Cyclic AMP Metabolism in Brain

by

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CYCLIC AMP METABOLISM IN BRAIN

Abstract

A method for measuring the rate of production of ^{14}C-labelled adenine nucleotides from ^{14}C-ATP was developed and used to study the relationship of adenyl cyclase to other membrane-bound ATP utilizing enzymes and cyclic 3',5'-nucleotide phosphodiesterase (PDE) in preparations of rat cerebral cortex and guinea-pig pancreas. It was shown that NaF and Mn^{++} increased cyclic AMP accumulation in these preparations, in part, by an inhibition of PDE activity. Adrenaline and ouabain stimulated cyclic AMP accumulation by a method not related to inhibition of other membrane-bound ATP utilizing enzymes or to inhibition of PDE activity. Adrenaline stimulation of cyclic AMP accumulation in the synaptic membrane preparation required the presence of Ca^{++} in the preparation media. Inhibition of a soluble PDE preparation from rat cerebral cortex was maximal in the presence of ATP and ADP and much lower in the presence of methylene analogs of ATP.

CYCLIC AMP METABOLISM IN BRAIN

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ABBREVIATIONS USED

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AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
cyclic AMP	adenosine 3',5'-monophosphate
PP_i	pyrophosphate
P_i	orthophosphate
PDE	cyclic nucleotide phosphodiesterase
ATPase	adenosine triphosphatase
Na/K ATPase	sodium-potassium dependent ATPase
glucose-6-P	glucose 6-phosphate
glucose-1-P	glucose 1-phosphate
Tris	tris(hydroxymethyl)aminomethane
EDTA	ethylenediaminetetraacetate
EGTA	1,2,-bis-(2-dicarboxymethylaminoethoxy)ethane
et al.	and others
mM	millimolar
μM	micromolar
M	molar
n	nano
m	milli
μ	micro
N	normal
ml	milliliter
g	gram
mg	milligram

dpm	disintegrations per minute
C_i	Curie
R_f	retardation factor
x g	acceleration of gravity
Km	Michaelis constant
^oC	degree centigrade
S.E.	standard error of mean
PEP	phosphoenolpyruvate
PK	pyruvate kinase
POB	phenoxybenzamine
TCA	trichloroacetic acid
α,β mATP	α,β-methylene-adenosine triphosphate
β,γ mATP	β,γ-methylene-adenosine triphosphate
I.U.	International Units.
DCI	dichloroisoproterenol

INTRODUCTION

Since the discovery of adenosine 3',5'-monophosphate (cyclic AMP) by Rall et al. (1957), evidence has accumulated indicating that this nucleotide is an intermediate in the action of a wide variety of hormones. The mechanism of activation of cyclic AMP accumulation by hormones or by non-specific agents such as NaF is unknown.

Systems used to study adenyl cyclase are invariably contaminated with other enzymes, particularly nucleotidases. Adenosine 5'-triphosphate (ATP) is a substrate for both ATPase and for adenyl cyclase, and therefore in these systems cyclic AMP accumulation is occurring at a time when substrate concentration is rapidly declining. It seemed possible that some of the breakdown products of ATP might affect the rate of cyclic AMP formation by influencing components of the system other than adenyl cyclase. It was not known whether the effects of certain hormones and other agents on cyclic AMP formation in broken cell preparations are achieved by a direct action on the enzyme adenyl cyclase or by an indirect action via an influence on ATP metabolism. An ATP regenerating system has been used by some authors, but in most studies it was not established that the regenerating system actually maintained ATP levels during the entire time of incubation (Rabinowitz et al., 1965; Streeto and Reddy, 1967; Dousa and Rychlik, 1968).

There is some evidence which suggests that activation of adenyl cyclase is associated with an alteration in the activity of other membrane-bound ATP utilizing enzymes, such as Na/K activated ATPase. Freidmann and Park (1968) have shown that glucagon stimulation of cyclic AMP accumulation in liver is associated with a very rapid, transient efflux of potassium ion. It has also been shown that the release of insulin from the β-cells of the

pancreas, believed to be a cyclic AMP mediated effect, can be stimulated by ouabain, which is presumed to be a specific inhibitor of Na/K ATPase (Milner and Hales, 1967). Ouabain has also been shown to stimulate cyclic AMP accumulation in brain slices (Shimizu et al., 1970c).

Cyclic nucleotide phosphodiesterase (PDE) activity has been found in almost all preparations used to study adenyl cyclase. Inhibitors of PDE activity, such as theophylline or caffeine, are generally used, but these compounds inhibit PDE activity by only 50% under standard experimental conditions (Cheung, 1966; Streeto and Reddy, 1967). There is some indication that cyclic AMP accumulation in these adenyl cyclase systems may be associated with an alteration in PDE activity. Cheung (1966) has shown that pyrophosphate (PP_i) and ATP are potent inhibitors of cyclic AMP destruction in rat cerebral cortex preparations, and agents such as insulin and nicotinic acid, which decrease cyclic AMP accumulation, are believed to stimulate PDE activity (Schultz et al., 1966; Senft et al., 1968a,b; Krishna et al., 1966).

It was decided to investigate the relationship between adenyl cyclase, other membrane-bound ATP utilizing enzymes and PDE. For this study a method that would allow the simultaneous determination of cyclic AMP, ATP, adenosine 5'-diphosphate (ADP) and adenosine 5'-monophosphate (AMP) was needed. There are a number of methods which measure cyclic AMP accurately: e.g., the isotope dilution method of Brooker et al. (1968), the protein binding assay of Gilman (1970), and the immunoassay of Steiner et al. (1969). Unfortunately, these and other sensitive assays for cyclic AMP do not allow the determination of ATP, ADP and AMP as well. Thus in the first part of this study a method was developed to measure the production of ^{14}C-labelled adenine nucleotides from ^{14}C-ATP with a high degree of

accuracy and sensitivity. In the next part of this work this method was used to study the relationship of adenyl cyclase to the activity of other membrane-bound ATP utilizing enzymes in preparations of rat cerebral cortex and guinea-pig pancreas. Finally, the effect of various agents on cyclic AMP destruction was investigated using these preparations of adenyl cyclase and a soluble preparation of PDE from rat cerebral cortex.

HISTORICAL REVIEW

PART 1

In 1957, Rall et al. reported increased formation of active glycogen phosphorylase in the presence of adrenaline or of glucagon in cell-free homogenates of liver. It was shown that the response of the homogenates to the hormones occurred in two stages. In the first stage a particulate fraction of homogenates produced a heat-stable factor in the presence of the hormones; in the second stage this factor stimulated the formation of active glycogen phosphorylase in supernatant fractions of homogenates in which the hormones themselves were inactive. Cook et al. (1957), while investigating the hydrolysis of ATP in the presence of barium hydroxide, isolated a product other than the major products, adenylic acid and PP_i. This product was crystallized (Lipkin et al., 1959) and shown to contain adenine, ribose and phosphate in the ratio of 1:1:1 and no monoesterified phosphate. The heat-stable factor reported by Rall et al. (1957) was isolated and characterized (Sutherland and Rall, 1958) and proved to be identical to the compound described by Lipkin et al. (1959). The compound was shown to be the mononucleotide of adenylic acid with the phosphate group esterified at carbon 3' and 5' of the ribose moiety, that is adenosine 3',5'-monophosphate (cyclic AMP).

A. PROPERTIES OF CYCLIC AMP:

Sutherland and Rall (1958), found that the ultraviolet spectrum and the molar extinction coefficient of cyclic AMP were similar to those of AMP. Cyclic AMP was shown to be very stable to acid and alkaline hydrolysis and was not attacked by many of the more commonly used phosphatases and phosphodiesterases (Sutherland and Rall, 1958).

The enzyme catalyzing the formation of cyclic AMP from ATP, adenyl cyclase, was found to be located in low-speed particulate fractions of the cell (Rall and Sutherland, 1958). PP_i was identified as the other product of the reaction (Rall and Sutherland, 1962). Mg^{++} was required, and the pH optimum of the reaction was between 7.2 and 8.2. The complete reaction is summarized in Figure 1.

Particulate preparations of liver collected at 2,000 x g catalyzed the formation of ^{14}C-cyclic AMP from ^{14}C-ATP with no decrease in the specific activity, indicating that the purine portion of the cyclic AMP produced was derived from the added ATP rather than from endogenous sources (Rall and Sutherland, 1958). In particulate preparations of skeletal muscle, cyclic AMP derived from α-labelled ATP (i.e. adenine-ribose-P^{32}-O-P^{31}-O-P^{31}) was shown to contain the isotope in good yield, but cyclic AMP derived from β-γ-labelled ATP (i.e. adenine-ribose-P^{31}-O-P^{32}-O-P^{32}) contained only trace amounts of the isotope (Sutherland and Rall, 1960); this indicated that the phosphate group in cyclic AMP was derived from the α-phosphate of the substrate, ATP.

B. DISTRIBUTION

a) Distribution in tissues. Adenyl cyclase was present in every animal tissue examined with the exception of dog erythrocytes (Sutherland et al., 1962). It was also present in all four of the phyla that have been investigated (Sutherland et al., 1962).

b) Cellular location. Sutherland and Rall (1960) found most of the adenyl cyclase activity in liver and heart preparations present in particulate fractions which sedimented at low gravitational forces (600 to

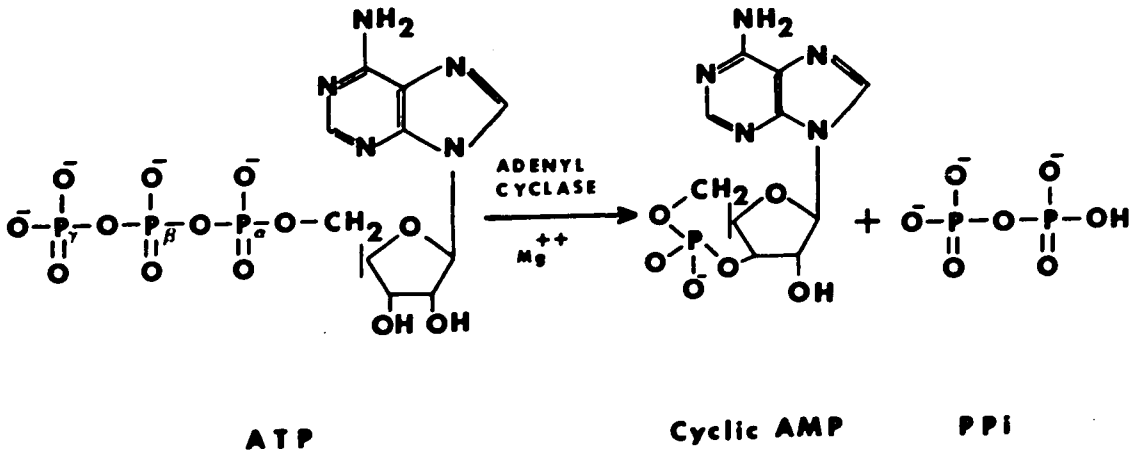


FIGURE 1: The Formation of Cyclic AMP from ATP

2,000 x g). Fractionation and repeated washings, as well as microscopic examination and use of mitochondrial markers, showed clearly that adenyl cyclase activity in these homogenates was not associated with the mitochondria nor with the microsomes but with some portion of the "nuclear fraction" (Sutherland et al., 1962). This fraction may have contained both nuclei and cell membranes, and it was concluded that these two cell components were the principal candidates for the location of adenyl cyclase. Sutherland et al. (1962) found that chicken and pigeon erythrocytes which contained nuclei also contained adenyl cyclase, but dog erythrocytes which were anucleate did not. This finding suggested that the nuclei contained the adenyl cyclase. Davoren and Sutherland (1963) separated nuclei from cell membranes in pigeon erythrocytes by a dispersion procedure which fragments the cell membranes without damaging the nuclei. They reported that most of the adenyl cyclase activity was present in the 78,000 x g precipitate which contained no nuclei and only a small fraction of the total DNA; this provided strong evidence that the adenyl cyclase was not associated with the nuclei. These workers concluded that all, or almost all, of the adenyl cyclase in the erythrocytes was located in the plasma membrane of the cell. Results of studies by Rodbell (1967) with homogenized fat cell ghosts, in which it was possible to separate nuclei and mitochondria from the plasma membrane, indicated that the plasma membrane contained most of the adenyl cyclase. Marinetti et al. (1969), who used the procedure of Coleman et al. (1967), showed adenyl cyclase activity to be specifically localized in a purified plasma membrane fraction of rat liver. This was confirmed by Pohl et al. (1969) in an isolated plasma membrane fraction of rat liver prepared by the procedure of Neville (1968). In studies by de Robertis et al. (1967),

adenyl cyclase activity in the rat brain was found to be concentrated in subfractions containing mainly synaptic membranes. Among the major fractions obtained by differential centrifugation of rabbit skeletal muscle homogenates, only the mitochondrial and microsomal fractions showed appreciable adenyl cyclase activity which apparently paralleled the distribution of relaxing factor in the muscle (Rabinowitz et al., 1965). A microsomal fraction from dog cardiac muscle that actively accumulates Ca^{++} exhibited adenyl cyclase activity (Entman et al., 1969). These workers proposed that this fraction represented fragments of the sarcoplasmic reticulum.

C. INACTIVATION

Sutherland and Rall (1958) found an enzyme capable of destroying cyclic AMP in preparations of dog and beef heart. This enzyme, PDE, was shown to be Mg^{++}-dependent and catalyzed the hydrolysis of the cyclic nucleotide at the 3'-position yielding AMP. It was found that the activity of this enzyme was stimulated by imidazole and inhibited by the methylxanthines, theophylline and caffeine (Butcher and Sutherland, 1959).

To date all tissues containing adenyl cyclase have shown PDE activity; nervous tissue has exhibited the highest amount (Weiss and Costa, 1968; Butcher and Sutherland, 1962). Experiments by Butcher and Sutherland (1962), Cheung and Salganicoff (1966) and de Robertis et al. (1967) showed that the enzyme was found in both supernatant and particulate fractions of tissue preparations. Several substances in addition to methylxanthines have now been shown to inhibit soluble PDE preparations from various tissues. They are: ATP, PP_i and citrate in rat brain (Cheung, 1966); ADP and ATP in rat

kidney (Dousa and Rychlik, 1970a); triiodothyronine in rat adipose tissue (Mandel and Kuehl, 1967); phenothiazines and reserpine derivatives in beef heart and rabbit brain (Honda and Imamura, 1968) and puromycin in rat diaphragm adipose tissue (Appleman and Kemp, 1966). Claims of PDE stimulation by nicotinic acid (Krishna et al., 1966) and insulin (Schultz et al., 1966; Senft et al., 1968a,b) in adipose tissue preparations could not be confirmed in studies by other workers (Kupiecki and Marshall, 1968; Blecher et al., 1968).

D. ACTIONS OF CYCLIC AMP

Following the initial discovery of a role for cyclic AMP in the activation of glycogen phosphorylase in liver and muscle, Haynes et al. (1959) found that cyclic AMP acted as an intermediate agent in ACTH stimulation of glycogen phosphorylase in adrenal tissue. At about this time other cases were discovered where the nucleotide functioned in regulatory processes not related to glycogenolysis. Mansour and Menard (1960) showed that the serotonin increase in phosphofructokinase activity in homogenates of the worm Fasciola hepatica was mediated by cyclic AMP. Berthet (1958) reported that the increased incorporation of acetate into ketone bodies by liver slices, which can be induced by adrenaline or glucagon, could be reproduced by the addition of cyclic AMP. The inhibitory effect of these hormones on amino acid incorporation could also be mimicked by the nucleotide (Pryor and Berthet, 1960). Studies by Orloff and Handler (1961) implicated cyclic AMP as the mediator of permeability changes in response to vasopressin in the toad bladder, and Butcher et al. (1965) found cyclic AMP to be the mediator of lipolysis in the epididymal fat pad.

