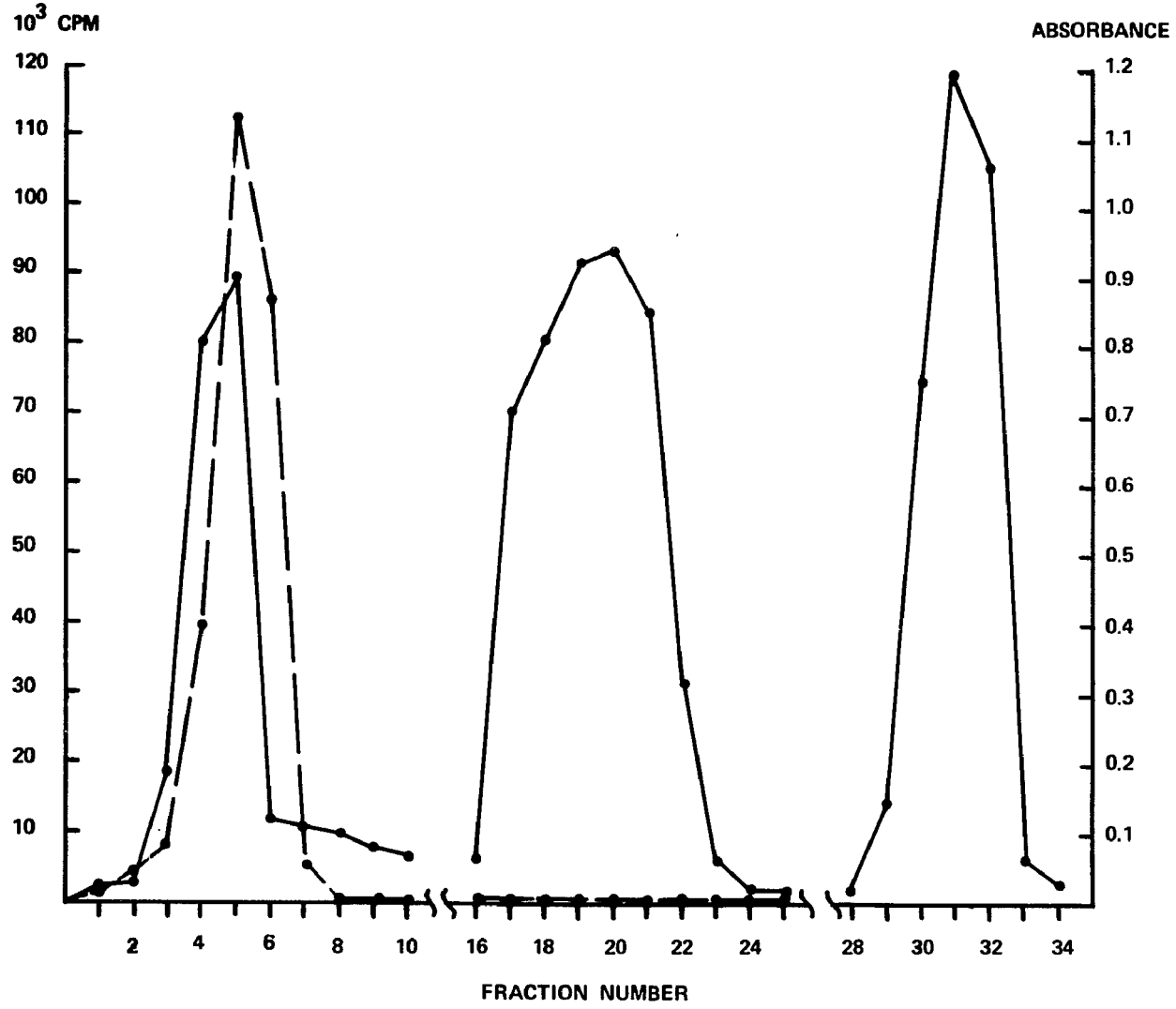
The last hypothesis, concerning the purity of the substrate, was proven in the following manner. It was considered that the most likely contaminates in the [^{3}H] cyclic AMP preparation, from a degradation point of view, would be either [^{3}H] 5'-AMP or [^{3}H] adenosine. An experiment was therefore designed to separate the above compounds chromatographically on Dowex 50-H^+ (Bio-Rad Laboratories: 50W-X4, 200-400 mesh) ion exchange columns using the method of Krishna, Weiss, and Brodie (19). An unlabeled authentic sample of each of the three compounds was first chromatographed in order to identify their respective elution

profiles (results not shown). An Isco monitor was employed to scan the effluent at 254 mμ. Water was used to elute the cyclic AMP and 5'-AMP, and 3 N HCl to elute the adenosine. These trials were followed by chromatography of the suspected [^{3}H] cyclic AMP preparation, including an addition of each of the three compounds, in unlabeled form, to serve as carrier. The eluate was collected in 1.0 ml fractions and counted for radioactivity in a liquid scintillation counter.

The results, shown in Figure 10, indicate 84.0% recovery of radioactivity in the cyclic AMP fractions and 0.6% recovery in the 5'-AMP fractions. Unfortunately, the adenosine fractions could not be counted due to very high quenching caused by the acidic eluate, HCl, which could not be corrected by neutralization.

Even though this experiment did not identify the contaminating substance, it did explain the phosphodiesterase reaction profiles contained in Figures 6 and 8. Since this experiment indicated that approximately 16% of the tritium in the [^{3}H] cyclic AMP preparation was not present as cyclic AMP (assuming 100% recovery of cyclic AMP), it seems reasonable to assume that the 23.4% and 24.7% radioactivity which was not precipitable after 20 minutes incubation in Figure 8 and the 19% which was not precipitated after 40 minutes in Figure 6 represented non-substrate tritium and that the phosphodiesterase reaction was

Figure 10. The determination of the purity of [^{3}H] cyclic AMP by chromatography on Dowex 50 - H^+ ion-exchange columns. The broken lines refer to the number of CPM present in each 1.0 ml fraction. The solid lines refer to the highest absorbance recorded on an Isco monitor (254 mμ) for each fraction. The absorbance curves represent cyclic AMP, 5'-AMP, and adenosine, respectively.


10^3 CPM
ABSORBANCE
120
110
100
90
80
70
60
50
40
30
20
10
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
2
4
6
8
10
16
18
20
22
24
28
30
32
34
FRACTION NUMBER

essentially complete. It was therefore concluded that the methods and procedures used in the phosphodiesterase assay were sound and could confidently be incorporated into the overall adenyl cyclase assay.

MEASUREMENT OF ADENYL CYCLASE ACTIVITY

Introduction

In any tissue where adenyl cyclase is found, the enzyme phosphodiesterase can be presumed to be also present. Thus, in most non-purified adenyl cyclase preparations, the activity of phosphodiesterase is probably sufficiently high to justify its inactivation if activity of adenyl cyclase is to be monitored by measuring the amount of cyclic AMP produced from ATP. Accordingly, the first part of this chapter will be devoted to experiments which test the effectiveness of several inhibitors of phosphodiesterase activity and their compatibility with the proposed adenyl cyclase assay. The second part of this chapter concerns the measurement of adenyl cyclase activity in myocardial and liver preparations in order to document the usefulness of the assay developed from the experimental results of this dissertation.

Materials and Methods

Preparation of Rat Myocardial Adenyl Cyclase

The myocardial adenyl cyclase was prepared by the method of Drummond and Duncan (12). Male Sprague-Dawley rats, weighing 200-250 g, were sacrificed by decapitation. The hearts were removed, sliced, rinsed in cold 0.9% saline, and placed in cold 10 mM Tris-HCL, (pH 7.5). The ventricular tissue was excised, blotted, and weighed. The tissue was homogenized in 10 volumes of the Tris-HCL buffer either with a Sorvall Omni-Mixer (four-15 second pulses) or with a tissue press followed by 15 strokes in a Potter-Elvehjem all glass homogenizer, all at 4^{o} C. Connective tissue was removed by straining through cheesecloth and the homogenate was centrifuged at 1,000 x g for 15 minutes at 4^{o} C. The precipitate was washed twice in the original volume of fresh buffer and then the pellet was suspended in 5 volumes of buffer (based on original tissue weight). The preparation was kept ice cold and used within 20 minutes.

Preparation of Liver Adenyl Cyclase

Liver adenyl cyclase was prepared using the modification by Ray and co-workers (30) of the procedure developed by Neville (23). Male Sprague-Dawley rats, weighing 200-250 g, were

decapitated. The livers were excised, rinsed in cold 0.9% saline, minced, weighed, and homogenized in 5-volumes of 1 mM $NaHCO_3$ using a Potter-Elvehjem all glass homogenizer (10 strokes). The homogenate was centrifuged at 1,500 x g for 10 minutes. The precipitate was washed 3 times in the original volume of 1 mM $NaHCO_3$ and then the pellet was resuspended in 1 volume (based on original tissue weight). The preparation was layered over a discontinuous sucrose gradient containing 6 ml each of 45%, 41.5% and 37% sucrose (sucrose concentrations determined with an Abbe refractometer). This was centrifuged at 25,000 RPM in an SW 25.1 head (90,000 x g) for 75 minutes using a Beckman Model L Ultracentrifuge. The membranes, which layered at the 37%/41.5% interface, were removed with a pasteur pipette and suspended in 10 ml/g original tissue weight of the 1 mM $NaHCO_3$. The preparation was kept ice cold and used within 1 hour.

Adenyl Cyclase Incubation Procedure

The adenyl cyclase incubate consisted of the following components in a total volume of 0.6 ml:

40 mM Tris-HCl (pH 7.5)

3 mM p-chloromercuriphenylsulfonic acid (Sigma Chemical Company)

5.5 mM KCl

20 mM phosphoenol pyruvate (Sigma Chemical Company)

15 mM $MgSO_4$

8 mM NaF

160 µg/ml pyruvate kinase (Calbiochem)

0.4 mM ATP (Sigma Chemical Company)

Enzyme preparation (75-150 µg of protein)

After the addition of all components except the enzyme, the incubation tubes (13 x 100 mm) were equilibrated at either 30° or 37° C for 2 minutes in an Aminco constant temperature water bath. The reaction was started by adding 0.2 ml of the adenyl cyclase preparation and the incubations were conducted with minimum shaking for 10 or 20 minutes at 30° or 37° C. The reaction was stopped by placing the tubes in a boiling water bath for 3 minutes. Control tubes contained all of the assay components except ATP. After the boiling water bath and cooling, 0.2 ml of 0.12 mM ATP in 10 mM Tris-HCl (pH 7.5) was added to the control tubes while 0.2 ml of the buffer was added to the experimental tubes. All tubes were then subjected to the boiling water bath for an additional 3 minutes. Denatured protein was removed by centrifuging at 2,000 x g for 20 minutes.

For each tube, duplicate determinations for cyclic AMP were performed by adding 0.2 ml aliquots of the supernatant to

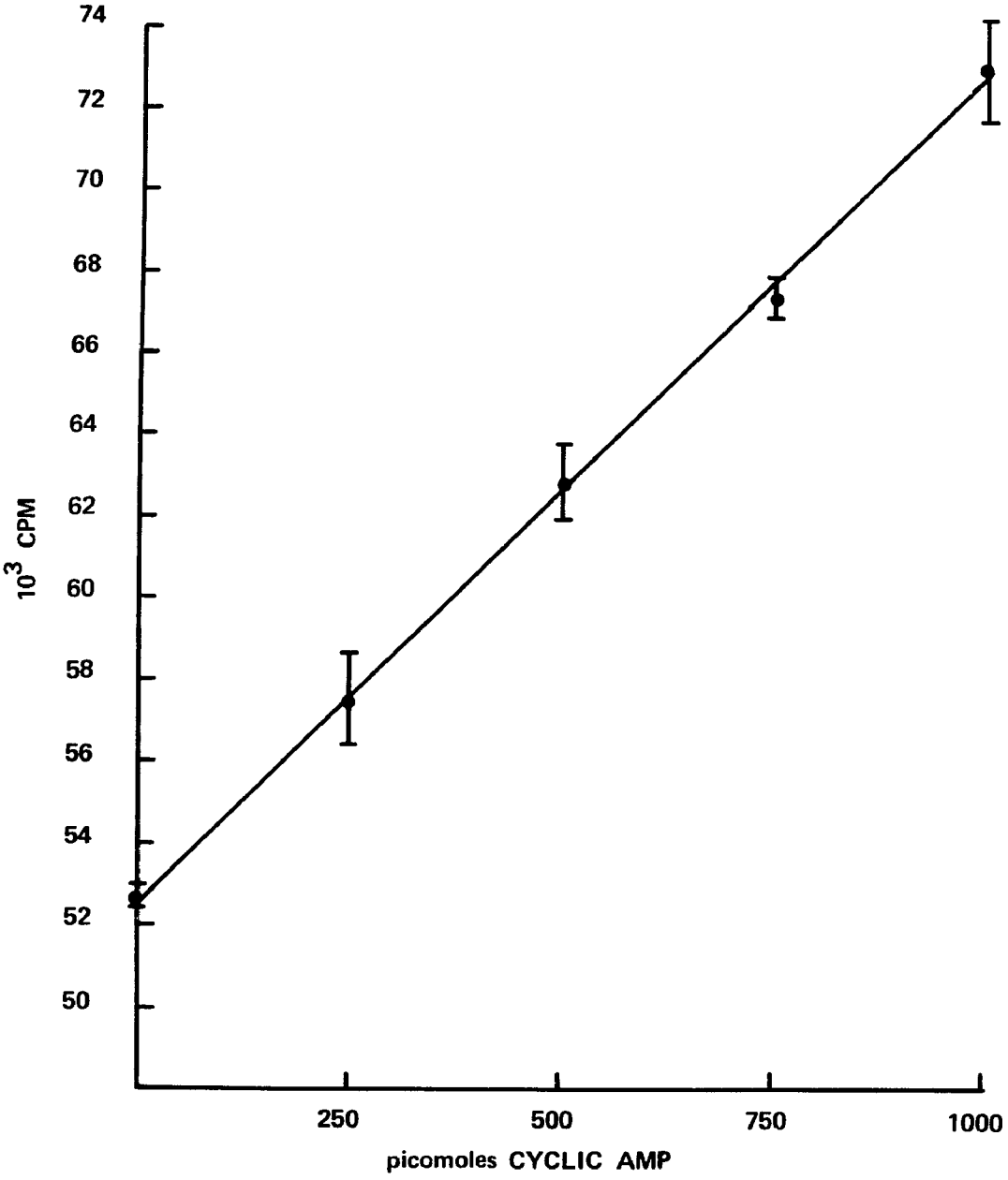
phosphodiesterase reaction tubes. As stated previously, the basis for the cyclic AMP determination rested upon non-isotopic dilution of [^{3}H] cyclic AMP thus reducing the amount of the latter converted to [^{3}H] 5'-AMP by the phosphodiesterase reaction. The quantitation of the amount of cyclic AMP transferred was accomplished by reference to a standard curve, produced by adding known amounts of unlabeled cyclic AMP to adenyl cyclase controls either before or after the incubation procedure. Standard curves were also produced by adding the unlabeled cyclic AMP to adenyl cyclase incubates in which the enzyme preparation had been inactivated by heating for 3 minutes in a boiling water bath. In a typical standard curve, the counts remaining in the supernatant of the phosphodiesterase reaction was plotted against the amount of unlabeled cyclic AMP transferred from the adenyl cyclase incubate standards.