Table 1, part of which is taken from Sutherland et al. (1968), gives a partial list of the many processes in which cyclic AMP has been implicated. All of these processes fulfill one or more of the four criteria for cyclic AMP involvement proposed by Sutherland et al. (1968). These criteria are: 1) adenyl cyclase in broken cell preparations should respond to the same hormones which are effective in the intact tissue; 2) the level of cyclic AMP in intact tissue should change in response to hormone stimulation; 3) hormones which stimulate adenyl cyclase should be potentiated by drugs which inhibit PDE activity; and 4) cyclic AMP or the dibutyryl analog (Posternak et al., 1962) should be able to mimic the physiological effect of the hormone.

E. ROLE OF CYCLIC AMP IN HORMONE MEDIATION:

In 1965, Sutherland et al. put forward the "second messenger" hypothesis, illustrated in Figure 2 (from Sutherland et al., 1968) to explain the role of cyclic AMP in hormone action. In this hypothesis the first messenger, a hormone, interacts with a membrane-bound adenyl cyclase leading to increased cyclic AMP synthesis within the cell. In this way the extracellular signal, through the mediation of cyclic AMP, initiates a series of intracellular reactions, the ultimate result of which depends on the enzymatic profile of the cell involved. This second messenger might also stimulate the formation of a third messenger, such as a steroid, which would be released from the specialized tissue to act elsewhere.

The complexity and diversity of the final response to a single second messenger made it seem likely that the second messenger had a fundamentally different mechanism of action within each specific cell type; this, however,

TABLE 1

Some Hormonal Responses Involving Cyclic AMP

Hormone	Response	Criteria* 1	2	3	4 a	4 b	References
Catecholamines	Glycogen phosphorylase activation (liver)	+	+	+	+	+	Sutherland and Rall (1960) Haugaard and Hess (1965) Sutherland and Robison (1966)
	Glycogen phosphorylase activation (heart)	+	+	+	±	+	Haugaard and Hess (1965) Drummond and Duncan (1966)
	Positive inotropic response	+	+	+	±	-	Sutherland and Robison (1966) Robison *et al.* (1967)
	Lipolysis	+	+	+	+	+	Butcher (1966) Butcher and Sutherland (1967)
	Neuromuscular transmission	+	+	+	+	-	Goldberg and Singer (1969)
	Glycogenolysis (muscle)	+	+	-	-	-	Lyon and Mayer (1969)
	Tyrosine α-ketoglutarate transaminase (liver)	+	+	+	+	-	Wicks (1969)
	Renin production (kidney)	-	+	-	+	-	Michelakis *et al.* (1969)
	Amylase secretion (parotid gland and pancreas)	-	-	+	+	-	Kulka and Sternlicht (1968) Bdolah and Schramm (1965) Rasmussen and Tenenhouse (1968)

(continued next page)

Hormone	Response	Criteria*					References
		1	2	3	4		
					a	b	
Glucagon	Glycogen phosphorylase activation (liver)	+	+	+	+	+	Sutherland and Rall (1960) Sutherland and Robison (1966)
	Insulin release	-	-	+	+	-	Lacy (1967) Sussman and Vaughan (1967)
	Tyrosine α-ketoglutarate transaminase (liver)	+	+	+	+	-	Wicks (1969)
ACTH	Steroidogenesis (adrenal cortex)	+	+	+	+	-	Haynes et al. (1960) Hilf (1965)
ICSH	Steroidogenesis (corpus luteum)	+	+	-	+	-	Hall and Koritz (1965) Marsh et al. (1966)
	Steroidogenesis (testes)	+	-	-	+	-	Sandler and Hall (1966) Murad et al. (1969)
Angeotensin	Steroidogenesis (zona glomerulosa)	-	-	+	+	-	Kaplan (1965)
Vasopressin	Permeability changes	-	+	+	+	-	Orloff and Handler (1967)
TSH	Thyroid hormone production	+	+	+	+	-	Gilman and Rall (1966) Pastan (1966) Ensor and Munro (1967)
MSH	Melanocyte dispersion	-	+	+	+	-	Bitensky and Burstein (1965)
Seretonin	Phosphofructokinase activation	+	+	-	-	+	Stone and Mansour (1967)

(continued next page)

Hormone	Response	Criteria*					References
		1	2	3	4		
					a	b	
Gastrin	HCl production	-	-	+	+	-	Robertson et al. (1950) Harris and Alonso (1965)
PTH	Gluconeogenesis (kidney)	+	+	-	+	-	Rasmussen et al. (1968) Nagata and Rasmussen (1970) Dousa and Rychlik (1968)
	Bone resorption	+	-	+	+	-	Chase et al. (1969)

*Criterion 1 = broken cell preparations; 2 = intact tissue; 3 = potentiation by methylxanthines; and 4 = production of response by cyclic AMP in a and b; a = intact tissue; b = broken cell preparation. A negative sign does not necessarily imply a negative result but only that the criterion has not been established.

TSH = thyroid-stimulating hormone; MSH = melanocyte-stimulating hormone; PTH = parathyroid hormone; ICSH = interstitial cell stimulating hormone; ACTH = adrenocorticotropic hormone.

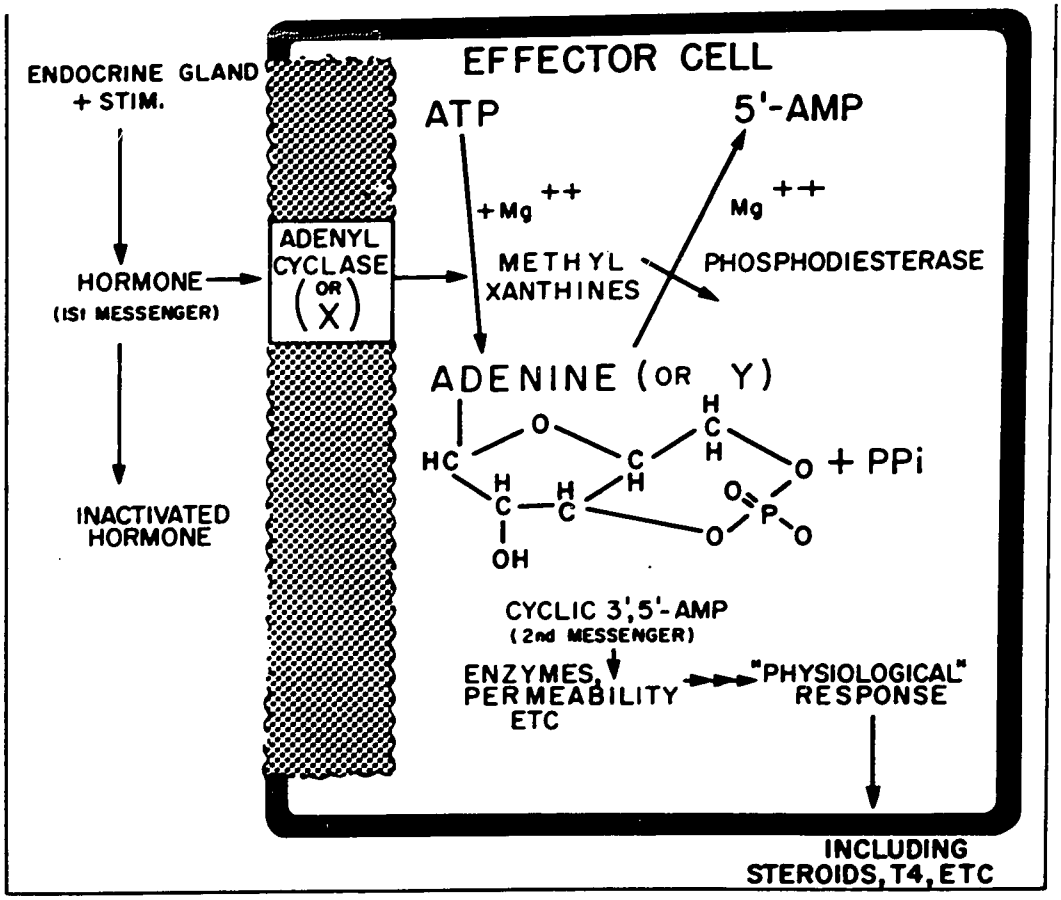


FIGURE 2: The "Second Messenger" Hypothesis

no longer appears to be the case. Recent work by Langan and Smith (1967), Rodnight (1967) and Ord and Stocken (1968) has led to the discovery of many phosphoproteins which exhibit a rapid turnover of their phosphate groups. Corbin and Krebs (1969), Kuo and Greengard (1969), Miyamoto et al. (1969) and Walsh et al. (1968) have demonstrated that in nearly all of the tissues in which cyclic AMP is a presumed second messenger, cyclic AMP-dependent protein kinases are present. This discovery has led to the hypothesis that all the effects of cyclic AMP are mediated by controlling the activity of this class of enzymes.

The widespread occurrence of both Ca^{++} (Douglas, 1968) and cyclic AMP as a coupling factor between excitation and response led to the investigation of their relationship by Rasmussen and Tenenhouse (1968) and Tenenhouse et al. (1969); Ca^{++} and cyclic AMP were shown to be involved in amylase secretion from the salivary gland and renal gluconeogenesis. Ca^{++} and cyclic AMP were also shown to be involved in peptide hormone action (Rasmussen and Nagata, in press), insulin and endocrine secretion (Malaisse et al., 1967; Yoshida et al., 1968) and neuromuscular transmission (Nayler et al., 1970; Kakiuchi et al., 1969). In nearly all these systems excitation of the cell is followed by a rise in cyclic AMP. This is usually accompanied by either an increased uptake of Ca^{++} by the cell, an absolute requirement for Ca^{++} in the external medium or both, in order for the normal physiological response to be observed (Douglas, 1968). Rasmussen (1970) proposed a hypothesis to explain the possible relationship between Ca^{++} and cyclic AMP. In this scheme Ca^{++} and cyclic AMP act as interrelated intracellular messengers. Activation of the cell leads to an increase in both intracellular cyclic AMP and Ca^{++}. The increase in cyclic AMP activates a phosphorylating enzyme or enzymes, the protein kinases. The

products of the protein kinase reaction are now sensitive to Ca^{++}. Their activation by Ca^{++} leads to either enzymatic activation, to a change in their structure (for example, contraction) to a change in their interations with the cellular constitutents (for example, Ca^{++} bridges between phosphate groups on vesicle or membranes or both) or to the determination of the membrane transport of Ca^{++}.

SUMMARY

Cyclic AMP was discovered in the course of investigations into the mechanism of the hyperglycemic action of adrenaline and glucagon. Since this discovery, evidence has accumulated indicating that this nucleotide is an intermediate in the action of a wide variety of hormones. The enzyme adenyl cyclase, which catalyzes cyclic AMP formation, and PDE, which inactivates it, has been found in all cell types thus far studied with the exception of the dog erythrocytes. A second messenger hypothesis has been proposed to explain the biological role of cyclic AMP hormone mediation. A further extension of this hypothesis to explain the role of Ca^{++} and protein kinases in the final response has recently been proposed. Further investigation of this latter system will lead to a better understanding of the role of cyclic AMP in hormone action.

PART 2 METHODS USED TO DETERMINE ADENOSINE 3',5'-MONOPHOSPHATE ACCUMULATION AND PHOSPHODIESTERASE ACTIVITY

A. METHODS OF DETERMINING CYCLIC AMP ACCUMULATION.

The many different methods available for measuring cyclic AMP in tissues reflects the difficulty of devising a simple assay procedure of high sensitivity and reliability. The analytical difficulties arise because of the extremely low concentrations of cyclic AMP in most mammalian tissues and the similarity of the cyclic nucleotide to other naturally occurring nucleotides present in several hundred to several thousand times the concentration of cyclic AMP (Goldberg et al., 1969).

a) Determination of endogenous tissue concentrations of cyclic AMP.

i) Phosphorylase kinase activation. This assay, introduced by Rall and Sutherland (1958), was based on the ability of cyclic AMP to activate liver glycogen phosphorylase by stimulating the activation of phosphorylase b kinase. Tissue extracts were added to a solution of $MgSO_4$, ATP, caffeine and Tris, pH 7.5. The reaction was started by the addition of an 11,000 x g supernatant fraction of dog liver homogenate, containing the phosphorylase b kinase and inactive glycogen phosphorylase. After a short incubation period the activation of glycogen phosphorylase was assayed by the addition of a solution of glucose 1-phosphate (glucose-1-P), NaF and glycogen. The reaction was terminated by the transfer of an aliquot to a solution containing I_2 and KI. After suitable dilution, the intensity of the resulting glycogen-iodine colour was measured in a colorimeter at 540 nm. The system has been used successfully to estimate cyclic AMP at concentrations as low as 5×10^{-7} M (Rall and Sutherland, 1961).

A number of modifications were subsequently introduced to increase the accuracy of this method. Enzymatic digestion of the heated tissue extract (Butcher et al., 1960), followed by ion-exchange chromatography (Haynes, 1958; Butcher et al., 1965) to purify the cyclic AMP, eliminated many potential contaminants prior to assay. The sensitivity of the assay was increased to 10^{-9} M by preincubating the total system involved in the first stage of the assay at $4^{\circ}C$ for 30 minutes and adding glycogen to this solution (Butcher et al., 1965).

ii) Measurement of cyclic AMP by enzymatic conversion to ATP. This method of measuring endogenous levels of cyclic AMP in intact mammalian tissues was developed by Breckenridge (1965). It was based on the enzymatic conversion of cyclic AMP to ATP and the subsequent augmentation of the triphosphate formed by enzymic cycling. In the first step, endogenous nucleoside phosphates present in the tissue extracts were degraded by apyrase and alkaline phosphatase. The phosphatases were then destroyed by digestion with pepsin. Cyclic AMP was converted to AMP by PDE and the AMP stoichiometrically converted to ATP in reactions catalyzed by adenylate kinase and pyruvate kinase. The ATP formed served as the rate-limiting component in the enzymatic dismutation between the hexokinase and pyruvate kinase reactions. The ultimate product, glucose 6-phosphate (glucose-6-P), was formed in millimolar concentrations and could be measured fluorimetrically or spectrophotometrically with TPN^{+} and glucose-6-P dehydrogenase. Sensitivity of the method was calculated as 2×10^{-8} M. In a modification of this procedure by Goldberg et al. (1969) cyclic AMP in tissue extracts was separated from 5'-adenosine nucleotides and other adenylate containing compounds by thin layer chromatography. This was considered superior to the

use of apyrase and alkaline phosphatase since it also eliminated inorganic phosphate and adenosine, two relatively potent inhibitors of the subsequent cycling reactions.

Johnson et al. (1970) described a method in which the ATP produced by the enzymatic conversion of cyclic AMP was determined by its luminescent reaction with firefly luciferase; cyclic AMP was converted to ATP according to Breckenridge (1965). An aliquot of this mixture was then injected into a solution containing $MgSO_4$, dithiothreitol, crystalline bovine serum albumin, crystalline luciferin and crystalline firefly (Photinus pyralis) luciferase, and the determination of ATP made with a luminescence biometer. Constant activity of luciferase could be maintained only by the inclusion of both bovine serum albumin and dithiothreitol in the luciferase reaction mixture. The response was linear with ATP concentration over 5 orders of magnitude with a maximum sensitivity of 2×10^{-11} M ATP. The following advantages of this method of measurement of ATP were cited by the authors: 1) firefly luciferase has been demonstrated to be highly specific for ATP (Hastings, 1968); 2) both crystalline luciferase and crystalline luciferin could be purchased from commercial sources; and 3) the sensitivity of determination of ATP made cycling reactions unnecessary and, accordingly, the assay involved fewer total steps.

iii) Measurement of cyclic AMP by chromatographic procedures. Bradham and Woolley (1964) described a spectrophotometric method for the quantitative determination of cyclic AMP formed in enzyme reaction mixtures. This method utilized ion-exchange column chromatography to remove impurities present in such mixtures, which interfere with the determination of cyclic AMP. The nucleotide was measured by ultraviolet absorbancy spectroscopy

at 260 nm. At this wavelength only one peak of absorption was observed and, when the material contained in this band was subjected to paper electrophoresis, only cyclic AMP was detected. This method was accurate for analysis of samples containing greater than 3×10^{-6} M cyclic AMP and was therefore less sensitive than other methods available.

Krishna (1968) reported a sensitive gas-liquid chromatography method for measuring nmole amounts of cyclic AMP as the trimethylsilyl ether. The application of this method to tissue sources has yet to be described.

b) Radioactive determinations of cyclic AMP.

i) Using labelled substrates. Streeto and Reddy (1967) described a method in which ^{14}C-ATP was used as substrate for adenyl cyclase in particulate fractions of various tissues. Following termination of the reaction, ^{3}H-labelled cyclic AMP was added and the mixture centrifuged. An aliquot of the supernatant was subjected to paper chromatography and, following a suitable development time, the cyclic AMP was located by u.v. light. This segment was counted for both ^{3}H and ^{14}C using double label counting techniques and the ^{14}C counts corrected to 100% on the basis of the ^{3}H recovery. This procedure separated several possible contaminants from cyclic AMP as the R_f values of ADP, ATP, AMP, adenine and caffeine in the solvent systems used were very different from that of cyclic AMP. This method could measure the formation of fractions of a nmole of cyclic AMP, the limit of sensitivity dependent on the specific activity of the ATP used.

Radioactive cyclic AMP determination by paper chromatographic isolation was the basis of methods described by Brodie et al. (1966); Rabinowitz et al.

(1965); Ho et al. (1967); Hirata and Hayaishi (1965) and Dousa and Rychlik (1970b), following essentially the same procedure as that outlined above.

Jungas (1966) reported a chromatographic assay of cyclic AMP in which AMP present in the assay medium was first removed by ion-exchange column chromatography. Cyclic AMP present in a sample of this eluant was then separated from other substances by thin layer chromatography.

Bär and Hechter (1969a) reported that attempts to separate cyclic AMP formed from ^{3}H, ^{14}C, ^{32}P-ATP and other by-products by one-dimensional chromatography invariably showed the cyclic AMP spot associated with high counting impurities. These overlapping contaminants could be reduced in part by two-dimensional cellulose thin layer chromatography using a variety of solvent systems. Anion-exchange thin layer chromatography was also found suitable for measuring trace amounts of cyclic AMP.

Krishna et al. (1968) described an assay for adenyl cyclase in which radioactive cyclic AMP formed from radioactive ATP was isolated by column chromatography followed by precipitation of all nucleotides and inorganic phosphates still present by $ZnSO_4$-$Ba(OH)_2$, leaving most of the cyclic AMP in the supernatant fluid. An aliquot of the supernatant fluid was then measured for radioactivity. Electrophoretic procedures verified that the radioactivity in the aliquot was cyclic AMP. The authors state that the method was sensitive enough to detect a 0.005% conversion of ATP to cyclic AMP with more than 70% of the cyclic nucleotide being recovered in relatively small volumes. The speed of the method was also cited as a desirable feature; the entire procedure could be completed in less than 3 hours.

When radioactive cyclic AMP determinations were applied to intact tissue preparations, radioactive adenine was used instead of ATP (Humes et al., 1969; Shimizu et al., 1969; Kuo and Renzo, 1969). Adenine freely penetrated the cells and, in the presence of glucose, was converted to ATP. Sufficient intracellular levels of radioactive ATP were produced so that radioactive cyclic AMP subsequently accumulated could be measured by any of the methods described above.

ii) Exchange reactions. In these methods endogenous cyclic AMP was measured by exchange reactions involving radioactive substances.

In a method by Turtle and Kipnis (1967), cyclic AMP in tissue extracts was first separated from other nucleotides by thin layer chromatography, then converted to AMP with PDE. In the presence of γ-labelled ^{32}P-ATP and myokinase the AMP was converted to ^{32}P-ADP which was then separated by thin layer chromatography and counted. In the method of Pauk and Reddy (1967) cyclic AMP formed in tissue homogenates was isolated by column and paper chromatography and then esterified with ^{3}H-labelled acetic anhydride via an acetylimidazole intermediate to form adenosine 3',5'-monophosphate 2'-acetate. This derivative was isolated by paper chromatography and counted. The high sensitivity of this procedure, 10^{-10} M, was due to the use of acetic anhydride of high specific activity.

A method described by Aurbach and Houston (1968) separated cyclic AMP from contaminating adenine nucleotides by column chromatography and $ZnSO_4$-$Ba(OH)_2$ precipitation. The cyclic AMP obtained was then hydrolyzed to AMP and subsequently converted to ATP as previously described (Breckenridge, 1965). The ATP was then measured by a radioactive phosphate exchange

reaction with the coupled enzymes phosphoglycerate kinase-glyceraldehyde phosphate dehydrogenase. The extent of the ^{32}P-ATP exchange reaction was linearly related to the amount of cyclic AMP present in the original sample between the range of 0.6 pmoles and 0.25 nmoles. In a method by Kaneko and Field (1969) ATP formed from cyclic AMP was used for the formation of ^{14}C-glucose-6-P from ^{14}C-glucose-1-P in an enzymic recycling system between hexokinase and pyruvate kinase. $^{14}CO_2$ evolved in a reaction with TPN^+, glucose-6-P dehydrogenase and 6-phosphogluconate dehydrogenase was then counted. The production of $^{14}CO_2$ under the standard conditions used was proportional to cyclic AMP concentration up to 100 pmoles.

iii) Radioisotope displacement. In 1968 Brooker et al. introduced radioisotope displacement, a modification of the isotope dilution principle, as a useful method for cyclic AMP assay; the rate of conversion of labelled cyclic AMP to AMP by PDE would be reduced by the introduction of nonradioactive cyclic AMP. Sufficient PDE preparation was added to a liquid scintillation vial to hydrolyze 30-40% of the ^{3}H-cyclic AMP present, and cyclic AMP isolated from tissue samples by ion-exchange column chromatography was added to this reaction mixture. AMP produced by the reaction of cyclic AMP with the PDE preparation was converted to the nucleoside by 5'-nucleotidase and separated from the unreacted substrate by treatment with anion-exchange resin; the unreacted nucleotide substrate was adsorbed by the resin and quenched, while the nucleoside produced was not bound by the resin and was detected in the liquid scintillation counting process. Numerical values were then assigned by reference to a standard curve. Results with this one-step method could be obtained within 1 hour after purification of the tissue sample.

iv) Radioimmunoassay of cyclic AMP. Steiner et al. (1969) described a method based on the competition of cyclic AMP with a labelled derivative of high specific activity for binding sites on an antibody specific for the cyclic nucleotide. The antibody to cyclic AMP was obtained by immunizing rabbits with an antigen prepared by conjugating succinyl cyclic AMP with human serum albumin. A high specific activity derivative of cyclic AMP was prepared by synthesizing succinyl cyclic AMP tyrosine methyl ester (SCAMP-TME) and iodinating the phenolic hydroxyl group of the tyrosine moiety with ^{125}I (^{125}I-SCAMP-TME). Free and antibody bound-^{125}I-SCAMP-TME were separated by precipitating the antibody bound-fraction with a second antibody. Displacement of ^{125}I-SCAMP-TME by unlabelled cyclic AMP, when plotted as a semi-logarithmic function, was linear over a concentration range of 2-100 pmoles. The specificity of the antibody was tested against structurally related nucleotides, nucleosides and purine bases; all had less than 0.005% of the potency of cyclic AMP in inhibiting ^{125}I-SCAMP-TME binding. The assay was thus sufficiently specific for cyclic AMP to eliminate the need for prior chromatographic separation of the cyclic nucleotide from other tissue nucleotides. This method permitted measurement of a large number of samples using as little as 10-20 mg tissue.

v) Protein binding assay of cyclic AMP. Gilman (1970) developed an assay that was based on competition for protein binding of the nucleotide to a cyclic AMP-dependent protein kinase from bovine muscle. A heat-stable protein, an inhibitor of the cyclic AMP-dependent protein kinase which increased the affinity of the cyclic nucleotide for the enzyme, was also prepared from bovine muscle. The standard reaction occurred in the presence of binding protein, saturating concentrations of ^{3}H-cyclic AMP and a maximally

effective concentration of the protein kinase inhibitor preparation. The amount of unknown cyclic AMP was then determined from the linear decrease in the total protein kinase-bound ^{3}H-cyclic AMP, defined by counting the nucleotide protein complex which was subsequently adsorbed to a millipore filter. The advantages of this method cited were: 1) as little as 0.10 pmole of cyclic AMP could be detected; 2) tissue purification was unnecessary due to the high specificity of the reaction; 3) the actual assay was very simple to perform, and 4) preparation of the two fractions required was not difficult and a preparation of each from 1 kg muscle yielded sufficient material for hundreds of assays.

B. METHODS OF DETERMINING PHOSPHODIESTERASE ACTIVITY.

a) Measurement of inorganic phosphate. Butcher and Sutherland (1962) described a method for PDE determination which measured inorganic phosphate (P_i) released from AMP produced following incubation of cyclic AMP with PDE preparations. Excess of 5'-nucleotidase from snake venom was used to hydrolyze the AMP, and P_i was measured spectrophotometrically by the method of Fiske and SubbaRow (1925). This method has been used by Cheung (1967), Müller-Oerlinghausen et al. (1968) and many others.

b) Measurement of AMP. Bär and Hechter (1969a) evaluated PDE activity by using carrier free ^{3}H-cyclic AMP as substrate and counting ^{3}H-AMP isolated by paper chromatography. Essentially the same procedure was used by Ho et al. (1967) and by Gilman and Rall (1968).

Cheung (1966) described a method for PDE determination in which AMP was assayed using the coupled reaction of myokinase, pyruvate kinase and lactate dehydrogenase (Adams, 1963).

c) Measurement of IMP. Drummond and Perrott-Yee (1961) described a method based on the conversion of AMP to 5'-IMP by adenylic acid deaminase. IMP has an absorbancy spectrum different from that of other nucleotides and thus afforded a spectrophotometric assay for PDE activity free from contamination.

d) Titrimetric assay. A method of PDE assay was recently described by Cheung (1969a) in which the rate of cyclic AMP hydrolysis was monitored by continuous titration. The hydrolysis of cyclic AMP to AMP was accompanied by a stoichiometric generation of protons and the rate of addition of an alkaline solution to the reaction mixture necessary to maintain a constant pH was thus stoichiometrically related to the rate of cyclic AMP hydrolysis. The results obtained with this method correlated well with results obtained with the conventional inorganic phosphate procedure, and the sensitivity of the two methods was essentially the same.

SUMMARY

The difficulty in measuring trace amounts of cyclic AMP can best be illustrated by the very large number of methods for cyclic AMP determination reported, none of which have found general acceptance. The original and most commonly used method for endogenous cyclic AMP determination based on glycogen phosphorylase activation (Rall and Sutherland, 1958), is less than ideal from the standpoint of absolute standardization, sensitivity and specificity. Potential activators of phosphorylase b kinase such as glycogen, heparin (Krebs et al., 1964) and Ca^{++} (Mayer et al., 1963) may be present in high enough concentrations in certain tissues to interfere with this assay (Aurbach and Houston, 1968). Although preliminary fractionation by ion-

exchange column chromatography (Butcher et al., 1965) has minimized the risk of contamination by these substances, Murad (1965) has reported a substance not separated by this technique which interfered with the assay. The coupled enzyme assay of Breckenridge (1965) presents the same problem; at each step one or more of the enzymes involved might be subject to interference by other substances that may be present in the complex mixtures used (Goldberg et al., 1969).

Attempts to separate cyclic AMP from other substances by one-dimensional thin layer or paper chromatographic methods have frequently shown the cyclic AMP spot to be associated with contaminants. Bär and Hechter (1969a) reviewed these systems and discarded them as impractical for the quantitative separation of such mixtures. These workers have found that ion-exchange chromatography allowed a more complete separation of cyclic AMP from impurities. Two-dimensional thin layer chromatography (Bär and Hechter, 1969a), in a variety of solvent systems, separated many of the contaminants from the cyclic AMP spot though adding considerable time to the assay procedure. With these thin layer and paper chromatographic methods it is also possible to separate ADP, ATP and AMP but this involves introduction of different solvent systems and possibly, rechromatography; in the procedure of Dousa and Rychlik (1970b) obtaining these substances in pure form involved the addition of more than 40 hours to the cyclic AMP assay procedure.

The Krishna method (Krishna et al., 1968) has been widely used. Bär and Hechter (1969a) reported that a significant number of counts were found, probably AMP, contaminating the cyclic AMP fraction prepared by this method; the amount of authentic cyclic AMP varied from 10 to 100% with a mean of 62%. Humes et al. (1969) reported the same difficulty with this method, the most

unreliable results being associated with low rates of cyclic AMP formation.

The phosphate exchange method of Aurbach and Houston (1968) appears rapid and specific and is linear over a wide range of cyclic AMP concentrations. This method, however, is less sensitive than others described. The steps required to separate cyclic AMP from contaminants are subject to the same criticism as the Breckenridge method (1965) although the authors claim that the use of the enzymes chosen afford specificity.

The isotope derivative method of Pauk and Reddy (1967) has been criticized (Aurbach and Houston, 1968) as being too cumbersome and insensitive and has not received wide application.

The isotope displacement method of Brooker et al. (1968) is receiving extensive trials. It is theoretically sound and deserves consideration as a useful method. The immunoassay method of Steiner et al. (1969) is also theoretically sound, although difficulty with preparing the necessary cyclic AMP derivatives and antibodies may limit its acceptance.

The method of Gilman (1970) involving competition for protein binding of the nucleotide on a cyclic AMP-dependent protein kinase probably will receive considerable attention in the future. It is a simple method with the protein kinase and the inhibitor protein easily prepared. The method is highly sensitive and, most important, sample purification by chromatographic separations prior to assay is unnecessary.

The most widely accepted PDE assay is the measurement of P_i produced from AMP by treatment with nucleotidase. Measurement of cyclic AMP destruction by the isolation of the AMP produced by thin layer or paper chromatography is a useful, though more time consuming, method and is only applicable if AMP is not

further hydrolyzed by a nucleotidase present in the assay medium. The measurement of IMP as a useful PDE assay has not received wide acceptance since it lacks sensitivity. The titrimetric assay of Cheung (1969a) is relatively new and has yet to receive extensive trials.

PART 3 PREPARATIONS OF ADENYL CYCLASE USED TO STUDY ADENOSINE 3',5'-MONOPHOSPHATE METABOLISM

Attempts to obtain mammalian adenyl cyclase in a highly purified form have been unsuccessful. The purification of this enzyme has been hampered by its association with particulate material and its ready inactivation by various procedures (Sutherland et al., 1962; Levey, 1970).

To date, five types of adenyl cyclase preparations have been used in the study of cyclic AMP metabolism.

A. LOW-SPEED PARTICULATE PREPARATIONS.

These were the earliest preparations in which cyclic AMP metabolism was studied. In this procedure, tissues were homogenized and centrifuged at 600-2,000 x g for 10-20 minutes, in either isotonic or hypotonic medium. The resulting pellet was resuspended and used for adenyl cyclase assay.

Sutherland and colleagues (Rall and Sutherland, 1958; Sutherland and Rall, 1958; Murad et al., 1962; Klainer et al., 1962; Sutherland et al., 1962) used this preparation in most of their early work in various animals to show NaF stimulation of cyclic AMP accumulation in liver, heart, skeletal muscle and brain, and adrenaline and glucagon stimulation of cyclic AMP accumulation in liver, heart and brain. Many studies using particulate preparations have confirmed these findings and extended them to other tissues. Work with this preparation has shown that adenyl cyclase responds to the same hormones that are effective in the corresponding intact tissue.