Results

Standard Curve for Cyclic AMP

Figure 11 demonstrates a standard curve obtained using a rat brain phosphodiesterase preparation at a concentration of 75 µg/ml (Lowry protein) and 60 picomoles [^{3}H] cyclic AMP/ml. The reaction was carried out at 30^{o} C for 10 minutes. The amount of

Figure 11. A standard curve for cyclic AMP. CPM refers to the number of CPM remaining in the phosphodiesterase incubate after the precipitation procedure. Picomoles cyclic AMP refers to the amount of the authentic unlabeled preparation included in the incubation. Incubation conditions: 10 minutes at 30° C. Each point represents the mean of 3 determinations and the vertical bars $\pm$ 1 S.D.


74
72
70
68
66
64
62
60
58
56
54
52
50
10^3 CPM
250
500
750
1000
picomoles CYCLIC AMP

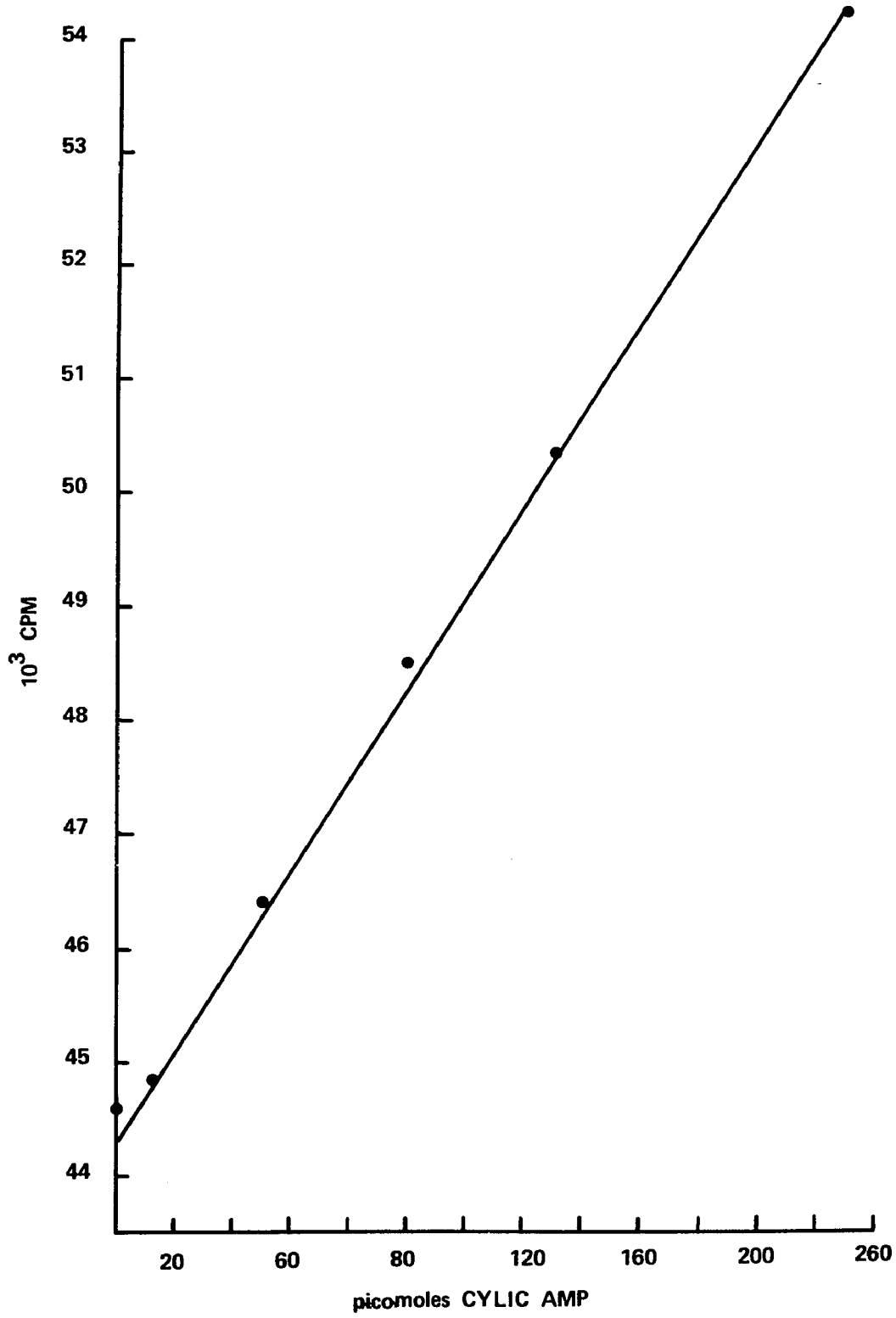
authentic unlabeled cyclic AMP added to the phosphodiesterase reaction varied between 0 and 1,000 picomoles. The result was a linear displacement of the [^{3}H] cyclic AMP from the reaction by the unlabeled cyclic AMP.

Figure 12 represents another standard curve which demonstrates the extreme sensitivity of this analytical procedure. As can be noted, amounts of cyclic AMP in the range of 20 to 250 picomoles are readily quantitated by this method.

Inhibition of Phosphodiesterase

Since Butcher and Sutherland (2) first noted the inhibitory effect of the methylxanthines on phosphodiesterase activity, theophylline has been routinely included in most adenyl cyclase determinations. However, incorporation of a phosphodiesterase inhibitor in the adenyl cyclase assay under consideration would require that it be either removable or selectively inhibitable in order to prevent interference with the second reaction (phosphodiesterase) in the assay sequence. It seemed reasonable that the dilution of theophylline achieved when an aliquot of the arrested adenyl cyclase reaction was added to the phosphodiesterase incubate might be adequate to reduce its inhibitory action to an acceptable level. To test this, 0.2 ml aliquots of 8 mM theophylline, the concentration commonly employed in adenyl

Figure 12. A standard curve for cyclic AMP. CPM refers to the average number of CPM for three determinations, remaining in the phosphodiesterase incubate after the precipitation procedure. Picomoles cyclic AMP refers to the amount of authentic unlabeled preparation included in the incubation. Incubation conditions: 10 minutes at 30° C. The vertical bars represent $\pm$ 1 S.D.

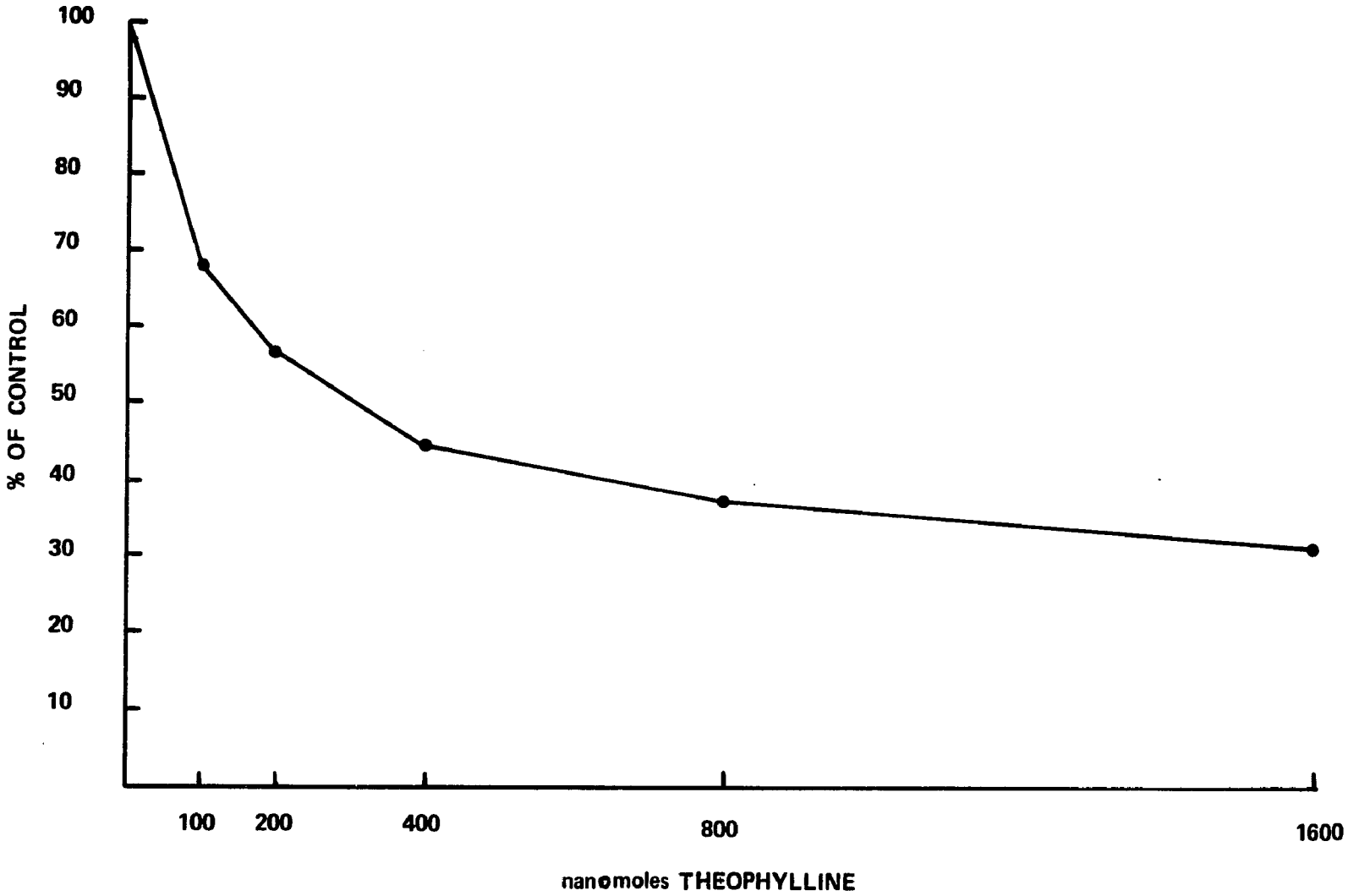

54
53
52
51
50
49
48
47
46
45
44
10^3 CPM
20
60
80
120
160
200
260
picomoles CYLIC AMP

cyclase incubations, were added to phosphodiesterase reaction tubes and incubated as described in the Materials and Methods section. This resulted in an approximately 60% inhibition of phosphodiesterase activity (data not shown).

The following experiment was designed to determine the extent to which the theophylline contained in the adenyl cyclase reaction medium would have to be diluted in order to reduce its inhibitory effect during the phosphodiesterase reaction to an acceptable level. 8 mM theophylline was diluted 1:2, 1:4, 1:8 and 1:16 with distilled water and 0.2 ml aliquots of each solution were added to tubes for the phosphodiesterase reaction. As shown in Figure 13, this approach was not feasible since only 100 nanomoles of theophylline (representing a 1:16 dilution of the adenyl cyclase incubate) reduced the phosphodiesterase activity to 70% of control values. Further dilution to eliminate theophylline inhibition would probably also lower the cyclic AMP concentration to unmeasurable levels.

Krishna, Weiss, and Brodie (19) reported that theophylline could be selectively precipitated by the $Ba(OH)_2$ - $ZnSO_4$ procedure. Since cyclic AMP is not precipitated, this was tested as a means of removing theophylline from the adenyl cyclase incubate. After determining the absorption maximum of this compound to be 272 mμ

Figure 13. The effect of varying amounts of theophylline on the phosphodiesterase reaction. Nanomoles theophylline refers to the amount present in each 0.6 ml incubation volume. Incubation conditions: 10 minutes at 30^{o} C.


100
90
80
70
60
50
40
30
20
10
% OF CONTROL
100
200
400
800
1600
nanomoles THEOPHYLLINE

on a Cary Model 15 Spectrophotometer, varying concentrations of theophylline were subjected to the precipitation procedure. Although a large portion of the theophylline was removed (Figure 14), the amounts remaining appeared sufficiently high to interfere with the phosphodiesterase reaction.

Cheung and Jenkins (11) reported in 1969 that phosphodiesterase was inhibited by mercurial reagents such as p-hydroxymercuribenzoate and that the inhibition could be reversed by β-mercaptoethanol. Accordingly, an experiment was conducted to evaluate the influence of three reagents -- iodoacetamide, p-chloromercuriphenylsulfonic acid, and phenylmercuric acetate (all from Sigma Chemical Company) -- on the activity of rat brain phosphodiesterase. The results, Figure 15, indicated that both p-chloromercuriphenylsulfonic acid (1 mM) and phenylmercuric acetate (1 mM) effectively but not completely inhibited phosphodiesterase. However, at the concentration tested, 1 mM, iodoacetamide had no measurable inhibitory effect on the phosphodiesterase reaction. Mercaptoethanol (5 mM) did not inhibit the reaction.

An experiment was then conducted to determine whether or not pretreatment of the enzyme preparation with 5 and 20 mM mercaptoethanol would abolish inhibitory action of the two

Figure 14. The effect of $Ba(OH)_2$ - $ZnSO_4$ precipitation on the removal of theophylline. The solid line refers to the absorbance at 272 mμ before the precipitation procedure, the broken line after the procedure. Absorbance values determined on a Beckman DU Spectrophotometer.