B. SLICE PREPARATIONS.

In these preparations the tissues to be studied were excised and slices of varying thickness prepared. The slices were then incubated in Krebs

medium or a similar solution. When a radioactive cyclic AMP assay was to be used radioactive adenine, in the presence of glucose, was pre-incubated with the preparation for a suitable time period. The stimulant was then added to the medium and cyclic AMP accumulation subsequently assayed.

Butcher et al. (1965) employed this preparation in studies with the rat epididymal fat pad and found cyclic AMP accumulation was increased by adrenaline. Dichloroisoproterenol (DCI) and phentolamine antagonized the effect of adrenaline. This preparation was used to show hormone stimulation in dog liver slices (Sutherland et al., 1965), dog kidney slices (Brown et al., 1963), beef and sheep thyroid slices (Gilman and Rall, 1968; Burke, 1970a) and other tissues. A useful application of these intact cell preparations was the ability to correlate cyclic AMP accumulation in these tissues with a physiological response, for example glycogen phosphorylase activation in the liver and heart (Sutherland et al., 1965; Namm et al., 1968; Namm and Mayer, 1968) and free fatty acid and glycerol release from the epididymal fat pad (Jungas, 1966; Butcher and Sutherland, 1967).

C. SOLUBLE PREPARATIONS

Sutherland et al. (1962) reported that particulate preparations from beef brain and heart and dog skeletal muscle, partially solubilized by treatment with the detergent triton-X-100, did not respond to adrenaline with increased formation of cyclic AMP. Addition of 10 mM NaF, however, produced an increase in cyclic AMP accumulation.

Levey (1970) found that the non-ionic detergent Lubrol-PX solubilized approximately 90-100% of the adenyl cyclase in cat heart homogenates. This preparation was responsive to NaF but not to the hormones that activated

particulate preparations of adenyl cyclase from heart.

D. ISOLATED CELL PREPARATIONS.

There have been two isolated cell preparations widely used in the study of cyclic AMP metabolism, the isolated rat fat cell preparation of Rodbell (1964) and the rat fat cell ghost preparation of Rodbell (1967). Using these preparations adenyl cyclase has been further characterized, in terms of hormone specificity and ion requirements.

a) Isolated fat cell preparation. Epididymal fat pads were removed and cut into small pieces. The pieces were further dispersed into small fragments by incubation with collagenase. Fat cells were liberated from the tissue fragments by gentle stirring. Fragments of tissue still remaining were removed and the cell suspension centrifuged at 400 x g for 1 minute. The fat cells floated to the surface and were removed, washed, centrifuged again at 400 x g for 1 minute and resuspended in incubation medium.

Butcher et al. (1968a,b) and Butcher and Baird (1968) found that cyclic AMP accumulation in the isolated fat cell preparation was stimulated by adrenaline, ACTH, TSH, glucagon and LH. When adrenaline in supramaximal concentration was added in combination with excess ACTH, glucagon or TSH, the resulting cyclic AMP accumulation was no greater than that with adrenaline alone, indicating that the various hormones were all stimulating a single cyclase system. The β-blocking agent pronethalol antagonized the action of adrenaline on cyclic AMP accumulation, but had no effect on ACTH or glucagon stimulation. Insulin and prostaglandin E_1, both shown to be inhibitors of lipolysis in the epididymal fat pad (Butcher and Sutherland, 1967; Steinberg and Vaughan, 1967), decreased cyclic AMP levels in fat cells exposed to

adrenaline, ACTH and glucagon. Nicotinic acid, also an inhibitor of lipolysis (Carlson and Bally, 1965), virtually eliminated adrenaline-induced cyclic AMP accumulation in the isolated fat cell preparation. Williams et al. (1968) studied the effects of metal ions on the accumulation of cyclic AMP in this preparation. They noted that little cyclic AMP was formed when no metal ions were added. The addition of 2.8 mM Mg^{++} significantly increased cyclic AMP accumulation. The degree of effectiveness of Mg^{++} in increasing cyclic AMP accumulation depended upon its concentration and its ratio to ATP present. Ca^{++}, 2.5 mM, inhibited cyclic AMP accumulation in the presence of 2.8 mM Mg^{++}. K^{+}, 4 mM, had little effect on cyclic AMP accumulation whether used alone or in the presence of Mg^{++}. NaF, 10 mM, produced no accumulation of cyclic AMP when no metal ions were present but caused a 10-fold increase in the presence of 2.8 mM Mg. NaF in the presence of Ca^{++}, 2.5 mM, caused no cyclic AMP accumulation, while in the presence of 2.8 mM Mg^{++} plus 2.5 mM Ca^{++} it produced a slight increase. Isopropylnoradrenaline augmented Mg^{++} (2.8 mM) increased cyclic AMP accumulation but its effect was abolished in the presence of 2.8 mM Ca^{++}. Kuo (1970) found that ACTH stimulated cyclic AMP accumulation in this preparation only in the presence of Ca^{++}. TSH and LH stimulation of cyclic AMP accumulation was augmented by Ca^{++} but glucagon and noradrenaline stimulation was unaffected. The addition of 2 mM EDTA in the presence of 0.6 mM Ca^{++} abolished the stimulatory effect of ACTH, TSH and LH on cyclic AMP accumulation but did not affect noradrenaline and glucagon stimulation.

b) Isolated fat cell ghosts. The object of this procedure was to isolate the plasma membrane of the fat cell in a form that would permit study

of the action of hormones on adenyl cyclase, in the absence of cytoplasmic components that could modify events occurring at the cell membrane. Isolated fat cells were incubated in hypotonic medium and underwent swelling. The cells were lysed by gentle agitation and the plasma membrane sacs ("ghosts") separated from most of the other particulate components of the cell by centrifugation at 900 x g.

It was shown that all hormones that stimulated cyclic AMP accumulation in isolated fat cells stimulated cyclic AMP accumulation in fat cell ghosts (Birnbaumer and Rodbell, 1969; Rodbell, 1967). Secretin (Rodbell et al., 1970) was also shown to stimulate cyclic AMP accumulation in this preparation. Bär and Hechter (1969b) and Birnbaumer and Rodbell (1969) showed that a combination of maximal doses of hormones failed to produce additive effects. Further studies were done to discern whether the fat cell possessed multiple adenyl cyclase enzyme systems with distinctive specificities for individual hormones, or a single cyclase system with broad specificity to a variety of hormones. In studies by Bär and Hechter (1969b) β-blocking drugs abolished adrenaline-induced cyclic AMP accumulation while not affecting the response to ACTH or glucagon. A minimal concentration of 0.1 μM Ca^{++} was required for ACTH stimulation of cyclic AMP accumulation, whereas glucagon and adrenaline exhibited no Ca^{++} requirement. Braun and Hechter (1970) illustrated that fat cell ghost preparations of adenyl cyclase from hypophysectomized and adrenalectomized rats had a decreased sensitivity to ACTH while the stimulating effects of other hormones were unchanged. Pretreating the rats with dexamethasone, a synthetic glucocorticoid, restored the response to ACTH. This effect of dexamethasone could be blocked by simultaneous administration of actinomycin D, a known inhibitor of protein synthesis.

Rodbell et al. (1970) showed that preincubation of fat cells with trypsin inhibited subsequent glucagon-induced cyclic AMP accumulation in fat cell ghosts, but did not affect the stimulation by adrenaline or NaF and only slightly affected the stimulatory effect of ACTH and secretin. Braun and Hechter (1970) and Rodbell et al. (1970) concluded from these studies that distinctive selectivity units for individual hormones were coupled to the same unit of adenyl cyclase in the fat cell membrane.

Birnbaumer et al. (1969) studied the effect of monovalent and divalent cations on cyclic AMP accumulation in the isolated fat cell ghost preparation in the presence and absence of ACTH and NaF. Li^+, 0.1 mM, inhibited ACTH and NaF stimulated activity while increasing basal activity. Na^+ and K^+ at 0.1 M also stimulated basal activity but had no significant effect on NaF stimulated activity. K^+, 0.01 M, slightly increased ACTH-induced cyclic AMP accumulation while 0.1 M Na^+ and 0.01 M K^+ together decreased ACTH activity and increased basal activity. Mn^{++}, at 5 mM, increased basal and NaF stimulated activity with no significant effect on ACTH stimulated cyclic AMP accumulation. The same amount of ACTH-induced cyclic AMP accumulation was observed with either Mn^{++}, Mg^{++} or a combination of these ions. Co^{++}, 5 mM, partially replaced Mg^{++}, though all activities were reduced relative to Mg^{++} alone. The most pronounced reduction in the presence of Co^{++} was the ACTH stimulated activity either in the absence or presence of 5 mM Mg^{++}. No significant adenyl cyclase activity was observed in the presence of Ca^{++}, Cu^{++} or Zn^{++}. Half maximal inhibition of Mg^{++}-dependent basal activity occurred with 2 mM Ca^{++}. ACTH and NaF stimulated activities were inhibited by lower Ca^{++} concentrations. It was found that concentrations of ATP exceeding that of Mg^{++} resulted in inhibition of adenyl cyclase

activity under all conditions tested. In no case did NaF or ACTH modify the apparent Km for ATP, indicating that the stimulatory effects of these substances could not be due to modification of the affinity of the enzyme for Mg-ATP at the catalytic site. Variation of the Mg^{++} concentration in the incubation medium at fixed concentrations of ATP revealed that ACTH and NaF increased the apparent affinity of adenyl cyclase for Mg^{++} 5- to 6-fold. In the absence of either ACTH or NaF, Mg^{++} had a significant stimulatory effect on the activity of the enzyme. No saturation of basal activity could be observed even when the Mg^{++} concentration exceeded 13 times that of ATP. As a consequence, high concentrations of Mg^{++} (80 mM) could substitute almost completely for ACTH or NaF in stimulating adenyl cyclase activity.

E. HIGH-SPEED PARTICULATE PREPARATIONS

Marinetti et al. (1969) prepared purified rat liver plasma membrane fractions using the method of Coleman et al. (1967). Livers were homogenized in isotonic sucrose and centrifuged at 1,000 x g for 10 minutes. The washed pellet was homogenized again and centrifuged at 1,000 x g. The resultant supernatant was then centrifuged at 3,000 x g for 10 minutes and the supernatant obtained by this procedure centrifuged at 21,000 x g for 30 minutes. The top portion of the resulting pellet was layered onto a discontinuous gradient of sucrose (density 1.13, 1.16, 1.18) and centrifuged at 100,000 x g for 2 hours. The material at the top of the 1.13 layer was collected as the final highly purified plasma membrane preparation.

Cyclic AMP accumulation in this preparation was stimulated by glucagon and adrenaline. The addition of 0.5 mM Ca^{++} to the preparation medium

increased cyclic AMP accumulation in controls as well as in the presence of adrenaline. A minimal concentration of 0.01 mM Ca^{++} was found necessary for the adrenaline stimulatory effect, whereas the stimulation of cyclic AMP accumulation by glucagon was reduced in the presence of Ca^{++}. NaF, from 1-10 mM, inhibited cyclic AMP accumulation in this preparation.

Pohl et al. (1969) isolated a highly purified plasma membrane preparation from rat liver parenchymal cells following the procedure of Neville (1968). In this preparation, livers were homogenized in hypotonic medium and centrifuged at 1,500 x g for 10 minutes. The washed pellet was rehomogenized and placed in 69% sucrose, overlaid with 42% sucrose and centrifuged at 90,000 x g for 2 hours. The pellet was suspended and placed onto a density gradient consisting of 3-37% sucrose on a "cushion" of 50% sucrose and centrifuged at 550 x g for 1 hour. The material at the 37% interface was removed for use.

In this study glucagon caused a significant stimulation of cyclic AMP accumulation while adrenaline did not. The lack of an adrenaline effect was ascribed to a selective destruction of the adrenaline stimulatory adenyl cyclase during the course of the preparation.

Entman et al. (1969) found that cyclic AMP accumulation in a microsomal fraction of dog heart, obtained by differential centrifugation using a 20-35% sucrose gradient as the final purification step, was significantly stimulated by 8 mM NaF and 0.5 mM noradrenaline.

Taunton et al. (1967) studied the adenyl cyclase activity of various particulate fractions of steroid-producing mouse adrenal tumours. All particulate fractions, including the 105,000 x g fraction, contained significant ability to accumulate cyclic AMP that was augmented by 10 mM NaF and 0.3 μM ACTH.

Burke (1970b) showed that a fraction of sheep thyroid homogenates obtained by 100,000 x g centrifugation in isotonic sucrose responded to 10 mM NaF with a 10-fold increase in cyclic AMP accumulation. No effects of hormones were reported in this study.

SUMMARY

The majority of studies on cyclic AMP accumulation have been done in low-speed particulate preparations. The results with these preparations showed the specificity of adenyl cyclase, in that only hormones capable of producing a physiological response in a given tissue, presumably through adenyl cyclase activation, stimulated cyclic AMP accumulation in that tissue. A slight variation in the molecular configuration of the enzyme system could account for this tissue specificity. The obvious imperfections of the low-speed particulate preparations, for example mitochondrial contamination and heterogeneous cell types, did not allow for any further characterization of adenyl cyclase.

The slice preparations also lack homogeneity, although investigations of possible relationships between changes in cyclic AMP concentration and physiological effects have made these preparations valuable research tools.

The relatively pure isolated fat cell and fat cell ghost preparations have afforded most of the information available on the nature of the adenyl cyclase enzyme, its specificity, ion requirements and relation to substrate. A multitude of hormones stimulate cyclic AMP accumulation in these preparations, which allows a comparative study of the parameters for cyclic AMP accumulation by these various substances to be made. These studies have shown that adenyl cyclase may possess at least three functionally distinguishable receptor

sites, that share a common catalytic site.

There are few reports in the literature describing hormone activation of high-speed particulate preparations of adenyl cyclase. This might be due to the difficulty in maintaining hormone responsiveness in these preparations (Robison, personal communication). The same is true for attempts to solubilize adenyl cyclase. As previously mentioned, adenyl cyclase has been shown to be bound to membranes and is thought to be localized in the plasma membrane (Davoren and Sutherland, 1963). In a study by Rodbell et al. (1968) phospholipase C was found to inactivate the enzyme. These studies suggest that the activity of adenyl cyclase is dependent on the membrane structure in which it is situated, that it itself is a complex lipoprotein or both (Birnbaumer et al., 1969). Purification or solubilization therefore probably destroy some aspect of this membrane-enzyme complex necessary for hormone stimulation. Why NaF-stimulated cyclic AMP accumulation appears little affected by these techniques remains unanswered.

PART 4 ADENOSINE 3',5'-MONOPHOSPHATE METABOLISM IN BRAIN

In a survey of the adenyl cyclase activity of various tissues, Sutherland et al. (1962) found the highest activity in particulate preparations of mammalian brain. Klainer et al. (1962) in this same preparation showed that adrenaline stimulated the accumulation of cyclic AMP as did isopropyl noradrenaline and noradrenaline. The very active adenyl cyclase in brain, coupled with the stimulatory effect of catecholamines, suggested some physiological role for cyclic AMP in CNS function. During the past few years extensive work has been carried out to further characterize cyclic AMP metabolism in brain.