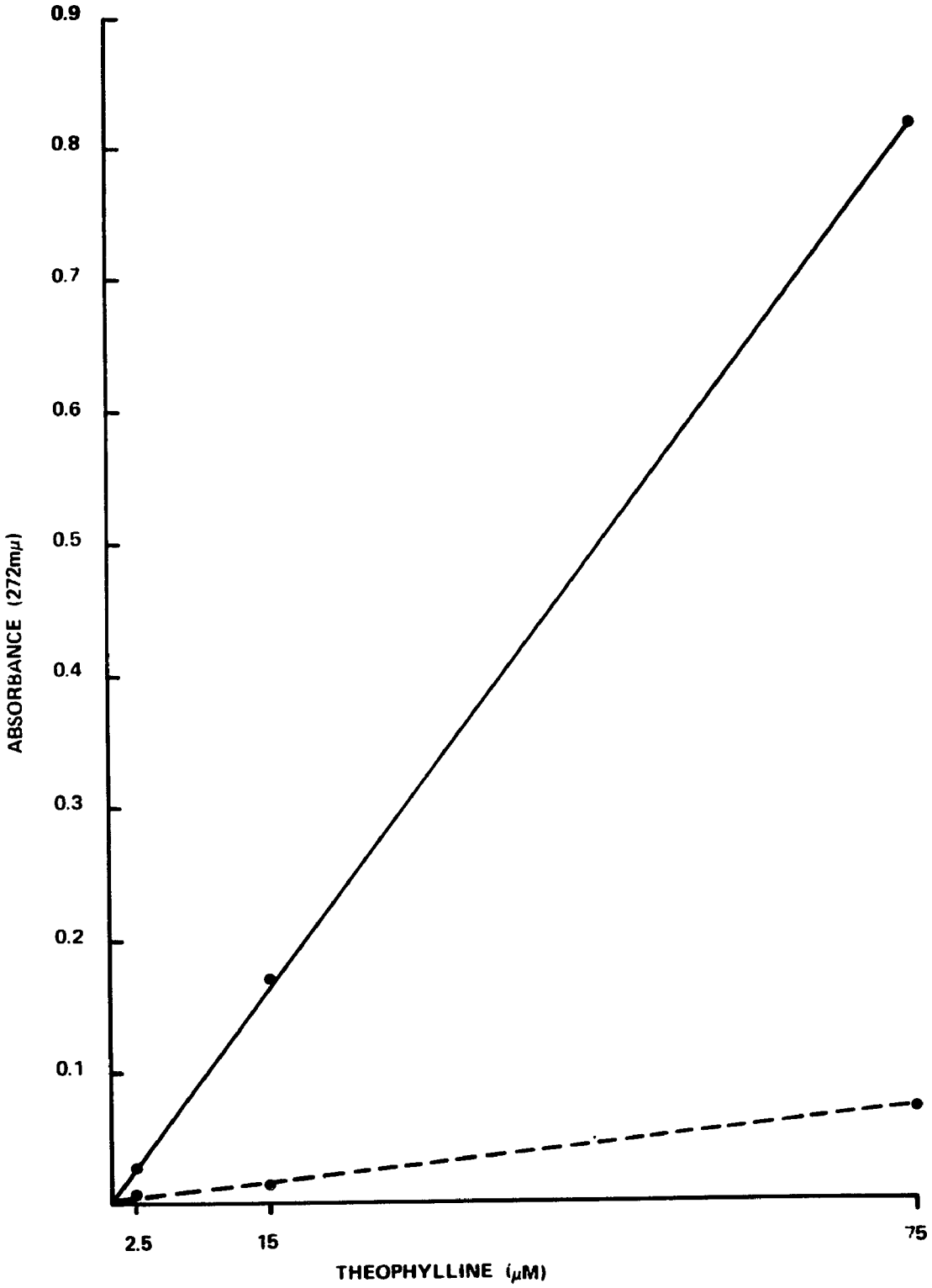

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE (272mμ)
2.5
15
75
THEOPHYLLINE (μM)

Figure 15. The effect of iodoacetamide, p-chloromercuriphenylsulfonic acid, and phenylmercuricacetate as inhibitors of phosphodiesterase. CPM refers to the average CPM $\pm$ S.D. of three determinations. Incubation conditions: 10 minutes at 30° C. All compounds added were in 10 mM Tris-HCl (pH 8.0).

A: "CPM check"

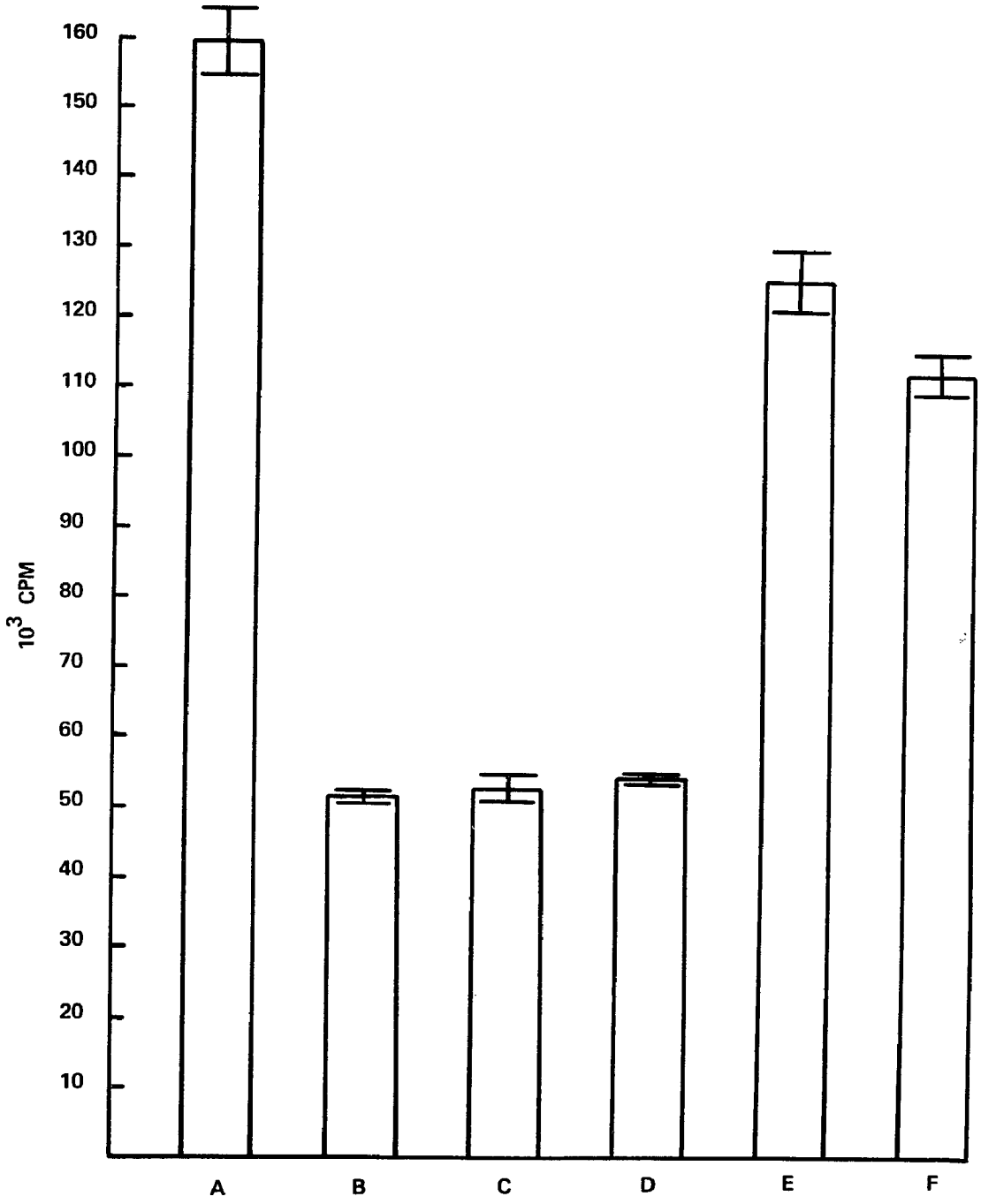
B: 5 mM - mercaptoethanol

C: Control (10 mM Tris-HCl (pH 8.0) added)

D: 1 mM iodocetamide

E: 1 mM p-chloromercuriphenylsulfonic acid

F: 1 mM phenylmercuricacetate


160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
10^3 CPM
A
B
C
D
E
F

effective compounds. As shown in Figure 16, the inhibitory effect of p-chloromercuriphenylsulfonic acid was effectively removed by pretreating the enzyme preparation with 20 mM mercaptoethanol. In contrast, the inhibitory effect of phenylmercuric acetate was only partially abolished by 20 mM mercaptoethanol. Therefore, p-chloromercuriphenylsulfonic acid was judged to be an inhibitor of phosphodiesterase apparently suitable for incorporation into the proposed adenyl cyclase assay.

In order to determine more precisely the concentration of p-chloromercuriphenylsulfonic acid required to inhibit the phosphodiesterase in a typical tissue adenyl cyclase preparation, the phosphodiesterase reaction was run with the myocardial adenyl cyclase preparation. The incubation was conducted for 10 minutes at 30^{o} C. The data (not shown) indicated that even in the absence of the inhibitor, phosphodiesterase activity in the myocardial preparation was not extremely high, as 88.2% of the initial [^{3}H] cyclic AMP was present at the end of the 10 minute incubation period. The series of tubes which contained 1.5 mM and 3.0 mM inhibitor retained 95.4% and 98.1%, respectively, of the initial [^{3}H] cyclic AMP. In order to assure accuracy of the assay, a 3.0 mM concentration of the mercurial was included in the adenyl cyclase incubations.

Figure 16. The influence of a pretreatment of the phosphodiesterase preparation with 5 mM (tubes B, C. and D) and 20 mM (tubes E, F, and G) mercaptoethanol on the inhibition of this enzyme by p-chloromercuriphenylsulfonic acid and phenylmercuricacetate. CPM refers to the average CPM $\pm$ S.D. of three determinations. Incubation conditions: 10 minutes at 30^{o} C.

A: "CPM check"

B: Control

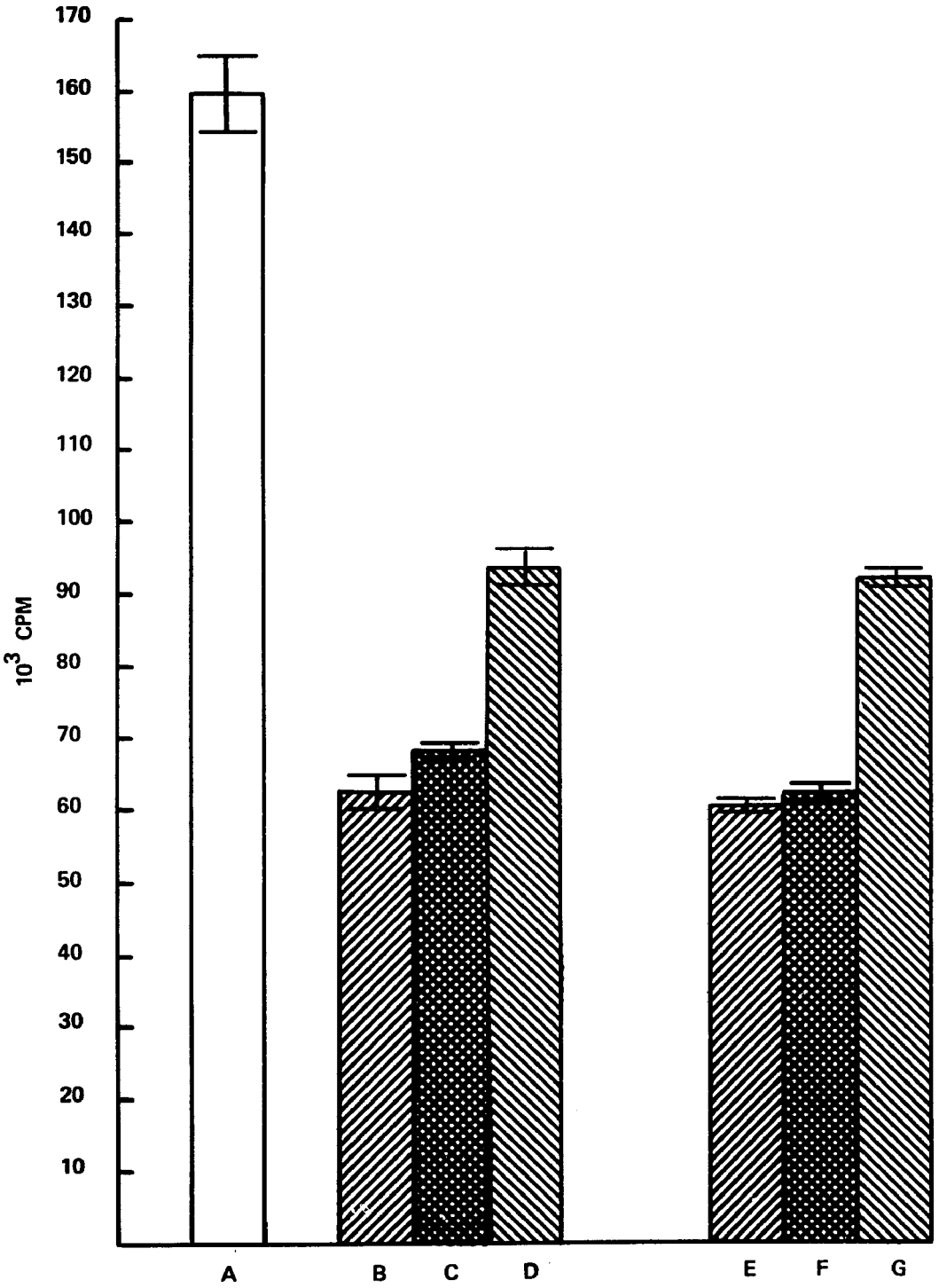
C: 1 mM p-chlormercuriphenylsulfonic acid

D: 1 mM phenylmercuricacetate

E: Control

F: 1 m M p-chloromercuriphenylsulfonic acid

G: 1 mM phenylmercuricacetate


170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
10^3 CPM
A
B
C
D
E
F
G

Determination of Myocardial Adenyl Cyclase Activity

The performance of the assay was checked in the following manner. Five myocardial adenyl cyclase preparations, each from a different rat, were incubated at 37° for 10 and 20 minutes as described in the materials and methods section. The activity, expressed in terms of picomoles cyclic AMP formed per minute per g wet weight is given in Table 7 along with the activity reported by Streeto and Reddy (33) and Drummond and Duncan (12) for comparison. Table 8 records the activity expressed in terms of tissue protein in the enzyme preparation. Activity for this tissue reported by McNeill, Muschak, and Brody (22) and Drummond and Duncan (12) is included for comparison.

The assay was also checked for linearity with respect to length of incubation. Figure 17 indicates the increase in production of cyclic AMP at 10 and 20 minute incubation periods for 5 myocardial preparations.

Table 9 indicates the production of cyclic AMP in myocardial tissue when the amount of tissue included in the incubation was varied. As may be noted, there was a direct correlation between the amount of tissue employed and the amount of cyclic AMP produced.

Table 7

Adenyl cyclase activity of rat myocardial tissue. The incubation was at 37° C and in the presence of 8 mM NaF. The activities were calculated from 10 minute incubations in preparations 1 through 5 and from 20 minute incubations in preparations 1a through 5a. Each value is the mean of two determinations. The adenyl cyclase activities reported for myocardial tissue by two other laboratories are shown for comparison.

Preparation	Activity (picomoles cyclic AMP formed per minute per g wet tissue)
1	909
2	761
3	866
4	763
5	1043
1a	957
2a	986
3a	843
4a	1193
5a	1260
Mean ± S.D.:	958 ± 168
Streeto and Reddy (33):	1060 - 1590 (30°, 10 mM NaF)
Drummond and Duncan (12):	4190 (37°, 8 mM NaF)

Table 8

Adenyl cyclase activity of rat myocardial tissue. The activity given in Table 7 is expressed on a mg protein basis. The concentration of NaF was 8 mM. Values given are the mean of two determinations. The activity of this tissue recorded by two other laboratories is shown for comparison.

Preparation	Activity (picomoles cyclic AMP formed per minute per mg protein)
1	22.7
2	18.5
3	19.3
4	16.4
5	24.2
1a	23.9
2a	23.9
3a	18.8
4a	25.6
5a	29.3
Mean $\pm$ S.D.:	22.3 $\pm$ 3.9
McNeill, Muschek, and Brody (22):	14.5 (30°, no NaF)
Drummond and Duncan (12):	86.4 (37°, 8 mM NaF)

Figure 17. Formation of cyclic AMP by rat myocardial tissue. Each bar represents the mean value (picomoles/mg protein) of cyclic AMP formed by five different adenyl cyclase preparations. Vertical bars represent 1 S.D. Minutes refer to the length of incubation at 37° C.

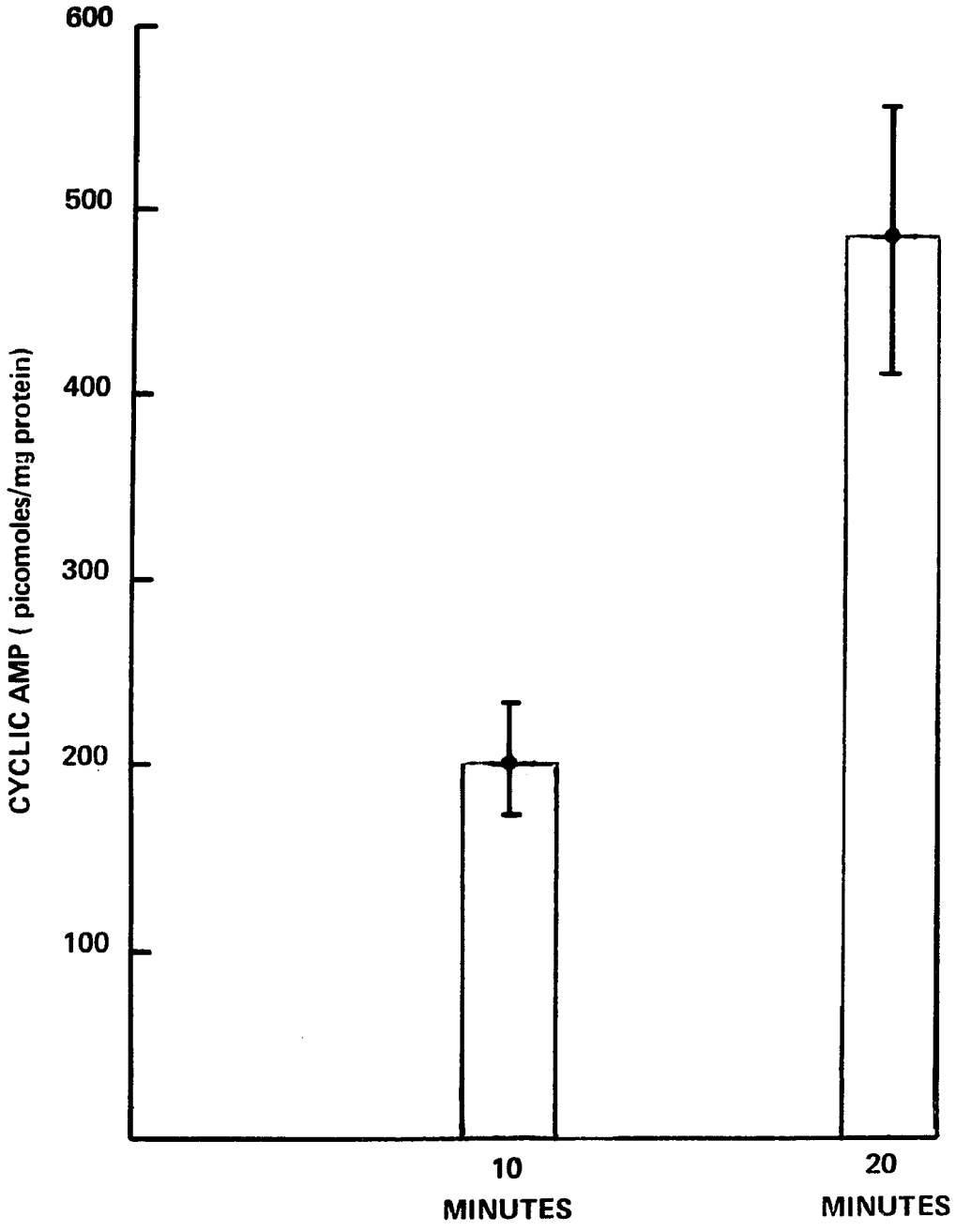

600
500
400
300
200
100
CYCLIC AMP (picomoles/mg protein)
10
MINUTES
20
MINUTES

Table 9

Myocardial adenyl cyclase activity using varying amounts of tissue. Each value is the mean of two determinations. Incubation: 10 minutes at 37° C.

Amount of tissue (mg) per incubate	Picomoles cyclic AMP formed
0.81	201
1.32	251
1.48	285

Determination of Liver Adenyl Cyclase Activity

Hepatic adenyl cyclase activity was determined on rat liver membranes prepared as described by Ray (30). This preparation had been shown by Pohl, Birnbaumer, and Rodbell (26) to be essentially free of phosphodiesterase activity. For this reason, the phosphodiesterase inhibitor, p-chloromercuriphenylsulfonic acid, was not included in the incubation medium. Also, 10 mM NaF and 3.2 mM ATP was employed to correspond with that used by Rodbell's group. The activity measured in three separate tissue preparations is given in Table 10 which includes for comparative purposes the data of Pohl, Birnbaumer, and Rodbell (26). The mean value of 1608 picomoles per mg protein per minute is in excellent agreement with their value of 1590.

Discussion

As may be noted in Table 7, the activity of adenyl cyclase in myocardial tissue determined by the proposed assay, was near the lower end of the range reported by Streeto and Reddy (33) but was only about 25% the value reported by Drummond and Duncan (12). However, Table 8 reveals that a higher activity was measured using the proposed assay, than that reported by McNeill, Muschak, and Brody (22). Some of these differences may be explained by the differences in incubation temperature or the

variations in the components of the incubation medium. However, since Pohl, Birnbaumer, and Rodbell (26) have just reported that p-chloromercuribenzoate inhibited adenyl cyclase in liver preparations, the inclusion of p-cholormercuriphenylsulfonic acid might account for the lower activity measured by the proposed method in myocardial tissue. Obviously, to be of general value in measuring adenyl cyclase activity in non-purified tissue preparations, alternative ways of blocking tissue phosphodiesterase must be found which can be shown not to affect adenyl cyclase activity.

However, as shown in Table 10, values for adenyl cyclase activity in partially purified rat liver preparations determined with the proposed assay (but without the phosphodiesterase inhibitor, p-chloromercuriphenylsulfonic acid) compared extremely well with that reported for this preparation by Pohl, Birnbaumer, and Rodbell. They also did not employ an inhibitor because of the extremely low phosphodiesterase activity in the purified preparation.

Therefore, it is suggested that this assay be limited to partially purified preparations where it can serve as an accurate, quick, and simple method to investigate many important characteristics of this enzyme. For example, <u>in vitro</u> effects of hormones on adenyl cyclase activity, by far the most extensive area of investigation in the adenyl cyclase-cyclic AMP field, could readily

Table 10

Activity of partially purified preparations of liver adenyl cyclase. The incubation condition was 30° C for 10 minutes. The concentration of NaF was 10 mM. Values given for each preparation are the mean of two determinations. The activity of this tissue recorded by Pohl Birnbaumer, and Rodbell is given for comparison:

Preparation	Activity (picomoles/mg protein/10 minutes)
1	1747
2	1425
3	1654
Mean ± S.D.:	1608 ± 166
Pohl, Birnbaumer, and Rodbell (26):	1590 (30°, 10 mM NaF)

be determined using the proposed assay. In addition, this assay would be of great value in studying kinetic parameters (e.g., K_m values) of adenyl cyclase. This would be difficult (and expensive) to do using the currently available assays which require labeled ATP as substrate.

In support of the more general usefulness of this assay in partially purified preparations of various tissues, it should be noted that the phosphodiesterase in the non-purified preparation of myocardial tissue degraded only 12% of the cyclic AMP present in 10 minutes. A partially purified preparation of this tissue would, in all probability, contain only negligible amounts of phosphodiesterase-allowing the proposed assay to be used without the inhibitor.

The only stipulation imposed on a general use of the proposed assay is that any partially purifed tissue preparation would have to be checked in order to confirm a non-interfering level of phosphodiesterase activity.

K_m VALUES OF RAT BRAIN PHOSPHODIESTERASE

Introduction

Since the development of Sutherland's second messenger concept and the resulting utopian role for cyclic AMP, many laboratories have investigated the mechanisms which control the level of this substance in a number of biological systems. A general overview of the literature reveals that major emphasis has been directed toward the formation of cyclic AMP by adenyl cyclase with much less attention devoted to the degradation of cyclic AMP by phosphodiesterase, a process of equal importance in determining cellular levels of this proposed messenger substance.

One essential bit of information relating to the catalytic nature of the enzyme, phosphodiesterase, which needs clarification concerns its K_m value. Brain has the highest phosphodiesterase activity of any tissue thus far studied (2, 13), and perhaps as a result of this, more estimates of K_m values have been reported

for this tissue than for all other tissues combined. However, as may be seen in Table II, the range of values reported, even for brain tissue from the same species, is unusually wide, suggesting the need for additional study.

Since rat brain phosphodiesterase has an essential role in the adenyl cyclase assay under consideration, it is important to have some idea of the relationship between the cyclic AMP concentrations employed in the standard curve and the K_m value. In addition, the sensitivity of the phosphodiesterase assay which has been developed in this study affords an opportunity to determine this kinetic parameter at lower substrate concentrations than employed by previous investigators. Accordingly, this chapter deals with determinations of the K_m value (or values) for rat brain phosphodiesterase.

Methods and Materials

The phosphodiesterase assay utilized to determine the K_m values of rat brain phosphodiesterase was that described in the Materials and Methods section which was initially developed as part of a new assay for adenyl cyclase. Nonetheless, as will be shown, it functions readily as an independent and very sensitive assay for phosphodiesterase. In all of the experiments to be described in this chapter, the rat brain phosphodiesterase

Table 11

A summary of K_m determinations of phosphodiesterase from brain of various species.

Source	K_m	Reference
fish brain	$8\text{-}10 \times 10^{-5}$ M	Yamamato and Massey (43)
mouse brain	2.6×10^{-4} M	Breckenridge and Johnson (7)
rat brain	$1\text{-}3 \times 10^{-4}$ M	Cheung (10)
rat brain	1×10^{-4} M and 5×10^{-6} M	Thompson and Appleman (40)
rat brain	1.3×10^{-4} M and 1×10^{-6} M	Brooker, Thomas, and Appleman (9)

enzyme preparation was prepared as described previously. Preliminary experiments (results not shown) suggested that incubation periods of either five or ten minutes at an enzyme concentration equivalent to 100 - 200 µg/ml of the purified enzyme protein would be satisfactory for the K_m determinations. In some of the experiments, substrate concentrations of cyclic AMP were varied between 1.6×10^{-8} M and 1.0×10^{-7} M by use of the [^{3}H] cyclic AMP preparation described earlier. Because of the high specific activity of this preparation, 10^{-7} M was the highest concentration employed in this particular series of determinations. This limitation was inserted only as a safety feature to keep the amount of radioactivity in each incubation tube within reasonable limits.

For other experiments, all experimental tubes contained the same amount of [^{3}H] cyclic AMP. The substrate concentration was varied by adding different amounts of unlabeled cyclic AMP. This procedure permitted K_m determinations at relatively high substrate concentrations while keeping the amount of radioactivity employed within a safe and workable range. True velocity values in this series were obtained by calculating the specific activity at each substrate concentration.

In all experiments, K_m values were calculated from the equations for the regression lines of Lineweaver-Burk plots of each

set of data. Using the slopes and the Y-intercepts of these regression equations, the K_m values were calculated from the equation, $K_m = V_{max} \cdot$ Slope.

Results

The results of these experiments suggests a relationship between the K_m value obtained and the range of substrate concentrations utilized to generate the data for its determination. The data shown in Figures 18, 20, 22, and 24 were all obtained from incubations in which the substrate concentration ranged between 1.6×10^{-8} M and 1.0×10^{-7} M. As shown in Figures 19, 21, 23, and 25, the K_m values obtained from the corresponding Lineweaver-Burk plots for these experiments ranged between 2.4×10^{-7} and 5.8×10^{-7} M with a mean value of 4.4×10^{-7} M. When the substrate concentration was elevated to the range of 4.6×10^{-7} M to 3.4×10^{-7} M (Figure 26) the K_m value derived was 7.4×10^{-6} M (Figure 27). At the highest range of substrate concentrations utilized in this series of experiemnts, 2.1×10^{-7} M to 5.9×10^{-6} M, the Michaelis-Menten plot shown in Figure 28 was obtained. The examination of the Lineweaver-Burk plot of those data (Figure 29), revealed an apparent anomaly; there appeared to be two linear portions of the curve. A comparison of the slopes of the two regression lines by a t-test rejected the null hypothesis that the two lines were identical at the $p < 0.001$ level

Figure 18. Michaelis-Menten plot of phosphodiesterase activity. Range of substrate concentrations: 1.6×10^{-8} M - 0.9×10^{-7} M. Incubation conditions: 5 minutes at 30° C.

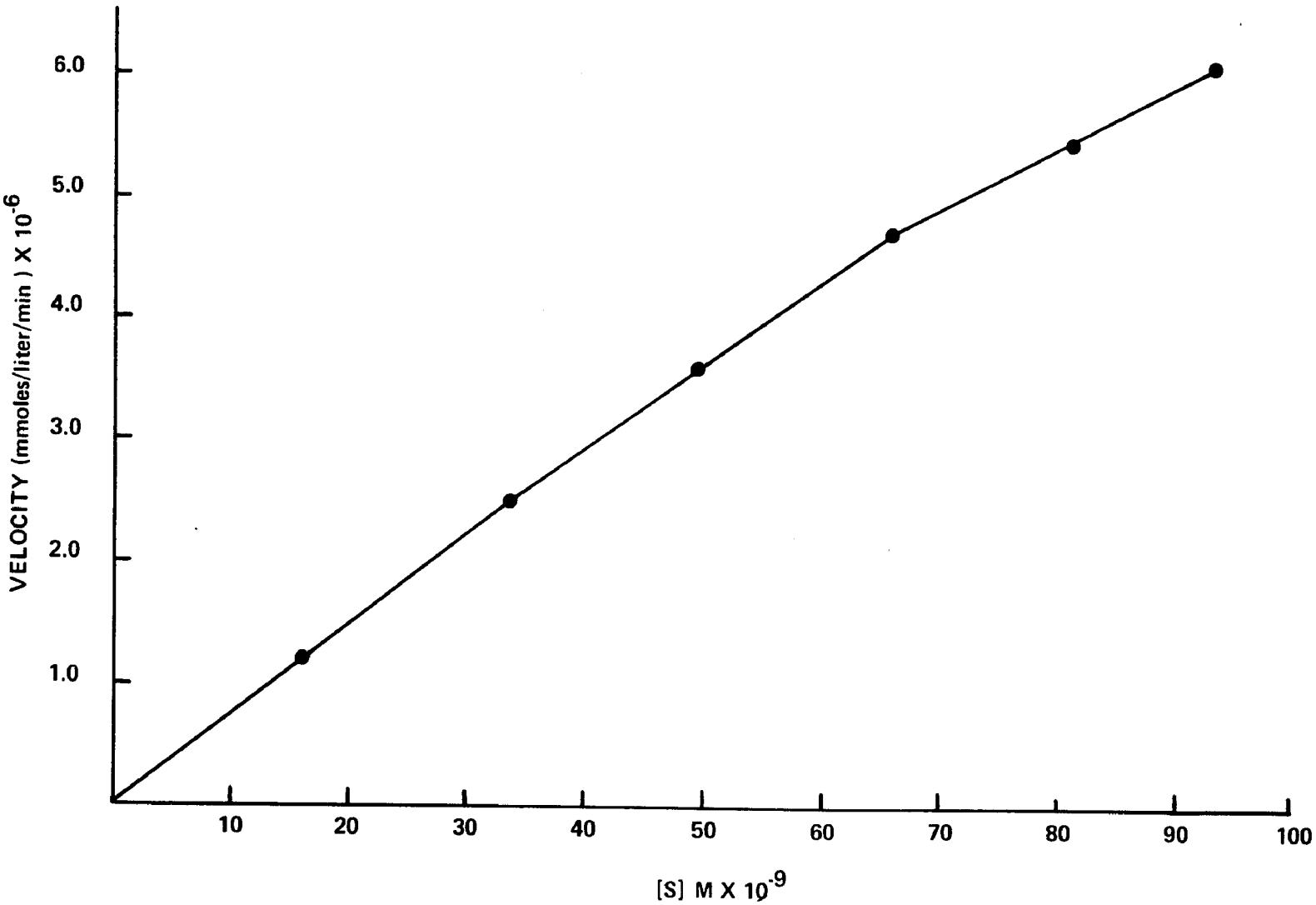

VELOCITY (mmoles/liter/min) X 10^{-6}
6.0
5.0
4.0
3.0
2.0
1.0
10
20
30
40
50
60
70
80
90
100
[S] M X 10^{-9}

Figure 19. Lineweaver-Burk plot of phosphodiesterase activity. Data from Figure 18. Range of substrate concentrations: 1.6×10^{-8} M - 0.9×10^{-7} M. K_m: 4.9×10^{-7} M.