A. REGIONAL AND SUBCELLULAR DISTRIBUTION OF ADENYL CYCLASE AND PHOSPHODIESTERASE IN BRAIN.

Weiss and Costa (1968) studied the distribution of adenyl cyclase and PDE in various regions of rat brain and found that enzyme activity was higher in grey matter (cerebral cortex) than in white matter (pons, medulla, and spinal cord). They found no correlation between the relative activities of adenyl cyclase and PDE in the various areas, although in most instances PDE activity exceeded the adenyl cyclase activity by almost 100 fold. It was also found that the distribution pattern of adenyl cyclase was different from that of the catecholamine concentration in the brain. Differential centrifugation of rat cerebellar homogenates indicated that most of the adenyl cyclase was particulate and occurred in the mitochondrial and microsomal fractions whereas most of the PDE was soluble. Drummond and Perrott-Yee (1961) found that PDE activity in rabbit brain homogenates was localized entirely in the 100,000 x g supernatant. De Robertis et al. (1967) and Voigt and Krishna

(1967) reported that 65% of the PDE activity and essentially all of the adenyl cyclase activity in rat cerebral cortex was particulate; the enzymes associated primarily with subfractions containing nerve endings, specifically synaptic membrane fractions.

B. PROPERTIES OF BRAIN ADENYL CYCLASE

Early reports by Klainer et al. (1962) and Sutherland et al. (1962) using low-speed particulate preparations of rat cerebral cortex showed cyclic AMP accumulation was stimulated by adrenaline, isopropylnor-adrenaline, noradrenaline and NaF. Stimulation by 10 mM NaF was maximal, while the hormone effects were variable and low. In the absence of added hormones or NaF, cyclic AMP accumulation was high, and extensive washing and pretreatment with reserpine were introduced in attempts to lower the endogenous catecholamine concentration. These attempts failed to increase hormone stimulation of cyclic AMP accumulation in this preparation. In a study of similar preparations of calf cerebral cortex, Bradham et al. (1970) noted that 10 mM NaF produced a 5 fold increase in cyclic AMP accumulation. A Mg^{++} concentration of 5 mM and a theophylline concentration of 5 mM were necessary to produce maximum accumulation of cyclic AMP in the presence of 2 mM ATP. The inclusion of 0.1 mM EGTA reduced the amount of cyclic AMP accumulated in the reaction mixture by more than 60%. This inhibitory effect of EGTA was reversed by equimolar concentrations of Ca^{++}. When Ca^{++} was added to the reaction mixture in the absence of Mg^{++} no cyclic AMP was produced, nor was there any stimulatory effect of Ca^{++} when it was added to the complete reaction mixture at a concentration of 0.1 mM or less. Higher concentrations of Ca^{++} were found to be inhibitory. Almost 80% inhibition of cyclic AMP

accumulation was obtained when Ca^{++} was included in the complete reaction mixture at concentrations of between 0.5 mM and 1 mM. Atomic absorption analysis revealed that the mean concentration of Ca^{++} present in a typical reaction mixture as a contaminant was 0.033 mM. This concentration of Ca^{++} appeared necessary for maximal accumulation of cyclic AMP and chelation of these ions by EGTA resulted in inhibition of the formation of the nucleotide.

Perkins (1970) in studies on low-speed particulate preparations of rat cerebral cortex showed that an ATP concentration above the Mg^{++} concentration was inhibitory to cyclic AMP accumulation, indicating that the true substrate of adenyl cyclase was an ATP-Mg complex. A higher than 1:1 concentration ratio of Mg^{++} or Mn^{++} to ATP further stimulated cyclic AMP accumulation, perhaps by binding to a second site on the enzyme. Ca^{++} was found to stimulate adenyl cyclase at low concentrations (0.05 mM) but inhibited activity at higher concentrations (above 0.1 mM). Na^{+} and K^{+} in concentrations up to 0.1 mM had no significant effect on the enzyme while concentrations above this inhibited cyclic AMP accumulation. Stimulation by 10 mM NaF was accompanied by an increase in Vmax with no effect on the apparent Km for ATP observed. No stimulation of cyclic AMP accumulation was observed in the presence of noradrenaline, adrenaline, histamine or isopropylnoradrenaline. Cohen and Bitensky (1969) and Voigt and Krishna (1967) also failed to show hormone stimulation of cyclic AMP accumulation in similar preparations of rat and cat cerebral cortex.

Kakiuchi and Rall (1968a) studied rabbit cerebellar slices incubated in Krebs medium and glucose and noted an increase in cyclic AMP accumulation in

the presence of 0.1 mM noradrenaline. Histamine and serotonin, in 10 μM concentrations also stimulated this preparation but to a lesser degree. The effects of these agents on cyclic AMP accumulation were additive, indicating that they stimulated adenyl cyclase through independent regulatory units. Phenoxybenzamine and diphenhydramine inhibited histamine stimulation of cyclic AMP accumulation specifically while DCI prevented noradrenaline stimulation. Chlorpromazine blocked both histamine and noradrenaline-induced cyclic AMP accumulation in this preparation. In rabbit cerebral cortex slice preparations, these workers (Kakiuchi and Rall, 1968b) showed that histamine caused an 8-fold increase in cyclic AMP accumulation, and that inclusion of theophylline increased this effect 3-fold. Noradrenaline, 0.1 mM, produced a 70% increase in cyclic AMP accumulation in this preparation but serotonin, acetylcholine and dopamine all failed to change cyclic AMP levels significantly. Shimizu et al. (1969,1970a) confirmed these results and showed as well that histamine analogs were capable of stimulating cyclic AMP accumulation in this preparation. These workers found (1970b) that, whereas histamine was a potent stimulator of rabbit cerebral cortex preparations, it was ineffective in guinea-pig and rat preparations. Further work by Shimizu et al. (1970c) illustrated that the depolarizing agents ouabain (0.05 mM), batrachotoxin (0.002 mM), veratridine (0.08 mM) and high K^+ (43 mM) were all capable of significantly stimulating cyclic AMP formation in guinea-pig cerebral slice preparations. A number of findings indicated that these agents stimulated adenyl cyclase by a pathway different from that acted upon by histamine and adrenaline. These are: 1) the elevation of cyclic AMP levels by the depolarizing agents required the presence of Ca^{++} in the

reaction mixture, while that caused by the biogenic amines did not; 2) whereas theophylline potentiated cyclic AMP accumulation in the presence of biogenic amines, the stimulation elicited by the depolarizing agents was inhibited by theophylline, and 3) there was no additive effect of the depolarizing agents on cyclic AMP accumulation, but the effects of these agents and the biogenic amines were additive.

It was noted that incubation of guinea-pig cerebral slices with depolarizing agents stimulated the release of acetylcholine and adenosine into the incubation medium (Shimizu et al., 1970b). Sattin and Rall (1970) showed that 0.1 mM-1.0 mM adenosine stimulated cyclic AMP accumulation in this preparation and that theophylline competitively blocked this increase. The adenosine effect on cyclic AMP accumulation was additive with that of the biogenic amines and did not require the presence of Ca^{++}. These authors suggested that depolarization resulted in the release of adenosine which then stimulated cyclic AMP accumulation, thus coupling electrical activity in the CNS with the formation of cyclic AMP. Kakiuchi et al. (1969) showed that electrical pulses applied to guinea-pig cerebral cortex slices led to significant increase in cyclic AMP accumulation. This effect, like that of adenosine, was reduced by theophylline and augmented by noradrenaline and histamine. Pretreatment of the animals with reserpine did not decrease the effect of electrical pulses on cyclic AMP accumulation, nor did diphenhydramine or DCI block this accumulation of cyclic AMP.

C. PROPERTIES OF BRAIN PHOSPHODIESTERASE.

Much of the definitive work in this field has been done by Cheung (1966,1967,1970) using PDE found in 30,000 x g supernatant fraction of rat

brain. The pH optimum of this preparation was 7.5-8.0 and the Km for cyclic AMP was 0.1 mM. Mg^{++} or Mn^{++} were required for full PDE activity; Co^{++}, Ni^{++} and Ba^{++} were less able to satisfy divalent ion requirements and Ca^{++}, Cu^{++} and Zn^{++} were all inhibitors of this preparation of PDE. Caffeine and theophylline were equipotent inhibitors of this preparation, maximal concentrations inhibiting PDE activity 50%. ATP and PP_i were shown to be potent inhibitors of brain PDE activity while other nucleotide triphosphates were less effective. Inorganic triphosphates and tetraphosphates produced almost total inhibition at 4 mM. It was postulated that the inhibitory action of these substances was due to their chelation ability, reducing the availability of ions for the metal-enzyme complex necessary for PDE activity.

When this preparation of PDE was further purified (Cheung, 1969b) it was active only in the presence of snake venom, the degree of activation depending on the time of exposure and the concentration of snake venom. The stimulating factor in the venom preparation was not isolated but was distinguished from the 5'-nucleotidase activity present in venom.

Honda and Imamura (1968) found that in addition to inhibition by xanthine derivatives, soluble preparations of rabbit brain PDE were significantly inhibited by 0.05 mM chlorpromazine, perphenazine, fluphenazine and prochlorperazine while imipramine, meprobamate, adrenaline, serotonin and cocaine were without effect. Shimizu et al. (1970b,c) showed that the various depolarizing agents and biogenic amines that stimulated cyclic AMP accumulation in rabbit cerebral cortex slice preparations of adenyl cyclase did not inhibit PDE activity in a soluble PDE preparation from rabbit brain, and adenosine inhibited PDE activity only slightly.

Roberts and Simonsen (1970) found that a particulate preparation of mouse

brain PDE was significantly stimulated by a variety of imidazole compounds. It was also shown that Mn^{++} was considerably more effective than Mg^{++}, on a molar basis, in activating PDE in this preparation. Maximal activation of PDE was achieved with 0.1 mM Mn^{++} and the addition of graded amounts of Mg^{++} to this concentration of Mn^{++} produced no further increase in PDE activity nor any inhibition, showing that no PDE activity in the preparation was activated exclusively by Mn^{++} or Mg^{++}.

SUMMARY

The high levels of adenyl cyclase and PDE found in the CNS have led to the proposal of a role for cyclic AMP in the regulation of brain function, either in the regulation of intracellular processes or in the chemical mediation of nerve impulses.

The localization of adenyl cyclase in synaptic nerve endings suggests a relationship of adenyl cyclase to transmitter function. The observation that cyclic AMP accumulation in brain slice preparations is stimulated by histamine and noradrenaline, two potential CNS transmitters and by electrical pulses and depolarizing agents substantiates this suggestion. The finding 1) that theophylline enhances histamine and noradrenaline induced cyclic AMP accumulation but not that of adenosine, the depolarizing agents or electrical stimulation and 2) that histamine and the depolarizing agents, electrical stimulation and adenosine produce additive effects on cyclic AMP accumulation indicates two different mechanisms leading to the ultimate stimulation of adenyl cyclase by these agents.

None of the agents shown to stimulate cyclic AMP accumulation in brain preparations appeared to do so by inhibiting the destruction of cyclic AMP.

On the other hand, it was shown that such intracellular substances as ATP and pyrophosphate significantly inhibited soluble preparations of brain PDE, indicating that this enzyme might be present in vivo in a greatly inhibited state. The more purified PDE preparation (Cheung, 1969b) required activation by snake venom, which indicates that brain PDE may be poised between the nucleotide inhibitors and the activator making PDE regulation a rather complicated and perhaps physiologically important regulatory system.

PART 5 RELATIONSHIP OF ADENYL CYCLASE TO OTHER MEMBRANE-BOUND ATP UTILIZING ENZYMES

Preparations in which cyclic AMP accumulation has been studied were contaminated with other membrane-bound enzyme systems. There was some indirect evidence that activation of this adenyl cyclase was associated with an alteration in the activity of other membrane-bound ATP utilizing enzymes, such as the Na/K ATPase.

Milner and Hales (1967) showed that the release of insulin from the pancreas, believed to be a cyclic AMP mediated effect, was stimulated by ouabain. The omission of K^+ from the incubation medium also stimulated insulin secretion, suggesting that inhibition of Na/K ATPase may be accompanied by a change in adenyl cyclase activity. This was further supported by the finding that stimulation of insulin secretion by ouabain was abolished by the omission of Na^+ from the incubation medium, suggesting that this stimulation was secondary to a rise in intracellular Na^+ concentration.

Ho et al. (1967) found that ouabain had a strong inhibitory action upon hormone stimulated lipolysis in isolated fat cells. Ouabain, however, did not inhibit lipolysis induced by dibutyryl cyclic AMP or caffeine, which suggested that it acted at the level of formation of cyclic AMP. The inhibitory action of ouabain could be reproduced by the removal of K^+ from the incubation medium. It was also shown that adenyl cyclase activity in tissue pretreated with ouabain was reduced significantly compared to that of untreated tissue. Similarly, the removal of K^+ from the incubation medium resulted in a marked decrease in cyclic AMP accumulation.

Freidmann and Park (1968) found that glucagon activation of cyclic AMP accumulation in liver slice preparations was associated with a very rapid and

transient efflux of K^+. It was also shown that noradrenaline and cyclic AMP produced K^+ efflux in this preparation.

It has been shown by Shimizu et al. (1970c) that ouabain, as well as other membrane depolarizing agents such as K^+, veratridine and batrachotoxin, were potent stimulators of cyclic AMP accumulation in rabbit brain slice preparations. Kakiuchi et al. (1969) found that electrical stimulation of this same preparation produced a significant stimulation of cyclic AMP accumulation. These agents were found to stimulate cyclic AMP accumulation through a pathway different from that by which histamine and noradrenaline stimulated cyclic AMP accumulation in this preparation. These workers suggested a relationship of membrane depolarization and transmitter release to cyclic AMP formation in the CNS.

Birnbaumer et al. (1969) reported that K^+ enhanced the stimulatory effect of ACTH on cyclic AMP accumulation in fat cell ghosts. The addition of Na^+ reversed this effect. Li^+, which previously had been found to inhibit active Na^+-K^+ transport in this preparation (Clausen et al., 1969), was also shown to inhibit NaF and ACTH stimulated adenyl cyclase. Li^+ was found to increase glucose utilization in fat cells (Clausen et al., 1968) as well, suggesting that it was acting in a non-specific manner affecting the general structure rather than the specific functional components in the plasma membrane.

NaF has been shown to be a potent stimulator of cyclic AMP accumulation in particulate preparations of adenyl cyclase (Sutherland et al., 1962). Weiss (1969) and other workers have shown that NaF is also a potent inhibitor of the ATPase activity of these preparations. In experiments by Weiss in the pineal gland, it was found that the concentration of NaF which increased

cyclic AMP accumulation 2-fold failed to inhibit ATPase activity. Increasing concentrations of NaF produced a smaller effect on cyclic AMP accumulation though producing a greater inhibition of ATPase activity. NaF was shown to increase the rate of accumulation of cyclic AMP over a wide range of substrate concentrations. These workers postulated that the effects of NaF at low ATP concentrations could be due in part to ATPase inhibition, but this could not explain NaF stimulation of cyclic AMP accumulation in the presence of saturating amounts of substrate. In experiments by Birnbaumer et al. (1969) in isolated fat cell ghosts, 10 mM NaF was found to inhibit ATPase activity by 57% while producing a linear time curve of cyclic AMP accumulation. Although NaF inhibited ATPase present in ghosts, it also appeared to have a direct effect on the activity of adenyl cyclase. This conclusion was based on the finding that in studies in which adenyl cyclase activity was determined at 30°C in the presence of an ATP regenerating system, conditions which maintained constant ATP concentrations, NaF still exerted its stimulatory effect. Under conditions in which ATP was rapidly hydrolyzed, addition of 10 mM NaF at any time during the incubation period resulted in an immediate stimulation of cyclic AMP accumulation; the rate of NaF stimulated cyclic AMP accumulation decreased after each successive period of incubation, at which time lower ATP levels were present. Dousa and Rychlik (1970b) found that the amount of cyclic AMP accumulated in rat kidney homogenates was directly related to the initial concentration of ATP in the incubation mixture. NaF increased cyclic AMP accumulation in this preparation in concentrations that inhibited ATP and ADP hydrolysis; the maximal stimulating effect on cyclic AMP

accumulation, however, occurred at lower concentrations than the maximal inhibitory effect on ATP hydrolysis. These workers proposed that NaF increased cyclic AMP accumulation in this preparation by maintaining substrate for adenyl cyclase as well as maintaining sufficient levels of ATP and ADP to inhibit PDE activity. NaF, however, also appeared to act by directly stimulating adenyl cyclase, since when an ATP regenerating system was used to maintain ATP levels to the same degree as NaF the amount of cyclic AMP accumulation was far less.