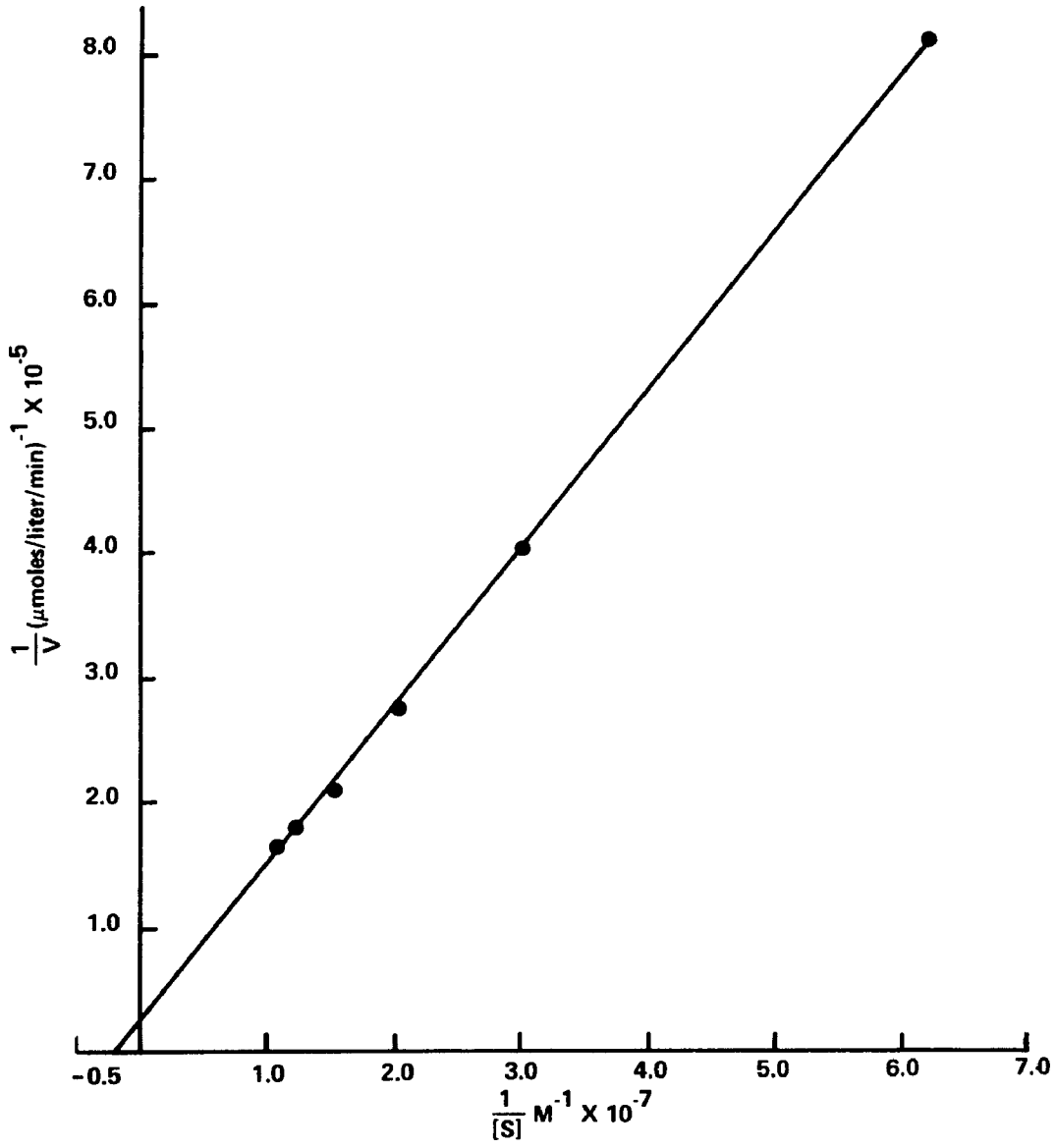

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
$\frac{1}{V}$ (μmoles/liter/min)$^{-1}$ X 10^{-5}
-0.5
1.0
2.0
3.0
4.0
5.0
6.0
7.0
$\frac{1}{[S]}$ M^{-1} X 10^{-7}

Figure 20. Michaelis-Menten plot of phosphodiesterase activity. Range of substrate concentrations: 1.7×10^{-8} M - 0.9×10^{-7} M. Incubation conditions: 10 minutes at 30° C.

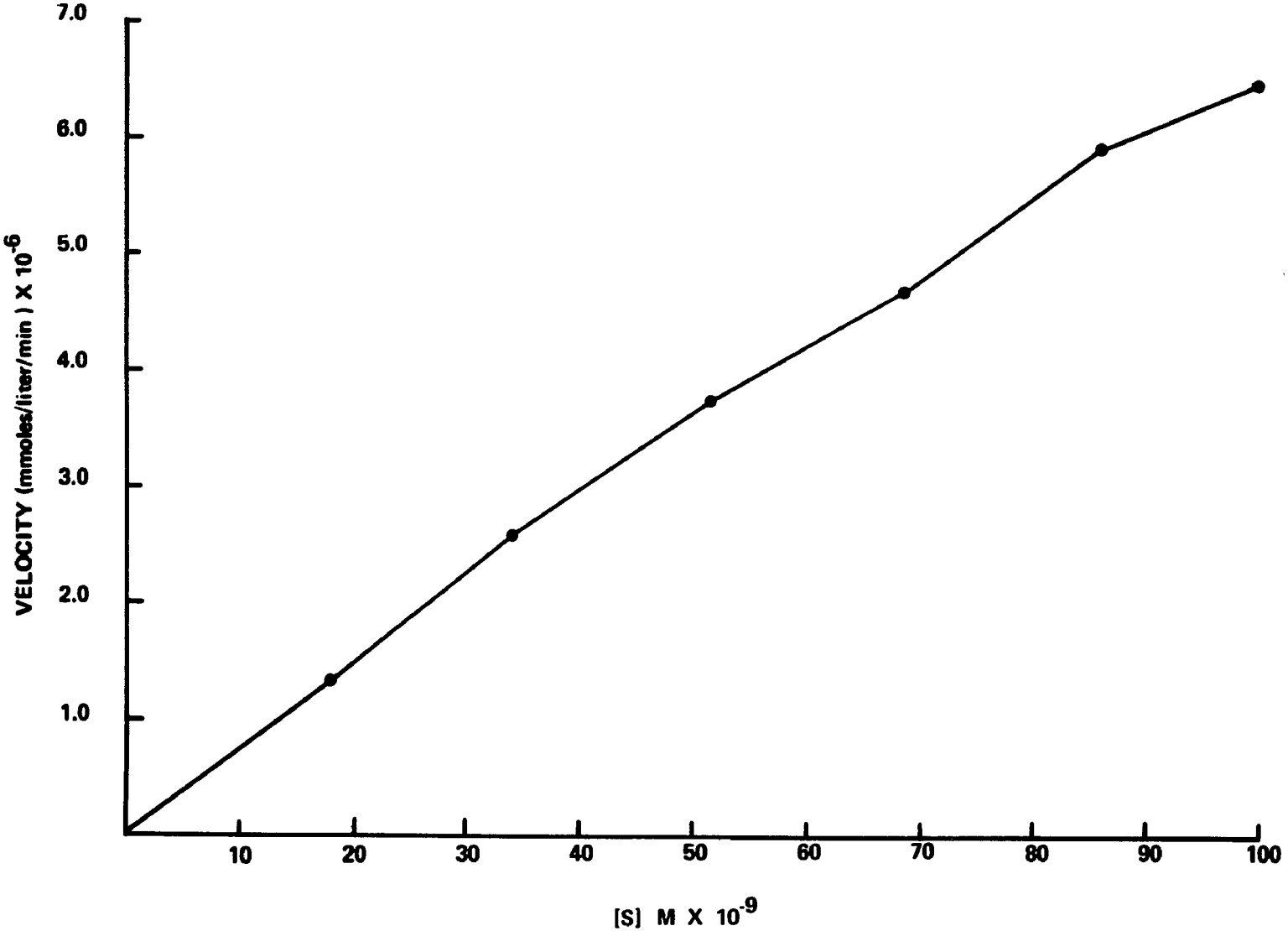

7.0
6.0
5.0
4.0
3.0
2.0
1.0
VELOCITY (mmoles/liter/min) X 10-6
10
20
30
40
50
60
70
80
90
100
[S] M X 10-9

Figure 21. Lineweaver-Burk plot of phosphodiesterase activity. Data from Figure 20. Range of substrate concentrations: 1.7×10^{-8} M - 0.95×10^{-7} M. K_m: 2.4×10^{-7} M.

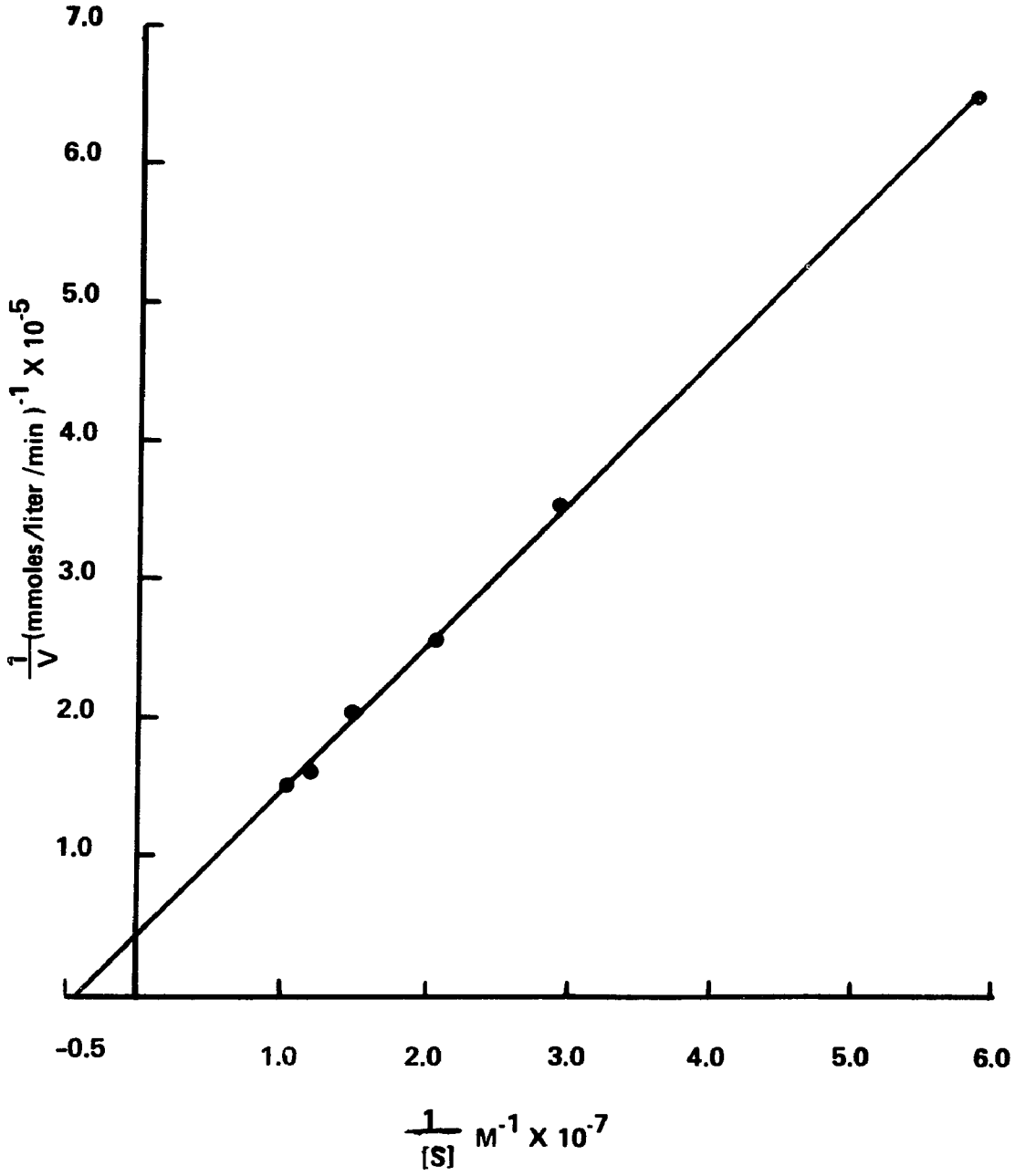

7.0
6.0
5.0
4.0
3.0
2.0
1.0
-0.5
1.0
2.0
3.0
4.0
5.0
6.0
$\frac{1}{V}$ (mmoles/liter /min)$^{-1}$ X 10^{-5}
$\frac{1}{[S]}$ M^{-1} X 10^{-7}

Figure 22. Michaelis-Menten plot of phosphodiesterase activity. Range of substrate concentrations: 1.8×10^{-8} M - 1.0×10^{-7} M. Incubation conditions: 5 minutes at 30° C.