SUMMARY

Details of the relationship of adenyl cyclase to other enzymes present in the plasma membrane are yet to be elucidated. A relationship of adenyl cyclase to ion transport has been suggested by observations that hormone-stimulated lipolysis, a reflection of adenyl cyclase activity in fat cells, was dependent on low concentrations of K^+ and that ouabain, an inhibitor of Na-K ATPase, inhibited the lipolytic action of several hormones as well as decreasing cyclic AMP accumulation (Ho et al., 1967). In contrast ouabain as well as other depolarizing agents were shown to stimulate cyclic AMP accumulation in brain slice preparations, by a mechanism believed to link depolarization, cyclic AMP accumulation and transmitter release (Shimizu et al., 1970c).

Since agents such as NaF and ouabain, known inhibitors of ATPase activity, also stimulated cyclic AMP accumulation, relationship of adenyl cyclase to ATPase activity was suggested. Experiments by Weiss (1969) and Birnbaumer et al. (1969) in the pineal gland and the fat cell ghost respectively indicated a dissociation of the NaF effect on ATPase inhibition

and the ability to stimulate cyclic AMP accumulation, though a clear dissociation of these two effects was not entirely achieved in all of their experiments.

Experiments by Dousa and Rychlik (1970b) showed that maintenance of ATP and ADP levels by NaF enhanced cyclic AMP accumulation by preserving substrate for adenyl cyclase as well as inhibiting PDE activity. In these experiments NaF also appeared to act by directly stimulating adenyl cyclase. Further studies appear necessary to determine the relationship of cyclic AMP accumulation to other membrane-bound systems and PDE activity.

METHODS AND MATERIALS

1. Preparation of Systems Used to Study Adenyl Cyclase Activity.

i) Rat cerebral cortex. Wistar rats (approximately 200 g) were guillotined and the cerebral cortices removed and transferred immediately into homogenizing medium which consisted of 2 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4 and 1.0 mM $MgSO_4$ at 4°C. All subsequent steps in the preparation were done at 4°C. The tissue was minced, and homogenized for 30 seconds in 4.5 volumes of homogenizing medium using a Potter-Elvehjem homogenizer. The homogenate was diluted in an equal volume of the same medium and centrifuged at 2,000 x g for 10 minutes in a Sorval RC-2B refrigerated centrifuge. The supernatant was discarded and the pellet resuspended in 10 volumes of homogenizing medium and centrifuged at 2,000 x g for 10 minutes. The pellet was then suspended in 40 mM Tris, pH 7.4 to give a concentration of approximately 60 mg protein/ml.

ii) Guinea-pig pancreas. Guinea-pigs (approximately 200 g) were guillotined and the pancreatic tissue removed and transferred immediately into homogenizing medium (2 mM Tris, pH 7.4 and 1 mM $MgSO_4$) at 4°C. All subsequent steps in the preparation were done at 4°C. The tissue was minced and homogenized in 4.5 volumes of homogenizing medium for 2 minutes. The homogenate was then diluted in an equal volume of the same medium and centrifuged at 3,000 x g for 10 minutes in a Sorval RC-2B refrigerated centrifuge. The supernatant was discarded and the pellet resuspended in 10 volumes of homogenizing medium and again centrifuged at 3,000 x g for 10 minutes. This pellet was then suspended in 40 mM Tris, pH 7.4 to give a concentration of approximately 20 mg protein/ml.

iii) Synaptic membrane preparation of adenyl cyclase. A modification of the method of De Robertis et al. (1967) was used. Wistar rats

(approximately 200 g) were guillotined and the brains removed into 0.9% saline at 4°C. All subsequent steps in the preparation were done at 4°C. The brains were transferred to a petri dish containing 0.32 M sucrose, 1 mM $MgSO_4$ and 0.05 mM Tris, pH 7.2. In some experiments equimolar amounts of Mn^{++} were substituted for Mg^{++} in the media used. The cerebral cortex was dissected away from the remainder of the brain, minced and homogenized in 4.5 volumes of the same medium using a Dounce hand homogenizer (25 ml volume). The homogenate was diluted with 2 volumes of the same medium and centrifuged for 10 minutes at 900 x g. The precipitate was washed twice by suspending it in 30 ml of the medium and centrifuged at 900 x g for 10 minutes to obtain the "nuclear fraction". The supernatants were pooled and centrifuged at 11,500 x g for 20 minutes. The precipitate was washed once in the same medium and centrifuged again. This precipitate was the "mitochondrial fraction". This fraction was resuspended in 9 volumes of a hypotonic medium consisting of 1 mM $MgSO_4$, pH 7.2 and homogenized using a Dounce hand homogenizer (50 ml volume). This homogenate was centrifuged at 20,000 x g for 30 minutes. The precipitate, called M_1, was resuspended in the preparation medium and layered on a discontinuous density gradient. The gradient consisted of 6 ml of 1.2 M sucrose (density, 1.15), and 5 ml each of 1.0 M (density, 1.13), 0.9 M (density, 1.11), and 0.8 M (density 1.10) sucrose and was prepared immediately before use. The equivalent of 3 g wet weight tissue in 10 ml of preparation medium was applied to the gradient, and centrifuged at 100,000 x g for 1 hour in a Beckman L65-B ultracentrifuge using an S.W. 27 swinging bucket rotor.

Figure 3 illustrates the subfractions of M_1 obtained by this procedure.

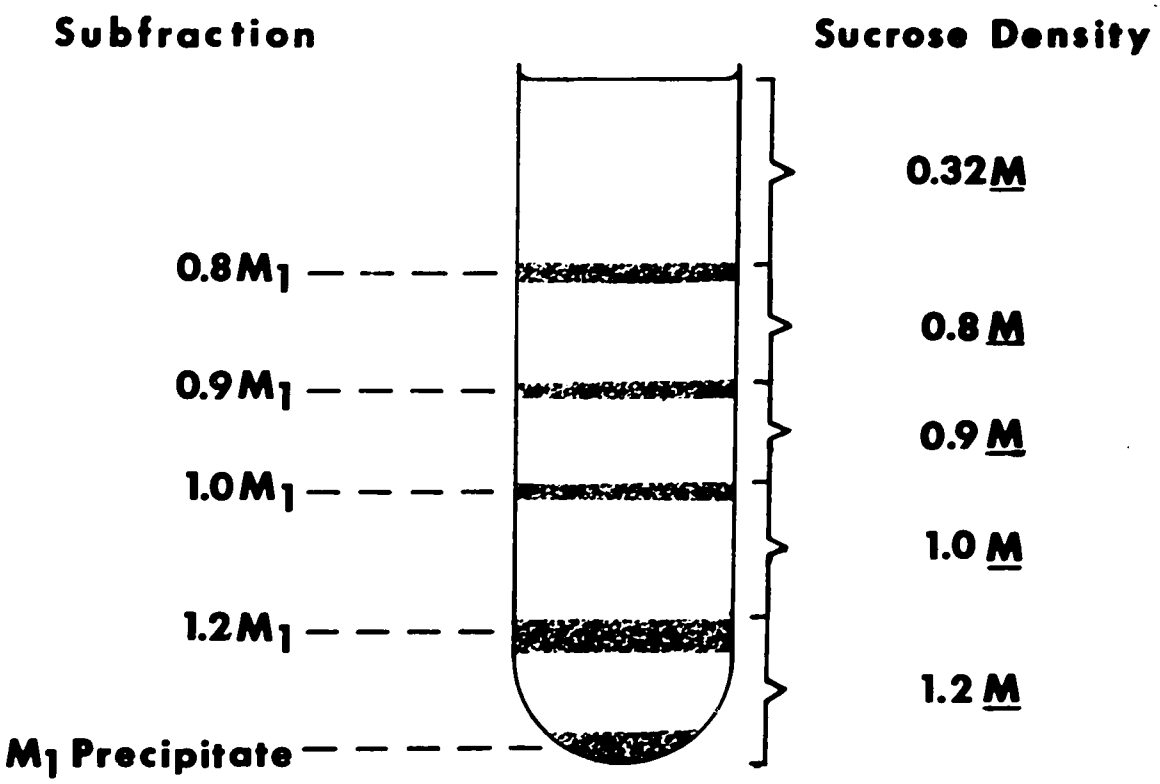


FIGURE 3: Subfractionation of M_1 Fraction of Rat Cerebral Cortex Homogenate by Sucrose Density Gradient Centrifugation

M_1 fraction of rat cerebral cortex homogenate was suspended in 0.32 M sucrose 1 mM $MgSO_4$, and 0.05 mM Tris, pH 7.2 and centrifuged into a discontinuous density gradient of sucrose for 1 hour at 100,000 x g; the subfractionations illustrated were obtained.

The ultrastructure of each subfraction was characterized by De Robertis et al. (1966) by electron microscopy. The layer of material at the 0.32 M - 0.8 M interface (0.8 M_1) was myelin; the next layer (0.9 M_1) consisted of synaptic membranes and myelin; the 1.0 M_1 and the 1.2 M_1 contained synaptic membranes; the precipitate contained all of the mitochondria.

The material at the top of the 1.2 M sucrose layer, denoted 1.2 M_1, was collected and suspended in 30 volumes of a medium containing 1 mM $MgSO_4$ and 2 mM Tris, pH 7.4 and centrifuged at 35,000 x g for 20 minutes. The precipitate was washed once by suspension in the same medium and centrifuged again. The resulting precipitate, which was the synaptic membrane preparation, was suspended in 40 mM Tris, pH 7.4 to give a concentration of approximately 9 mg protein/ml.

The distribution of adenyl cyclase in the subfractions 0.9 M_1, 1.0 M_1 and 1.2 M_1 was determined; the results of these experiments are summarized in Table 2. The 1.0 M_1 fraction contained the highest specific activity of adenyl cyclase but the 1.2 M_1 fraction was chosen for use because the total adenyl cyclase activity was greatest in this fraction. NaF, as expected, stimulated cyclic AMP accumulation in all fractions.

Figure 4 illustrates an electron micrograph of this 1.2 M_1 fraction and it shows the fraction is rich in membrane fragments and free from mitochondria. De Robertis et al. (1967) used monoamine oxidase as a mitochondrial marker and found that it was not present to any appreciable extent in the 1.2 M_1 fraction.

2. Preparation of Partially Purified Phosphodiesterase.

The method of Cheung (1966) was used. The brains of 200 g Wistar

TABLE 2

Distribution of Adenyl Cyclase in Subfractions of M_1 Separated by Sucrose Density Gradient Centrifugation

Fraction	Sucrose Concentration (M)	Ultrastructure	Protein mg/g tissue	Adenyl Cyclase Activity nmoles/g tissue		Adenyl Cyclase Specific Activity nmoles/mg protein	
				C	NaF	C	NaF
M_1 0.9	0.9 (2)	Synaptic membranes and myelin	1.12 ± 0.18	0.85 ± 0.16	2.60 ± 0.10	0.50 ± 0.20	1.90 ± 0.95
M_1 1.0	1.0 (4)	Synaptic membranes	1.22 ± 0.07	1.00 ± 0.11	3.10 ± 0.65	0.75 ± 0.16	2.40 ± 0.90
M_1 1.2	1.2 (4)	Synaptic membranes	2.24 ± 0.14	1.40 ± 0.40	3.50 ± 0.72	0.62 ± 0.22	1.60 ± 0.41

A freshly prepared subfraction M_1 was resuspended in medium containing 0.32 M sucrose, 1 mM $MgSO_4$ and 0.05 mM Tris, pH 7.2 and layered on the sucrose gradient illustrated by Fig. 3. After centrifugation at 100,000 x g for 1 hour, the subfractions were separated and analyzed for adenyl cyclase activity in the presence and absence of 10 mM NaF as described in text; protein was measured by the biuret method. In the table tissue weight refers to wet weight tissue and numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E.

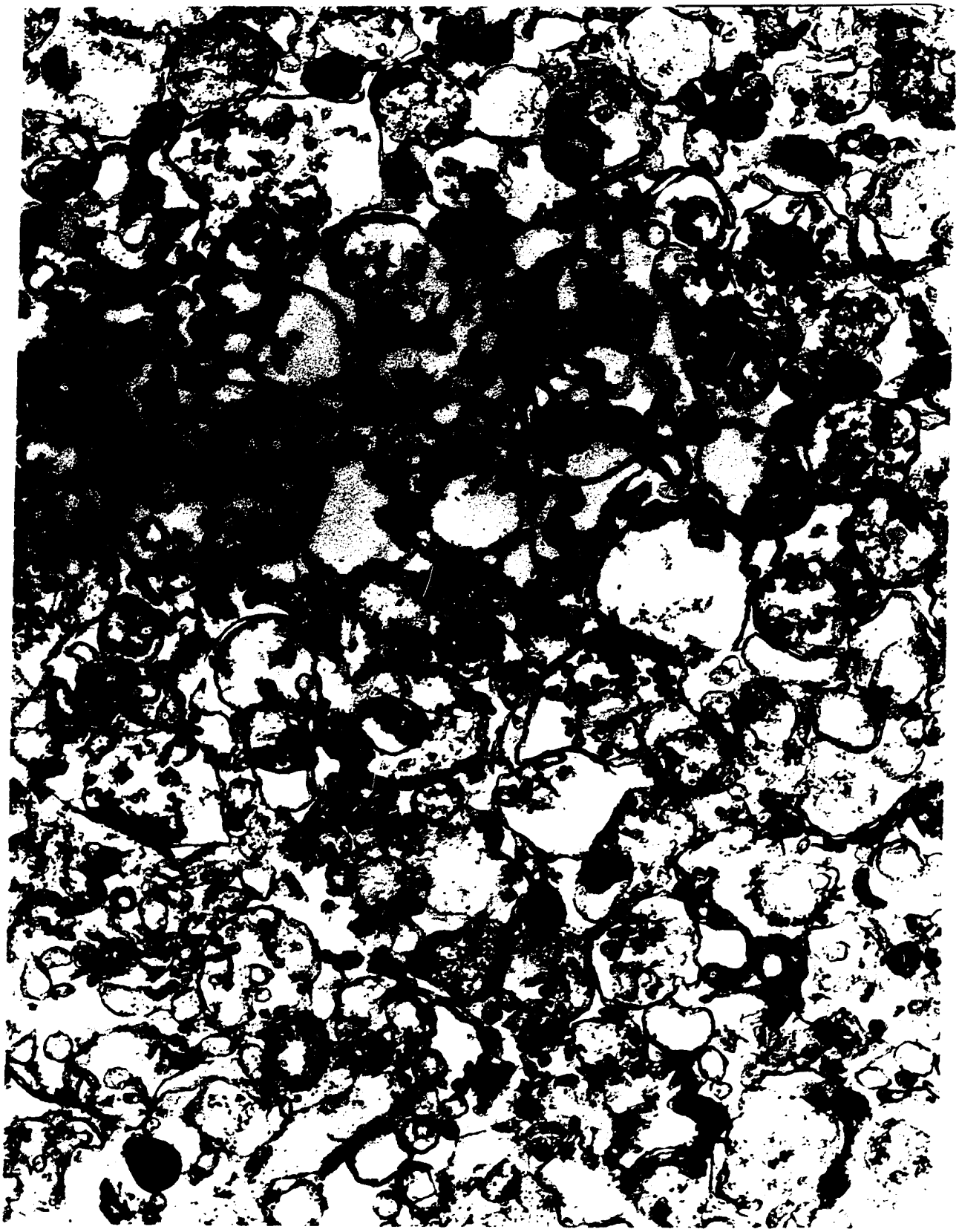
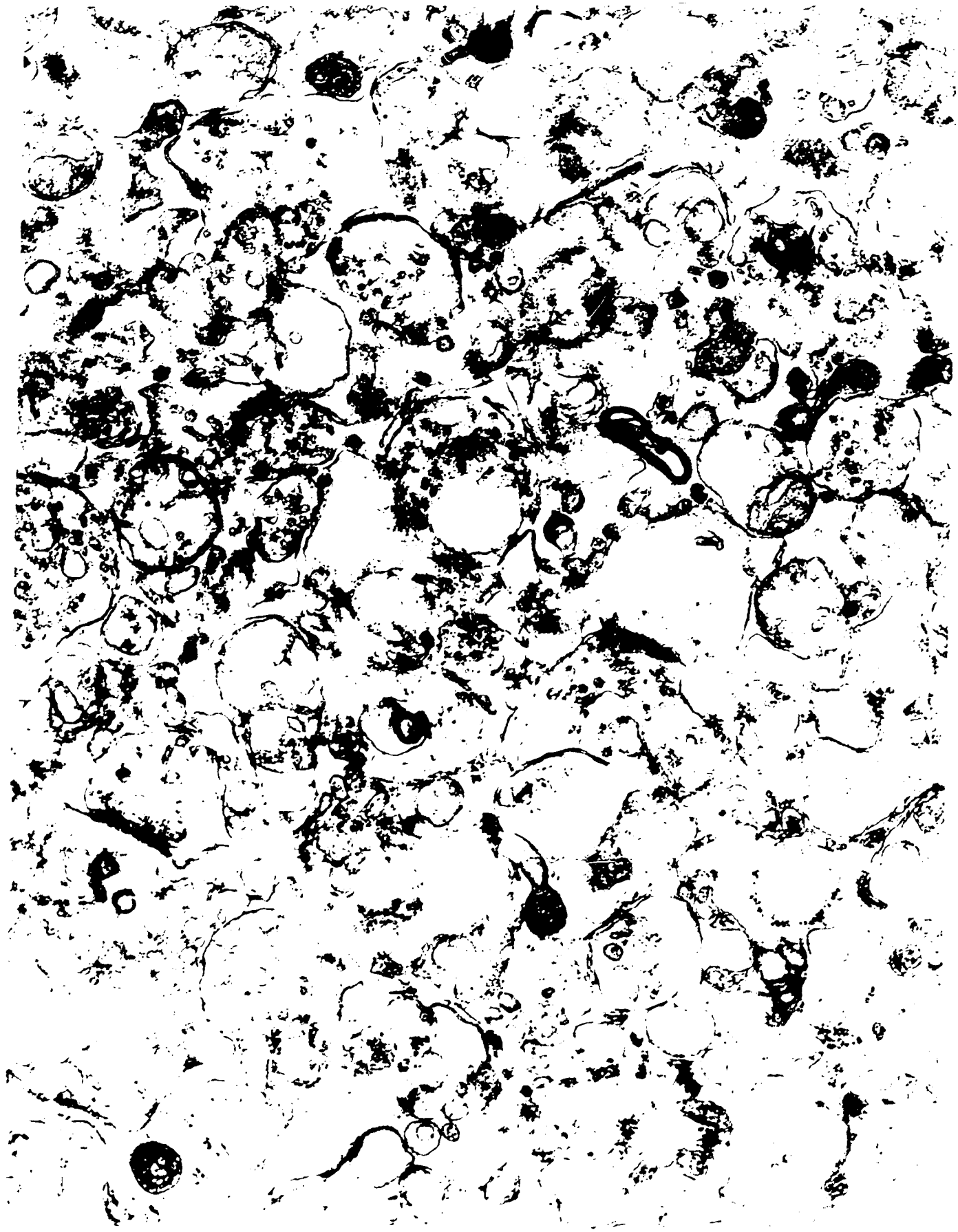


FIGURE 4: Electron Micrograph of 1.2 M_1 Fraction of Rat Cerebral Cortex Homogenate

The 1.2 M_1 subfraction, prepared as described in methods, was analyzed by electron microscopy at a magnification of 35,154 X.



rats were removed into distilled water and the cerebral cortex dissected away from the remainder of the brain and homogenized for 1 minute in 5 volumes of distilled water. The homogenate was dialyzed overnight against 200-400 volumes of 20 mM Tris, pH 7.5. The medium was changed once during this period. The dialyzed extract was centrifuged at 30,000 x g for 30 minutes in a Sorval RC-2B refrigerated centrifuge and the supernatant was divided into 5 ml aliquots and stored at -20°C until use. This preparation could be stored for up to 4 weeks without significant loss in PDE activity.

3. Assay of Adenyl Cyclase and other Membrane-Bound ATP Utilizing Enzymes.

In the standard assay the adenyl cyclase preparation was incubated in a Warner-Chilcott incubation bath at 37°C. The total incubation volume of 2 ml contained: 40 mM Tris (pH 7.4), 3.5 mM $MgSO_4$, 6.67 mM theophylline and 2.1 mM ^{14}C-ATP (0.250 µCi/µmole). When an ATP regenerating system was used it consisted of 10 mM phosphoenol pyruvate (PEP), and 25 µg/ml pyruvate kinase (PK). In the assay of adenyl cyclase in brain synaptic membrane preparations, 5.0 mM $MgSO_4$ was used instead of 3.5 mM. After 5 minutes preincubation for temperature equilibration, the reaction was started by adding the ^{14}C-ATP. Unless otherwise stated, the time of incubation was 10 minutes. The incubation was terminated by adding cold 45% trichloroacetic acid (TCA) to give a final concentration of 7.5%. Tracer amounts of ^{3}H-labelled AMP (0.340 µCi), ADP (0.310 µCi), ATP (0.410 µCi), and cyclic AMP (0.210 µCi) were added to each sample. Prior to use the radiochemicals were analyzed for purity by the anion-exchange column chromatography method to be described. While it was found

that the ^{14}C-adenine nucleotides contained no contaminants, the ^{3}H-adenine nucleotides contained up to 10% contamination, primarily due to the breakdown of these compounds during storage. This contamination was taken into account in the quantitation of the ^{3}H-adenine nucleotides added to the incubation media. These nucleotides were stored together in 1 M ammonium acetate, pH 4.4 and were added to the samples in a volume of 0.1 ml. The samples were then mixed and centrifuged at 4,000 x g in a Sorval RC-2B refrigerated centrifuge to remove the TCA precipitates. The supernatants were extracted three times with 5 volumes of water-saturated ether to remove residual TCA, the aqueous phases evaporated to dryness in a Buchler Rotary Evapo Mix and stored at -20°C. The ^{14}C-adenine nucleotides present in the samples were determined by the ion-exchange column chromatography method to be described.

4. Assay of Phosphodiesterase Activity.

i) Assay of partially purified preparation of PDE. Enzyme preparation containing 3 mg of protein was incubated for 10 minutes at 37°C in 1 ml of a medium containing 40 mM Tris, pH 7.5, 1.8 mM $MgSO_4$ and 2 mM ^{14}C-cyclic AMP (specific activity 0.012 µCi/µmole). After 5 minutes preincubation for temperature equilibration, the reaction was started by adding the ^{14}C-cyclic AMP. The reaction was terminated by adding cold 45% TCA to give a final concentration of 7.5%. ^{3}H-AMP (0.310 µCi) and ^{3}H-cyclic AMP (0.210 µCi) were then added to the samples, and the samples were then mixed and centrifuged at 4,000 x g in a Sorval RC-2B refrigerated centrifuge. The supernatants were extracted 3 times with 5 volumes of water-saturated ether and the aqueous phases evaporated to dryness in a

Buchler Rotary Evapo Mix and stored at -20°C. The PDE activity was determined by the recovery of ^{14}C-AMP and ^{14}C-cyclic AMP present in the sample by the ion-exchange column chromatography method to be described.

ii) Assay of PDE activity of the rat cerebral cortex preparation of adenyl cyclase. The assay procedure was the same as that described for the partially purified PDE preparation except that the assay medium contained 3.5 mM $MgSO_4$ and 1 μM ^{14}C-cyclic AMP (specific activity 0.250 mCi/μmole). In all assays of PDE activity in this preparation, 250 mg wet weight tissue was used. The PDE activity was determined by measuring the amount of ^{14}C-cyclic AMP remaining after the incubation period by the ion-exchange column chromatography method to be described.

iii) Assay of PDE activity of the synaptic membrane preparation of adenyl cyclase. The assay procedure was the same as that described for the partially purified preparation except that the assay medium contained 5.0 mM $MgSO_4$ and 0.1 μM ^{14}C-cyclic AMP (specific activity 2.50 mCi/μmole). Three mg protein was used in all assays of PDE activity with this preparation. PDE activity was determined by measuring the amount of ^{14}C-cyclic AMP remaining after the incubation period by the ion-exchange column chromatography method to be described.

5. Preparation of Ion-Exchange Resin for Column Chromatography.

AG-1 x 8 (200-400 mesh) in the chloride form was purchased from Calbiochem Ltd. From 200-250 g of resin was washed twice with 2N NaOH at room temperature and then washed with purified water until the pH of the wash was 7.0-7.5. The resin was then washed once with 2 N acetic acid and again with purified water to neutral pH. The resin was finally

suspended in 1 M ammonium acetate, pH 4.4, and stored at $4^{\circ}C$ until used.

6. Preparation of Columns for Adenine Nucleotide Determination.

The columns (24 cm x 0.5 cm) were packed by gravity and washed with 1 M ammonium acetate (pH 4.4) for 12-15 hours before use. The bottles containing the eluting buffer were placed at a height 3-4.5 feet vertically above the columns so that the initial flow rate was 15-18 ml/hour.

7. Double Label Isotope Scintillation Counting.

Radioactivity in each of the samples eluted from the columns was determined in a Picker 220 liquid scintillation counter using standard methods for double label isotope counting. Quench correction was made by an external standard-channels ratio method. The scintillation fluid used contained 4 g 2,5 -diphenyloxazole, 50 mg 1,4-bis(2-(4-methyl-5-phenyloxazolyl))-benzene 1000 ml toluene and 600 ml ethylene glycol monomethyl ether. The maximum counting efficiency was: 15% for ^{3}H and 39% for ^{14}C; the spill of ^{3}H into the ^{14}C channel was nil and that of ^{14}C into the ^{3}H channel was 12%. Samples were counted for a time that was long enough to yield a standard error of less than 5%.

8. Quantitation of Nucleotides Recovered.

The counting data were processed using a PDP 8L computer (Digital Equipment Corp.). The computer was programmed to compute the DPM for both isotopes (corrected for spill-over and quench) and the $^{3}H/^{14}C$ ratio of each sample. A second program corrected the recovery of each ^{14}C-nucleotide to 100% by calculating the recovery of the corresponding ^{3}H-labelled nucleotide; the quantity of each ^{14}C-nucleotide present in each sample was

then calculated in μmoles.

9. Protein Determination.

The biuret method (Kabat and Meyer, 1964) for protein determination was used.

10. Statistics.

Student's t test for unpaired data was used to test the significance of the difference between results; two-tailed tests were always used. Variation of results is expressed as ± standard error (S.E.).

MATERIALS

a) Resin: AG 1-X8, 200-400 mesh chloride form, Bio-Rad Laboratories.

b) Reagents: All reagents of analytical grade.

ethyl ether, anhydrous, Mallinkrodt Chemical Works.

sodium hydroxide, J.T. Baker Chemical Co.

trichloroacetic acid, J.T. Baker Chemical Co.

acetic acid, glacial, Anachemia Chemicals Ltd.

ammonium hydroxide, McArthur Chemical Co. Ltd.

sodium fluoride, J.T. Baker Chemical Co.

manganese chloride, Fisher Scientific Co.

magnesium sulphate, J.T. Baker Chemical Co.

calcium chloride, J.T. Baker Chemical Co.

tris(hydroxymethyl)aminomethane (Trizma Base), Sigma Chemical Co.

dextrose, J.T. baker Chemical Co.

c) Components of Scintillation Fluid:

1,4-bis(2-(4-methyl-5-phenyloxazolyl))benzene, scintillation grade, Nuclear-Chicago Corp.

2,5-diphenyloxazole, scintillation grade, Nuclear-Chicago Corp.

ethylene glycol monomethyl ether, certified reagent, Fisher Scientific Co.

toluene, analytical grade, J.T. Baker Chemical Co.

d) Radioactive Agents: all agents from Schwarz Bioresearch Inc.

(8-^{14}C) adenosine 5'-triphosphate tetrasodium (35-50 mCi/mmole)

(8-^{14}C) adenosine 5'-monophosphate diammonium (35-50 mCi/mmole)

(8-^{14}C) adenosine 3',5'-cyclic phosphate (35-50 mCi/mmole)

(8-^{3}H) adenosine 3',5'-cyclic phosphate (12-15 Ci/mmole)

(8-^{3}H) adenosine 5'-monophosphate diammonium (12-15 Ci/mmole)

(8-^{3}H) adenosine 5'-triphosphate tetralithium (12-15 Ci/mmole)

(8-^{3}H) adenosine 5'-diphosphate trilithium (12-15 Ci/mmole)

e) Other Agents:

theophylline, K & K Laboratories, Inc.

phospho(enol)pyruvate trisodium salt, hydrate, Sigma Chemical Co.

pyruvate kinase, type II from rabbit skeletal muscle, Sigma Chemical Co.

adenosine 5'-triphosphate disodium, Schwarz Bioresearch Inc.

adrenaline bitartrate hydrate, Calbiochem Co.

ouabain, K & K Laboratories, Inc.

phenoxybenzamine hydrochloride, Smith, Kline & French Co.

pronethalol, ICI Ltd.

carbachol, K & K Laboratories, Inc.

pancreozymin, sterile powder, Boot Pure Drug Co. Ltd.

secretin, Gift from Dr. J. Dupré.

α,β-methylene-adenosine 5'-triphosphate, Miles Laboratories Inc.

β,γ-methylene-adenosine 5'-triphosphate, Miles Laboratories Inc.

RESULTS

PART 1. DEVELOPMENT OF A METHOD FOR ADENINE NUCLEOTIDE DETERMINATION

In these experiments a procedure was developed for measuring the production of ^{14}C-labelled adenine nucleotides, including cyclic AMP, from ^{14}C-ATP. The procedure was based on the separation of each of the adenine nucleotides in pure form by anion-exchange column chromatography using AG 1-X8 resin in the acetate form. The nucleotides were eluted by increasing the molar concentration of the eluant, ammonium acetate, at constant pH (4.4). The recovery of the ^{14}C-labelled nucleotides was determined by measuring the recovery of the ^{3}H-labelled nucleotides added to each incubation vessel immediately after the reaction had been terminated. The ratio of ^{3}H/^{14}C in the eluates was a measure of the purity of each nucleotide recovered, and only samples with a constant ratio (less than 5% variation) were used in the final quantitation of each nucleotide.