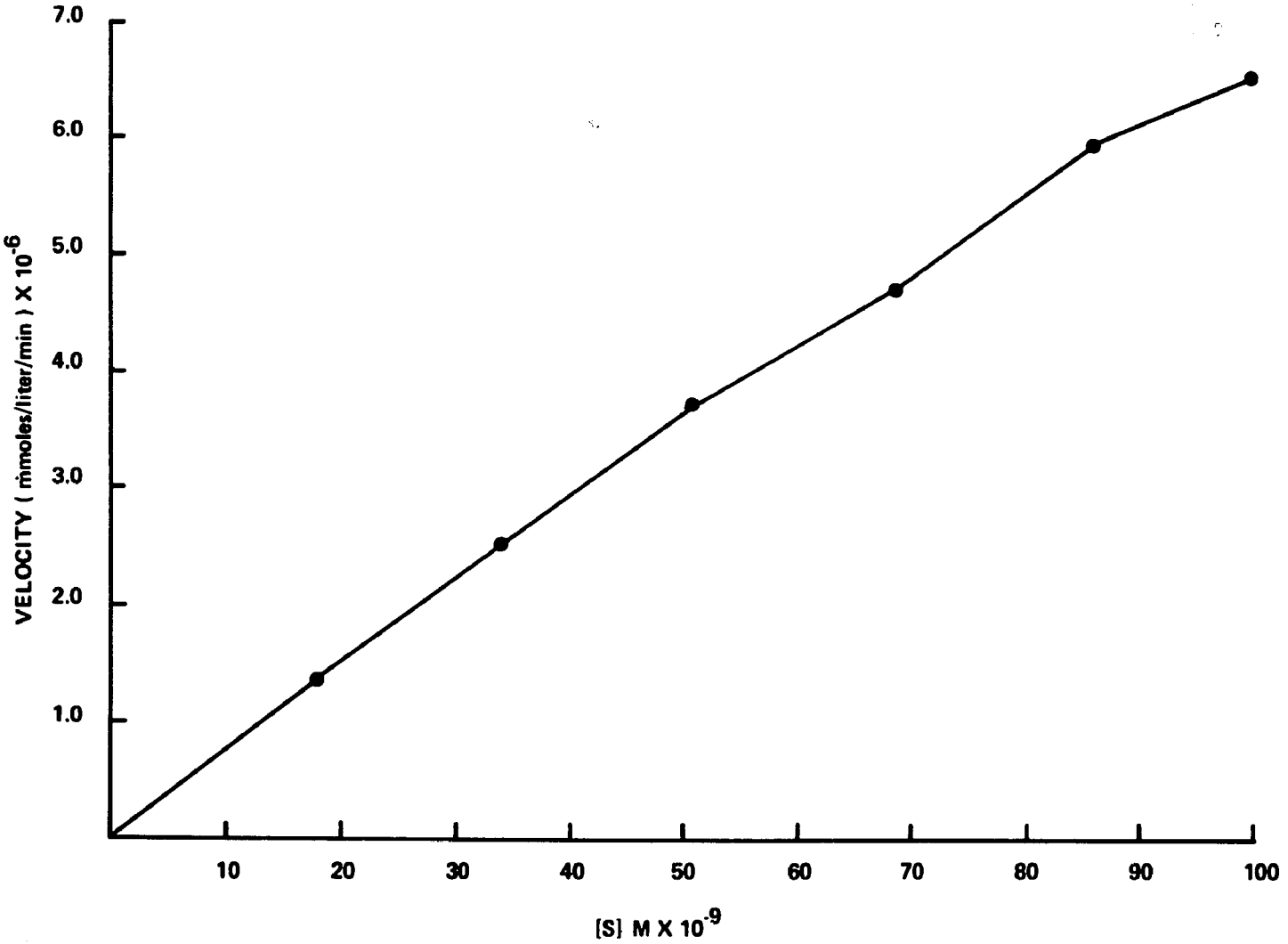

7.0
6.0
5.0
4.0
3.0
2.0
1.0
VELOCITY (mmoles/liter/min) X 10-6
10
20
30
40
50
60
70
80
90
100
[S] M X 10-9

Figure 23. Lineweaver-Burk plot of phosphodiesterase activity. Data from Figure 22. Range of substrate concentrations: 1.8×10^{-8} M - 1.0×10^{-7} M. K_m: 4.3×10^{-7} M.

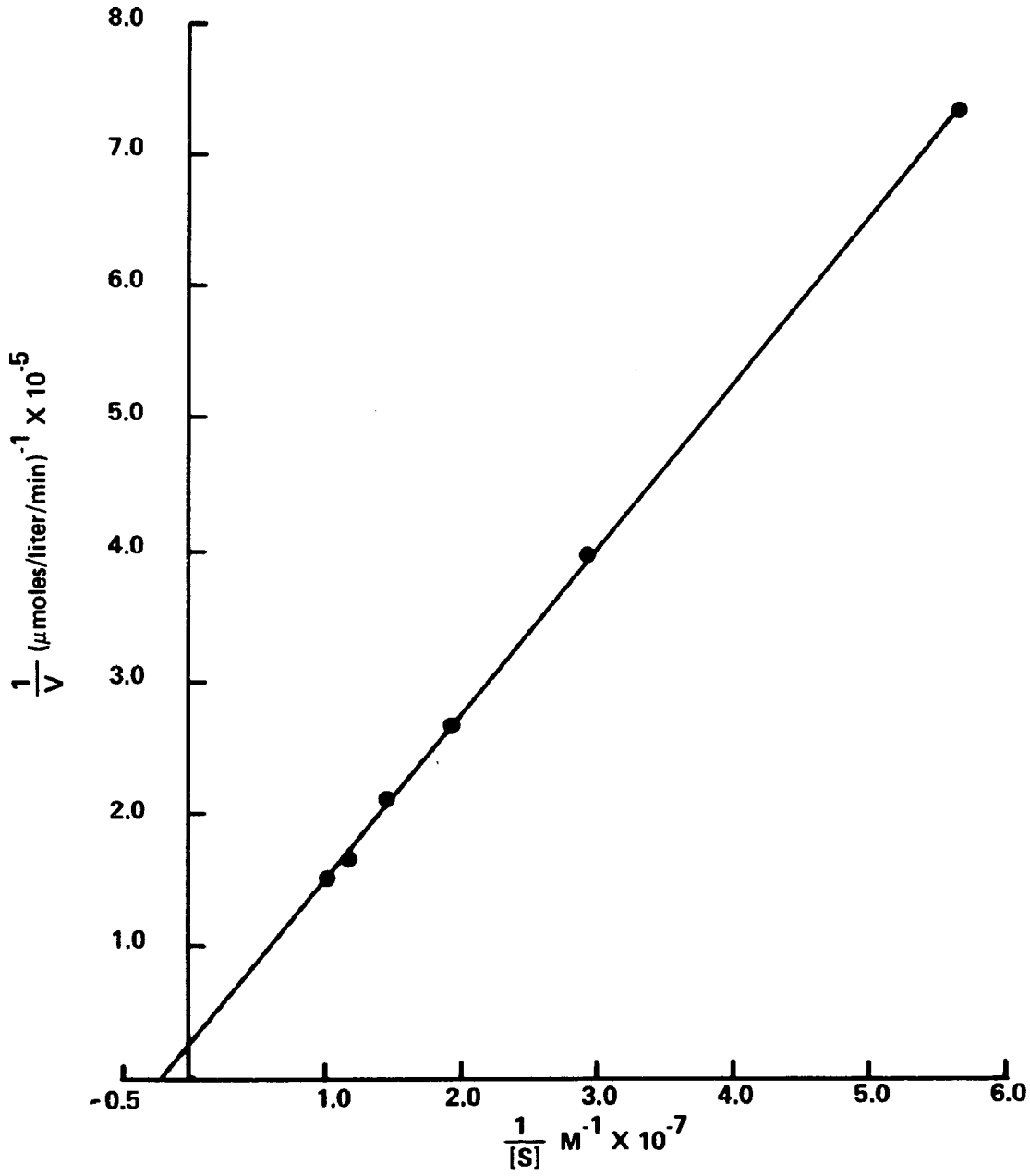

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
-0.5
1.0
2.0
3.0
4.0
5.0
6.0
$\frac{1}{V}$ (μmoles/liter/min)$^{-1}$ X 10^{-5}
$\frac{1}{[S]}$ M^{-1} X 10^{-7}

Figure 24. Michaelis-Menten plot of phosphodiesterase activity. Range of substrate concentrations: 1.8×10^{-8} M - 1.0×10^{-7} M. Incubation conditions: 10 minutes at 30° C.

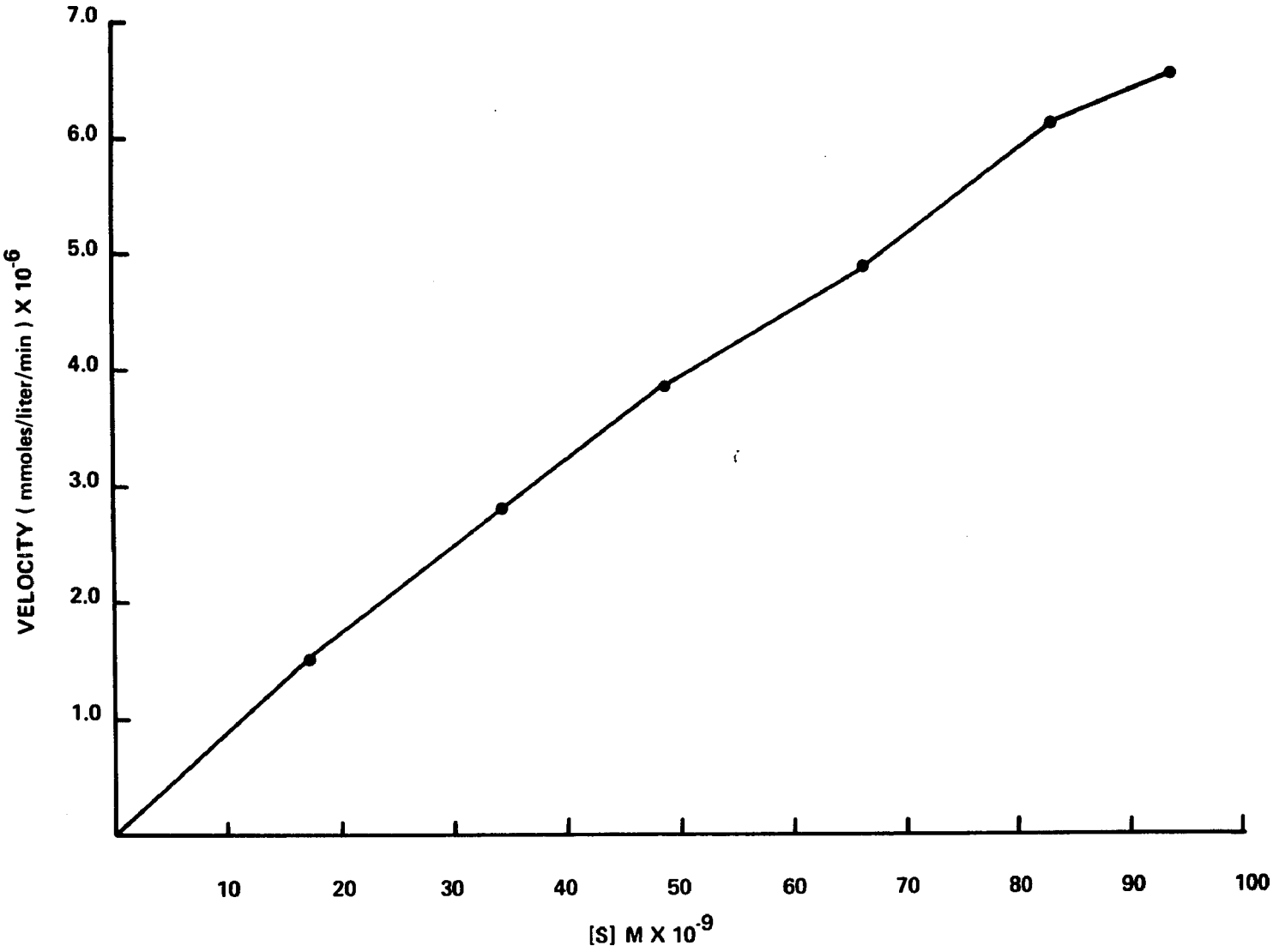

7.0
6.0
5.0
4.0
3.0
2.0
1.0
VELOCITY (mmoles/liter/min) X 10-6
10
20
30
40
50
60
70
80
90
100
[S] M X 10-9

Figure 25. Lineweaver-Burk plot of phosphodiesterase activity. Data from Figure 24. Range of substrate concentrations: 1.8×10^{-8} M - 1.0×10^{-7} M. K_m: 5.8×10^{-7} M.

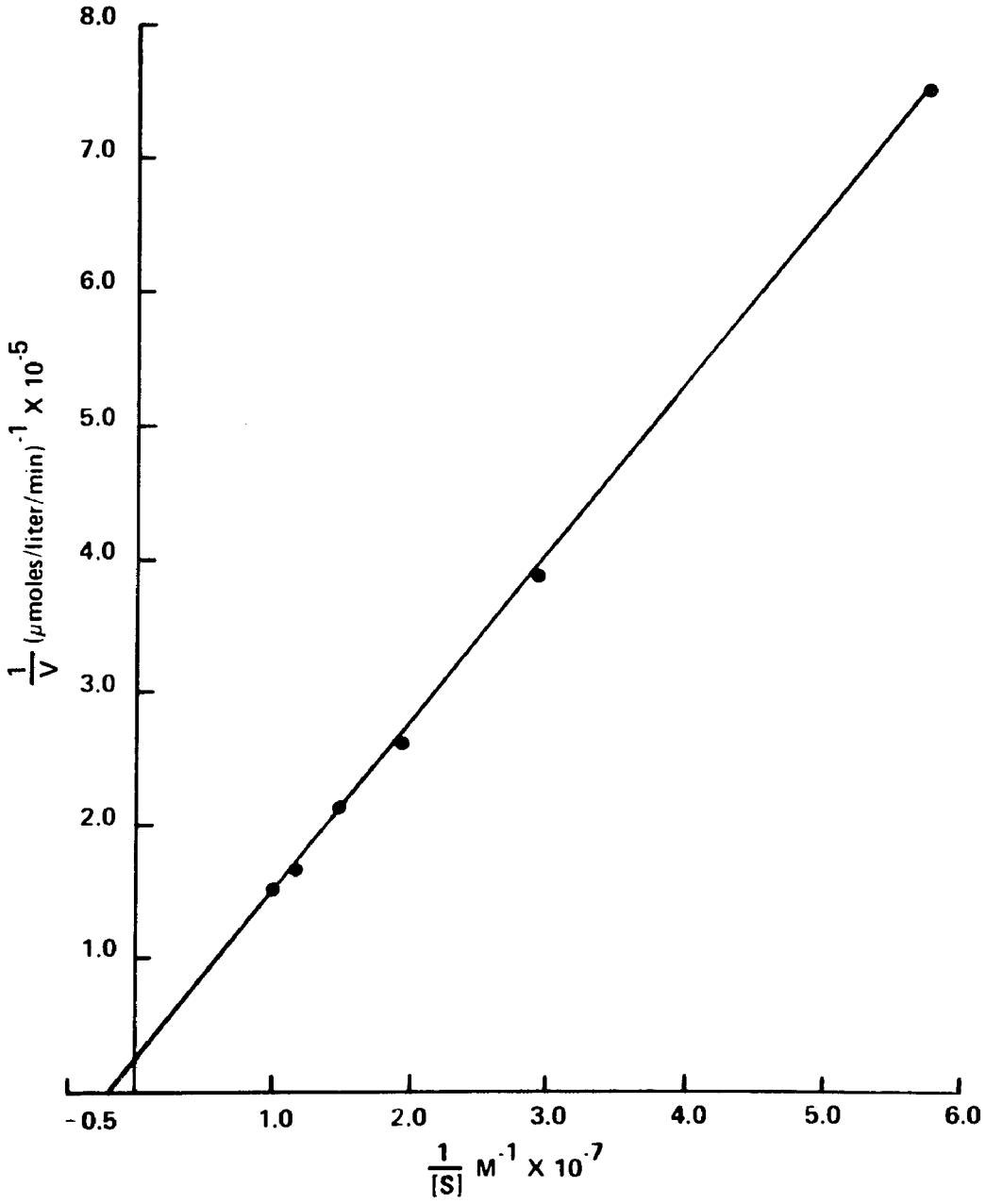

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
$\frac{1}{V}$ (μmoles/liter/min)$^{-1}$ X 10^{-5}
-0.5
1.0
2.0
3.0
4.0
5.0
6.0
$\frac{1}{[S]}$ M^{-1} X 10^{-7}

Figure 26. Michaelis-Menten plot of phosphodiesterace activity. Range of substrate concentrations: 4.6×10^{-7} M - 3.4×10^{-6} M. Incubation conditions: 10 minutes at 30^{o} C.

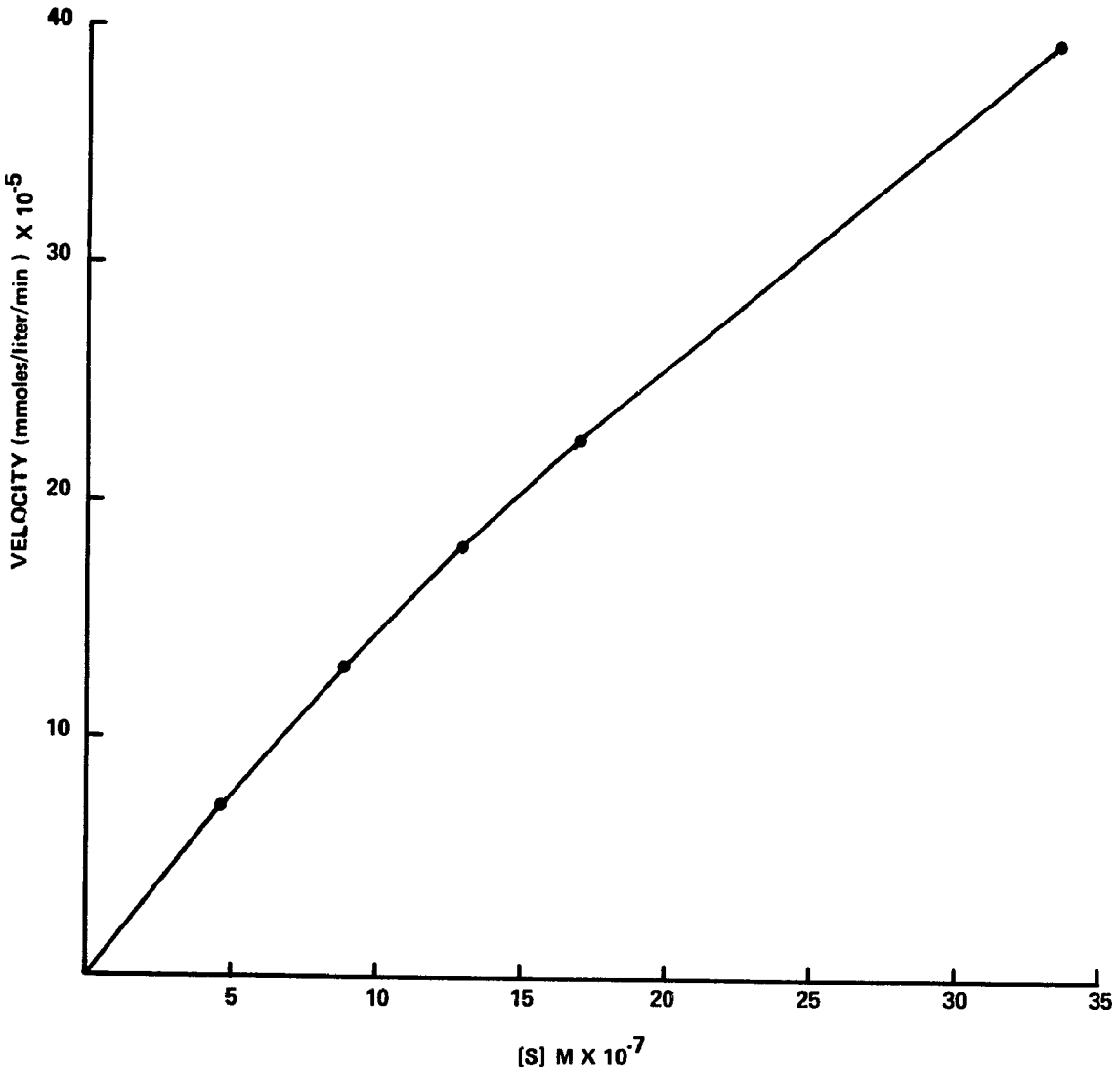

40
30
20
10
VELOCITY (mmoles/liter/min) X 10^{-5}
5
10
15
20
25
30
35
[S] M X 10^{-7}

Figure 27. Lineweaver-Burk plot of phosphodiesterase activity. Data from Figure 26. Range of substrate concentrations: 4.6×10^{-7} M - 3.4×10^{-6} M. K_m: 7.4×10^{-6} M.

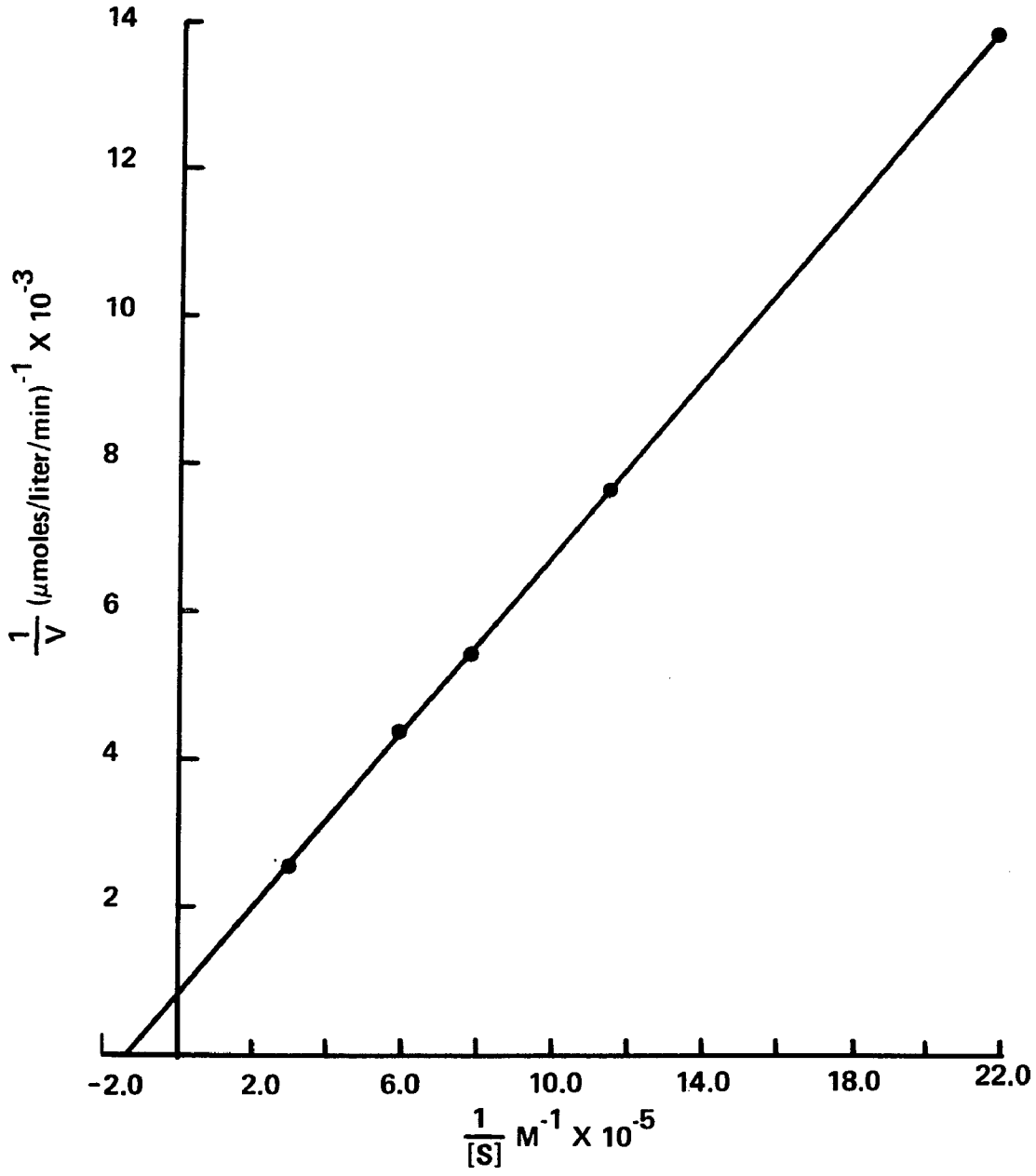

14
12
10
8
6
4
2
$\frac{1}{V}$ (μmoles/liter/min)$^{-1}$ X 10^{-3}
-2.0
2.0
6.0
10.0
14.0
18.0
22.0
$\frac{1}{[S]}$ M^{-1} X 10^{-5}

Figure 28. Michaelis-Menten plot of phosphodiesterase activity. Range of substrate concentrations: 2.1×10^{-7} M - 5.9×10^{-6} M. Incubation conditions: 10 minutes at 30° C.

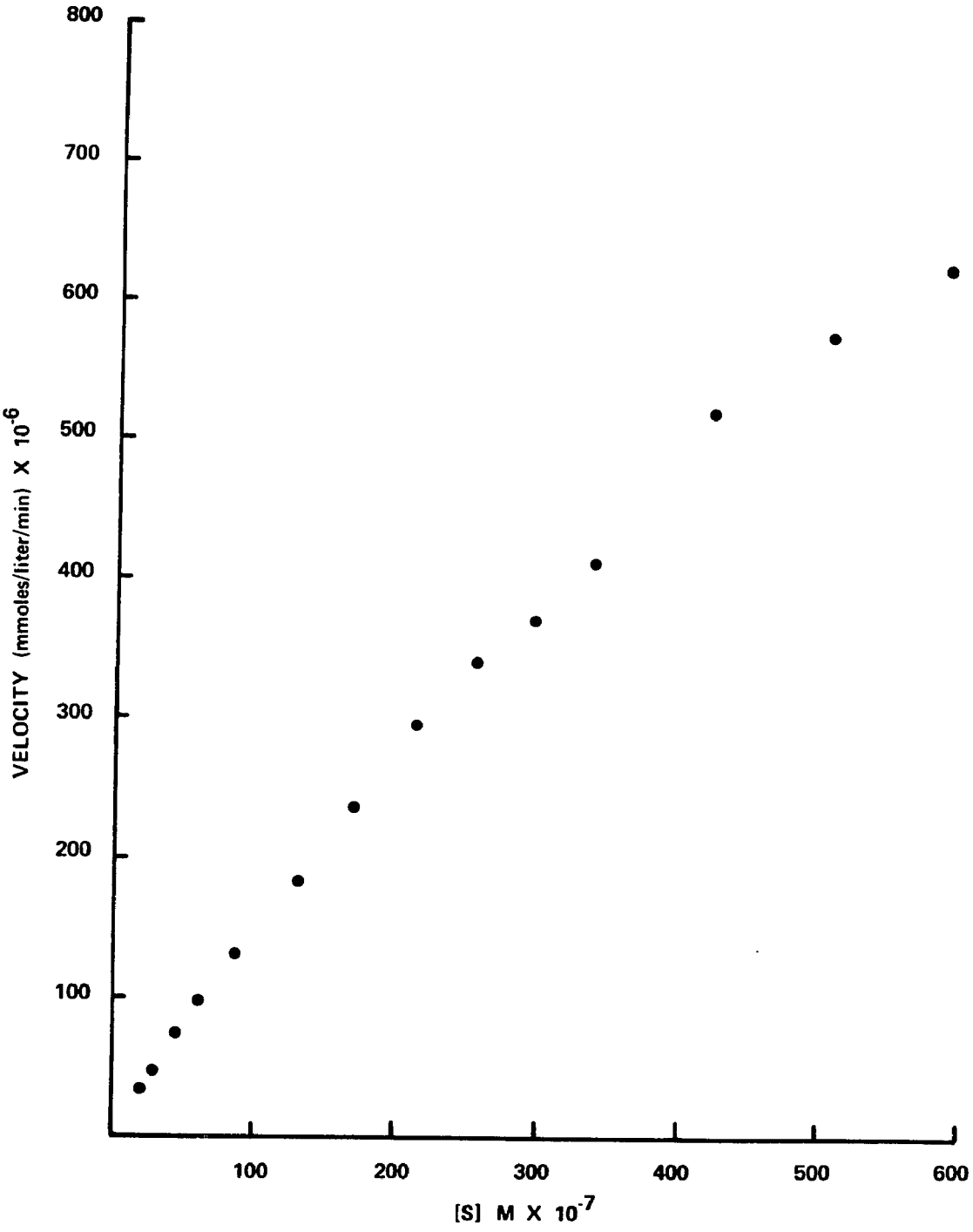
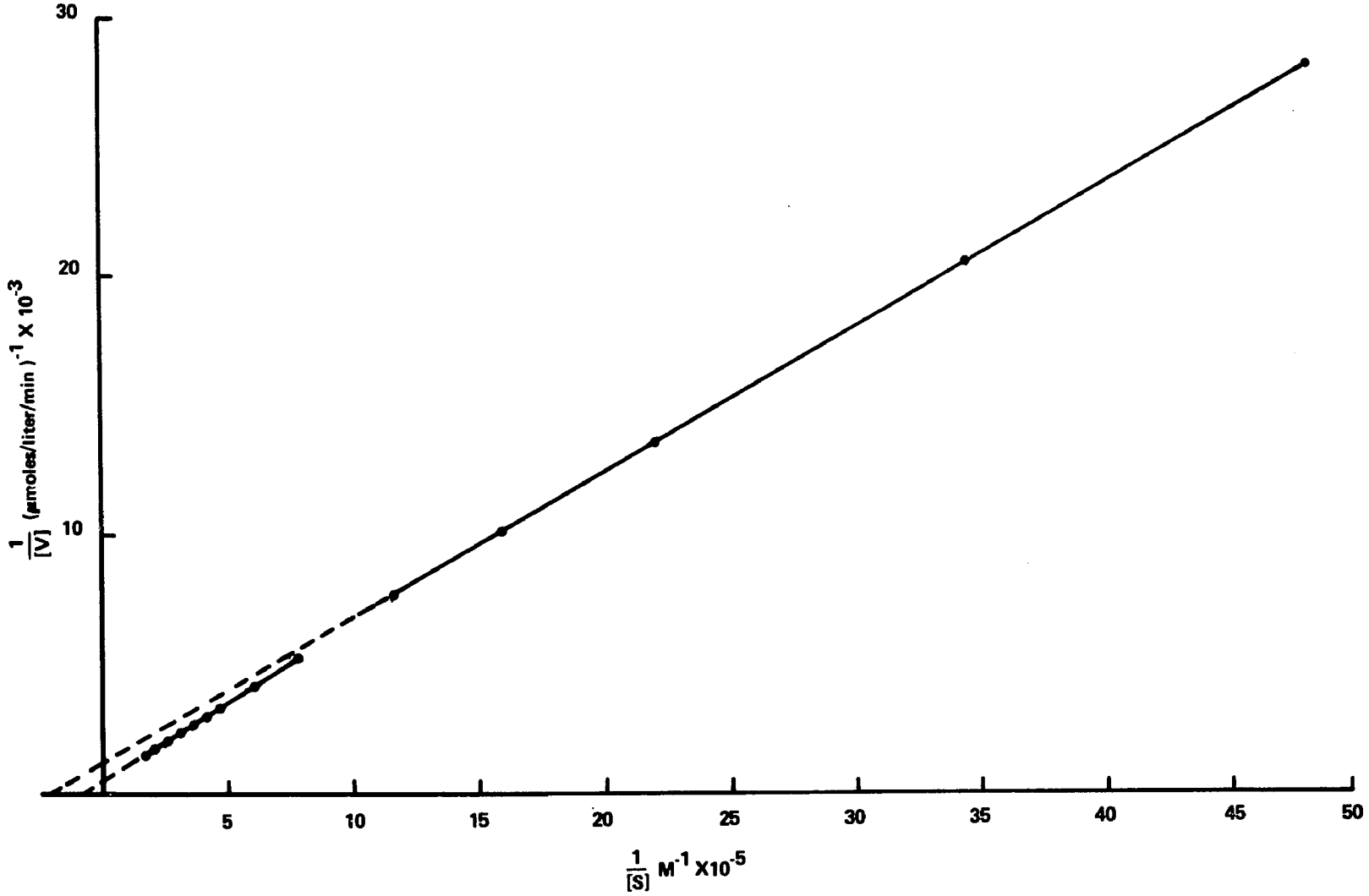

800
700
600
500
400
300
200
100
VELOCITY (mmoles/liter/min) X 10^{-6}
100
200
300
400
500
600
[S] M X 10^{-7}

Figure 29. Lineweaver-Burk plot of phosphodiesterase activity. Date from Figure 28.

Range of substrate concentrations: 2.1×10^{-7} M - 5.9×10^{-6} M.

K_m values: 1.2×10^{-5} M and 5.3×10^{-6} M.


30
20
10
$\frac{1}{[V]}$ (μmoles/liter/min)$^{-1}$ X 10^{-3}
5
10
15
20
25
30
35
40
45
50
$\frac{1}{[S]}$ M^{-1} X10^{-5}

of significance. K_m values calculated from the regression data of the two lines gave apparent K_m values of 5.3×10^{-6} M and 1.2×10^{-5} M.

Discussion

Brooker, Thomas, and Appleman (9), in 1967, reported an anomalous Lineweaver-Burk plot for rat brain phosphodiesterase (Figure 30). The apparent K_m values calculated from their data were 1.3×10^{-4} M and 1.0×10^{-6} M. In 1971, Thompson and Appleman (40) also reported an anomalous Lineweaver-Burk plot for phosphodiesterase using a rat brain preparation. However, after Agarose gel filtration, two active fractions were obtained: (1) a higher molecular weight fraction with an apparent K_m of 1×10^{-4} M, and (2) a lower molecular weight fraction with an apparent K_m of 5×10^{-6} M. These K_m values were identical to those obtainable from the anomalous Lineweaver-Burk plot which had been determined using an unchromatographed enzyme preparation. Thompson and Appleman interpreted the anomalous Lineweaver-Burk plot as "representing two enzyme systems with separate affinties for cyclic AMP rather than displaying the kinetics of one enzyme with multiple subunit sites of different apparent affinities."

The wide disparity among the K_m values reported for rat brain phosphodiesterase (Table 12) plus the findings of

Figure 30. A reporduction of Figure 4 in the paper by Brooker, Thomas, and Appleman (9). This represents the first anamalous Lineweaver-Burk plot reported for rat brain phosphodiesterase.

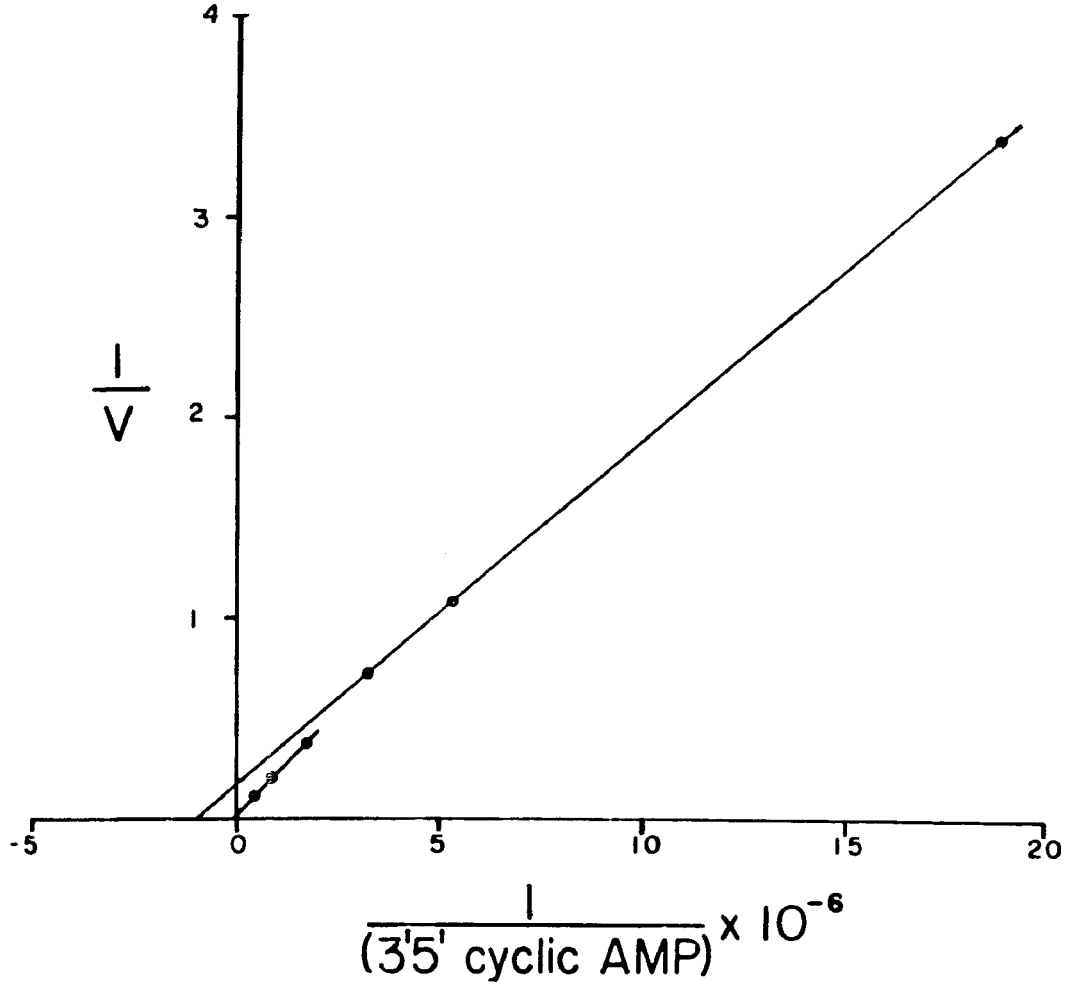

4
3
2
1
1/V
-5
0
5
10
15
20
1/(3'5' cyclic AMP) x 10^-6

Table 12

A summary of K_m values reported for rat brain phosphodiesterase. The concentration of cyclic AMP at which each was determined is included. Where two K_m values are given for the same reference (2 and 3, 4 and 5, 6 and 7), this refers to information derived from anomalous Lineweaver-Burk plots.

Number	K_m	Substrate Range (cyclic AMP)	Reference
1	$1.0\text{-}3.0 \times 10^{-4}$ M	$3.0 \times 10^{-4}\text{-}2.0 \times 10^{-3}$ M	Cheung (10)
2	1.3×10^{-4} M	$5.0 \times 10^{-7}\text{-}2.0 \times 10^{-6}$ M	Brooker, Thomas & Appleman (9)
3	1.0×10^{-6} M	$5.7 \times 10^{-8}\text{-}3.1 \times 10^{-7}$ M	
4	1.0×10^{-4} M	$5.0 \times 10^{-6}\text{-}1.0 \times 10^{-4}$ M	Thompson and Appleman (40)
5	5.0×10^{-6} M	$1.1 \times 10^{-6}\text{-}3.3 \times 10^{-6}$ M	
6	1.2×10^{-5} M	$1.7 \times 10^{-6}\text{-}5.9 \times 10^{-6}$ M	Figure 29
7	5.3×10^{-6} M	$2.1 \times 10^{-7}\text{-}1.3 \times 10^{-6}$ M	
8	7.4×10^{-6} M	$4.6 \times 10^{-7}\text{-}3.4 \times 10^{-6}$ M	Figure 27
9	4.3×10^{-7} M	$1.8 \times 10^{-8}\text{-}1.0 \times 10^{-7}$ M	Figure 23
10	5.8×10^{-7} M	$1.8 \times 10^{-8}\text{-}1.0 \times 10^{-7}$ M	Figure 25
11	2.4×10^{-7} M	$1.7 \times 10^{-8}\text{-}0.95 \times 10^{-7}$ M	Figure 21
12	4.9×10^{-7} M	$1.6 \times 10^{-8}\text{-}0.94 \times 10^{-7}$ M	Figure 19

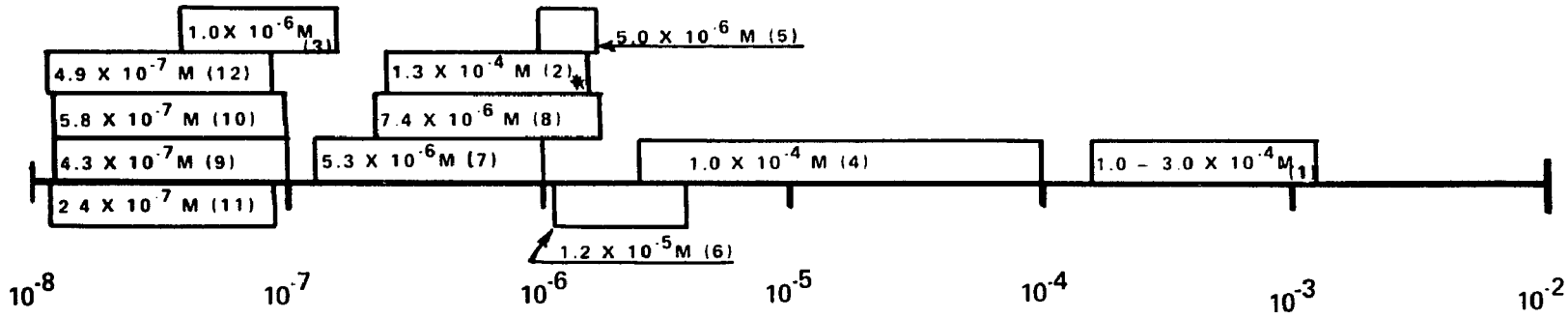
Thompson and Appleman (40) suggest the following hypothesis:

> Rat brain tissue contains at least three phosphodiesterase enzymes whose activity may be represented by the apparent K_m values--- 1.0×10^{-4} M, 5.0×10^{-6} M, and the least as low as approximately 4.4×10^{-7} M.

Assuming this to be correct, the K_m value obtained in a particular determination would depend on the range of substrate concentrations utilized. For example, if substrate concentrations were limited to the first order part of a Michaelis-Menten curve for the enzyme with the highest K_m value, then the activity of the enzymes with the lower K_m values would be concealed. In like manner, a range of substrate concentrations which spanned the first order parts of Michaelis-Menten plots for two of the enzymes would yield an apparent K_m in between the true values for those enzymes. Finally, a range of substrate concentrations limited to the first order part of a Michaelis-Menten plot for the enzyme with the lowest K_m value would give no experimental indications of the presence of a higher K_m.

In support of this hypothesis, the information in Table 12 is plotted in Figure 31. In this figure, the range of substrate concentrations is denoted by the rectangles. The observed K_m value for each range of concentrations is given inside

Figure 31. A visual representation of the range of substrate concentrations used to determine the K_m values reported for rat brain phosphodiesterase in Table 12. The length of each rectangle refers to the range of substrate concentrations employed in each K_m determination. The K_m value for that range of substrate concentrations is given within each rectangle with its reference to Table 12.


1.0X 10⁻⁶M (3)
4.9 X 10⁻⁷ M (12)
5.8 X 10⁻⁷ M (10)
4.3 X 10⁻⁷M (9)
2 4 X 10⁻⁷ M (11)
1.3 X 10⁻⁴ M (2)
7.4 X 10⁻⁶ M (8)
5.3 X 10⁻⁶M (7)
5.0 X 10⁻⁶ M (5)
1.0 X 10⁻⁴ M (4)
1.0 - 3.0 X 10⁻⁴M (1)
1.2 X 10⁻⁵M (6)
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²

the rectangle along with its reference in Table 12. As may be seen, 11 out of the 12 observations are compatible with the above mentioned hypothesis. The single observation which does not fit the scheme is denoted by an asterisk; it (No. 2 of Table 12) was obtained from an anomalous plot. One of the values (No. 5 of Table 12) which "fits" the hypothesis less closely than the remaining 10 was computed from only three substrate concentrations and thus might be suspect.

On the basis of this hypothesis it seems reasonable that the previously unreported K_m value in the 10^{-7} M range (No. 9, 10, 11, and 12 of Table 12) is real. The fact that this low K_m value for rat brain phosphodiesterase has not been observed by previous investigators might be related to:

(1) failure to utilize sufficiently low substrate concentrations or

(2) the unavailability of an assay procedure sufficiently sensitive to measure these low substrate concentrations.

SUMMARY

A new method for the in vitro measurement of adenyl cyclase activity has been developed. The major advantages of this assay are:

(1) The assay is simple -- common biochemical procedures are employed.

(2) The assay is relatively quick. An entire assay may be accomplished in one man day, as no time-consuming procedures are involved.

(3) An ATP-regenerating system is included which should preclude errors in measurement of activity due to changes in substrate concentration.

(4) Unlabeled ATP is used as substrate for the reaction -- thus allowing the procedure to be readily employed in determining kinetic parameters of adenyl cyclase (such as K_m) without the expense which accompanies the use of labeled ATP.

The assay was tested by determing adenyl cyclase activity in rat myocardial and liver preparations. The activity recorded using this assay fell within the range of activities reported for

myocardial tissue by other investigators and compared very favorably with the activity reported for liver tissue which used a very similar tissue preparation.

Additionally, a new method for measuring phosphodiesterase activity has been developed. This procedure was originally developed as a component of the adenyl cyclase assay but has proven to function well as an independent assay. The assay is very sensitive and relatively simple. Its usefulness was demonstrated in experiments concerning the kinetic parameters of phosphodiesterase.

Finally, a previously unreported K_m value for rat brain phosphodiesterase was recorded. An hypothesis for the correlation of this value with other reported K_m values for this enzyme was submitted.

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