The concentration gradient of ammonium acetate used to separate the adenine nucleotides was determined using a nine chamber gradient device (Technicon Corp.). All the chambers of this device were in a hydrostatic equilibrium which was constantly being reestablished in response to the removal of liquid from the chamber at one end of the series. The solvent in each chamber flowed out according to the following equation: (Hori, 1967).

$$\frac{C}{L} = \frac{(N-1)!}{(N-n)!(n-1)!}\left(1-\frac{v}{V}\right)^{N-n}\left(\frac{v}{V}\right)^{n-1}$$

in which C is the concentration emerging from the mixer at any point, L is the concentration of the solution which is placed in the chamber of number n, N is the total number of chambers in the system, v is the volume of liquid

which has emerged up to that point and V is the total volume of liquid originally in the mixer. The calculated contributions of individual chambers in a nine-chambered system are shown in Table 3 (from Peterson and Sober, 1959). In a typical experiment, summarized in Table 4, chambers were filled with concentrations of ammonium acetate (pH 4.4) increasing from 1.0 M-3.0 M, and 1 μmole of AMP, ADP, cyclic AMP and ATP placed individually on columns of AG 1-X8 (prepared as described in methods) were eluted. Aliquots of 5.0 ml fractions of eluants were read spectrophotometrically (Carl Zeiss) at 260 nm and 280 nm. The concentration of eluant at which the peak activity of each nucleotide was noted was determined from Table 3. These concentrations were: AMP, 1.272 M; cyclic AMP, 1.757 M; ADP, 2.250 M; and ATP, 2.962 M.

Since it was found impractical to continually use the nine-chamber gradient device in standard experimental procedures, an alternative method using a stepwise gradient of ammonium acetate was developed. In this procedure the selection of ammonium acetate concentrations was based on the results shown in Table 4. They were: 1.0 M, 10.0 ml; 1.6 M, 30.0 ml; 2.0 m, 40.0 ml; 2.5 M, 50.0 ml; and 5.0 M, 70.0 ml. Preliminary experiments determined the elution pattern of the nucleotides under these conditions Samples of ^{3}H-labelled nucleotides (0.18 μCi-0.32 μCi) were dissolved in 0.2 ml of 1.0 M ammonium acetate and applied to the columns; effluent was collected at 8 minute intervals and the radioactivity in 200 μl aliquots determined by liquid scintillation counting. Figure 5 shows the sequence, molar concentration and volume of each buffer used along with the elution pattern of the nucleotides. The nucleotides were eluted in the following order: AMP, cyclic AMP, ADP and ATP. Adenosine (not shown in Fig. 5) was

TABLE 3

Calculated Contributions of Individual Chamber in Systems Comprising Nine Chambers

	9 Chambers[c]					
	1	2	3	4		
v/V[b]	9	8	7	6	5	v/V[b]
0.00	0.000	0.000	0.000	0.000	0.000	1.00
0.05	0.000	0.000	0.000	0.000	0.000	0.95
0.10	0.000	0.000	0.000	0.000	0.005	0.90
0.15	0.000	0.000	0.000	0.003	0.018	0.85
0.20	0.000	0.000	0.001	0.009	0.046	0.80
0.25	0.000	0.000	0.003	0.023	0.086	0.75
0.30	0.000	0.001	0.010	0.047	0.136	0.70
0.35	0.000	0.003	0.022	0.081	0.187	0.65
0.40	0.001	0.008	0.041	0.123	0.233	0.60
0.45	0.002	0.016	0.070	0.171	0.263	0.55
0.50	0.004	0.031	0.109	0.218	0.273	0.50
0.55	0.008	0.055	0.157	0.257	0.263	0.45
0.60	0.017	0.090	0.209	0.279	0.233	0.40
0.65	0.032	0.137	0.259	0.279	0.187	0.35
0.70	0.058	0.198	0.297	0.254	0.136	0.30
0.75	0.100	0.266	0.312	0.207	0.086	0.25
0.80	0.168	0.336	0.293	0.147	0.046	0.20
0.85	0.272	0.385	0.238	0.084	0.018	0.15
0.90	0.430	0.383	0.149	0.033	0.005	0.10
0.95	0.663	0.279	0.051	0.005	0.000	0.05
1.00	1.000	0.000	0.000	0.000	0.000	0.00

[b] Scale at left is to be used for chamber numbers appearing at top of column, that at right for chamber numbers appearing at bottom. Each column thus describes a pair of mirror images or, in some cases, a symmetrical curve.

[c] Numbers refer to chamber initially containing solution having a concentration of unity. Initial concentration in other chambers of a given series is zero.

TABLE 4

Elution of adenine nucleotides from AG 1-X8 anion-exchange columns using a nine-chamber gradient device

Chamber Number	Ammonium Acetate Concentration M	v/V	Emerging Concentration M	Emerging Nucleotide
1	1.0	0.10	1.272	AMP
2	1.3	0.30	1.727	Cyclic AMP
3	1.8	0.60	2.250	ADP
4	2.0	0.75	2.700	
5	2.0	0.90	2.962	ATP
6	2.0	0.95	2.989	
7	3.0			
8	3.0			
9	3.0			

Samples (1 μmole) of AMP, ADP, ATP and cyclic AMP were placed on AG 1-X8 columns (prepared as described in methods) and eluted with increasing concentrations of ammonium acetate (pH 4.4) using a nine chamber device. Aliquots of 5.0 ml eluant fractions were read spectrophotometrically at 260-280 nm and the emerging concentration of each nucleotide determined as described in the text. In the Table v refers to the volume of liquid which has emerged up to that point and V is the total volume of liquid originally in the mixer.

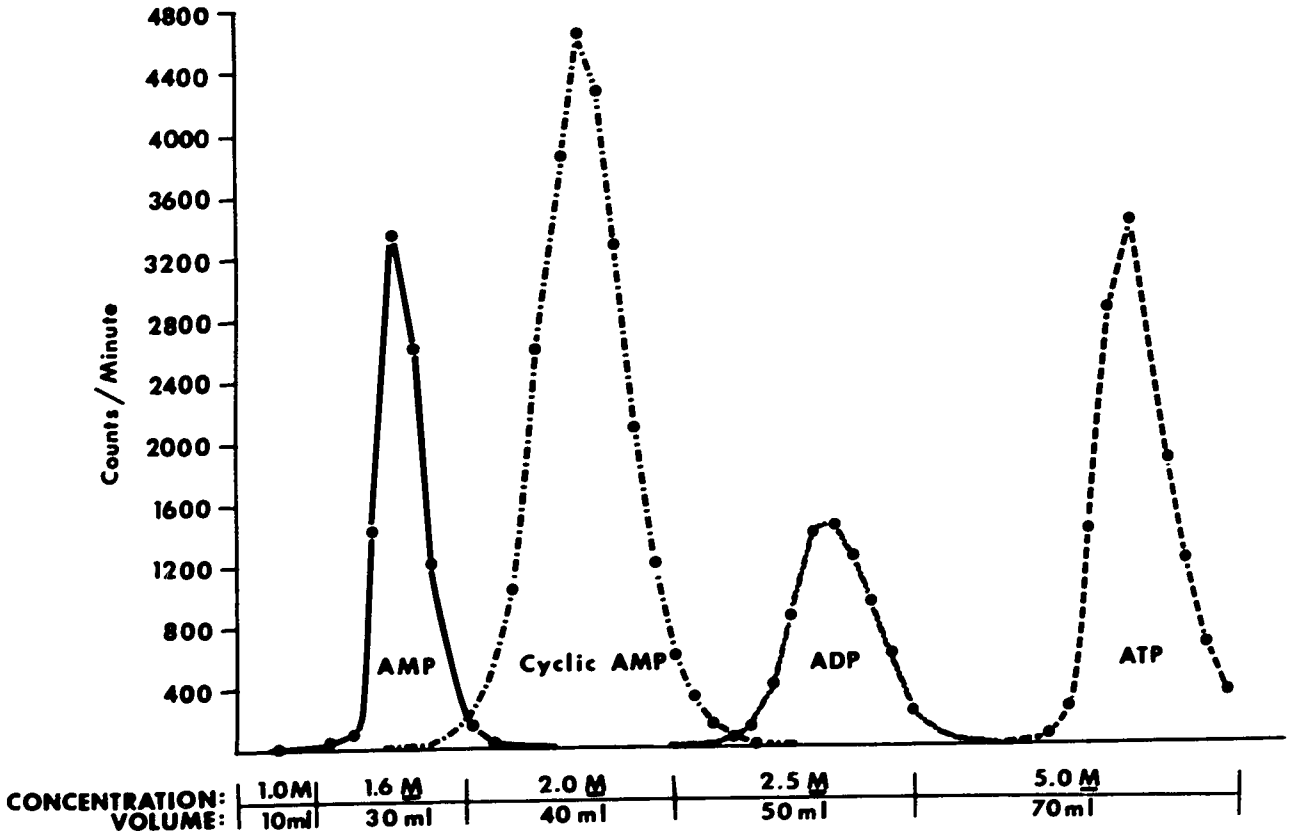


FIGURE 5: Elution Pattern of AMP, ADP, ATP and Cyclic AMP from AG 1-X8 Anion-Exchange Columns Using a Stepwise Concentration Gradient of Ammonium Acetate

Samples of ^{3}H-labelled nucleotides (0.18 µCi-0.32 µCi) were applied to AG 1-X8 columns (prepared as described in methods) and eluted using a stepwise concentration gradient of ammonium acetate (pH 4.4). Effluent was collected at 8 minute intervals and the radioactivity of 200 µl aliquots determined by liquid scintillation counting. The sequence of ammonium acetate concentrations and the volume of each used is shown at the bottom of the elution pattern.

not retained by the column under these conditions and could be quantitatively eluted, free of other adenine compounds, with 1.0 M ammonium acetate. This elution pattern was very consistent and the time of appearance of each peak could be predicted with accuracy.

In practice, the freeze-dried samples containing the adenine nucleotides to be determined (prepared as described in methods) were dissolved in 0.2 ml of 1.0 M ammonium acetate (pH 4.4) and applied to the columns. Eluates were not collected continuously, but samples were collected and monitored for ^{3}H just before the peak activity of each nucleotide was expected, as determined from the elution pattern shown in Fig. 5. At the appearance of each peak, 8-10 samples were collected directly into the vials for scintillation counting. For each sample the eluant was collected for 90-105 seconds, depending on the flow rate of the columns, to give a sample volume of not greater than 0.35 ml. The samples were counted for 0.4 minutes immediately after their collection and samples were collected until there was a definite reduction in ^{3}H counts/minute. Following the experiment 4-6 of these peak samples were counted for longer time periods. These samples contained from 10-20% of the ^{3}H-labelled nucleotide used. Although it was possible to recover up to 70% of each nucleotide in relatively pure form, recovery of this smaller amount lessened the possibility of cross-contamination, particularly of cyclic AMP by AMP.

Table 5 shows the results of a typical experiment designed to test the accuracy of the method for measuring small quantities of cyclic AMP. In this experiment known quantities (0.01-0.10 μmoles) of ^{14}C-cyclic AMP (specific activity: 0.250 μCi/μmole) were added to the standard incubation

TABLE 5

Recovery of cyclic AMP from AG 1-X8 column

Cyclic AMP Added µmoles	Cyclic AMP Recovered µmoles	% Recovery
0.010	0.011	110
0.020	0.021	105
0.030	0.030	100
0.040	0.039	98
0.050	0.047	94
0.100	0.098	98

A known amount of ^{14}C-cyclic AMP (specific activity: 0.250 µCi/µmole) was added to the standard incubation medium (no ATP), the reaction stopped immediately and cyclic AMP recovery determined as described in text.

medium (no ATP present). The reaction was stopped immediately with 45% trichloroacetic acid and a known amount of ^{3}H-cyclic AMP added to the incubation vessels. The samples were treated as described in the methods, and applied to the columns. The recovery of ^{14}C-cyclic AMP was then determined as described above. The recovery of cyclic AMP was between 94-110%. In most of the experiments to be described in this thesis samples contained cyclic AMP equal to or greater than 0.01 µmoles, with the exception of some control groups in which the accumulation rarely exceeded 0.006 µmoles. The error in measuring these low control values was approximately 15%. The amounts of AMP, ADP and ATP under all conditions tested generally exceeded 0.1 µmoles and the error in the determination of these nucleotides was less than 5%.

In other experiments, of which typical results are summarized in Table 6, the recovery of known amounts of cyclic AMP in the presence of a large excess of AMP was studied. A known quantity of ^{14}C-cyclic AMP (0.005-0.050 µmoles; specific activity: 0.250 µCi/µmole) was applied to the columns together with 1 µmole of ^{14}C-AMP (specific activity: 0.240 µCi/µmole) and the amount of cyclic AMP determined. There was little contamination of the cyclic AMP peak by the added AMP. Accuracy of the method in the determination of cyclic AMP in the presence of an excess of AMP was therefore verified, as 1000 times more AMP than cyclic AMP failed to contaminate the recovery of the latter.

DISCUSSION: DEVELOPMENT OF A METHOD FOR ADENINE NUCLEOTIDE DETERMINATION.

To facilitate the study of the relationship of adenyl cyclase to other membrane-bound ATP utilizing enzymes and PDE, a method was developed that would allow the measurement of the production and destruction of AMP, ADP,

TABLE 6

Recovery of known amounts of cyclic AMP in the presence of a large excess of AMP

Cyclic AMP Added µmoles	Cyclic AMP Recovered µmoles	% Recovery
0.0050	0.0057	114
0.010	0.011	110
0.025	0.026	104
0.050	0.049	98

A known amount of ^{14}C-cyclic AMP (specific activity: 0.250 µCi/µmole) was applied to the AG 1-X8 anion-exchange columns together with 1 µmole of ^{14}C-AMP (specific activity: 0.240 µCi/µmole). Cyclic AMP recovery was determined as described in the text.

ATP and cyclic AMP in a single-step procedure. The use of ^{3}H-labelled nucleotides to monitor the recovery of the ^{14}C-labelled nucleotides provided a simple means of quantitation of recovery.

The sensitivity of the procedure in the determination of cyclic AMP was approximately 0.5 nmoles with the specific activity of ATP used. The presence of a large excess of AMP did not greatly interfere with the recovery of these small amounts of cyclic AMP.

In the following studies, this method for adenine nucleotide determination was used to elucidate the relationship of cyclic AMP accumulation to the activity of other membrane-bound ATP utilizing enzymes in preparations of rat cerebral cortex and guinea-pig pancreas.

PART 2. ADENOSINE 3',5'-MONOPHOSPHATE ACCUMULATION IN ADENYL CYCLASE PREPARATIONS FROM RAT CEREBRAL CORTEX AND GUINEA-PIG PANCREAS.

A. RAT CEREBRAL CORTEX PREPARATION.

Figure 6 shows the results of a typical experiment that tested the relationship of cyclic AMP accumulation to tissue concentration of the rat cerebral cortex preparation of adenyl cyclase. From 100 mg wet weight tissue to 550 mg wet weight tissue was incubated at 37°C for 10 minutes in the presence and absence of 10 mM NaF. The amount of cyclic AMP accumulated in the absence of NaF was very small over the range of tissue concentrations tested. In the presence of 10 mM NaF, cyclic AMP accumulation was greatly increased with maximal accumulation, 26.5 nmoles, at a tissue concentration of 450 mg wet weight tissue (approximately 32.0 mg protein).

A tissue concentration of 450 mg wet weight tissue was used in all further experiments with the rat cerebral cortex adenyl cyclase preparation.

Table 7 summarizes the effects of varying concentrations of NaF on the metabolism of ATP in this preparation of adenyl cyclase. All three concentrations of NaF tested significantly inhibited both ATP hydrolysis ($P<0.001$ for 50 mM and 10 mM NaF and $P<0.05$ for 5 mM NaF) and AMP accumulation ($P<0.001$ in all three cases). There was also a significant accumulation of ADP at all three concentrations of NaF ($P<0.001$ in all cases). The highest concentration of NaF (50 mM) caused the greatest inhibition of ATP hydrolysis and AMP accumulation. In contrast, 10 mM NaF produced the highest level of cyclic AMP accumulation. The amount of cyclic AMP accumulated in the presence of 10 mM NaF was significantly greater than that accumulated in the presence of either 50 mM ($P<0.02$) or 5.0 mM NaF ($P<0.05$). Although the accumulation of cyclic AMP in the presence of 5.0 mM NaF was significantly greater than

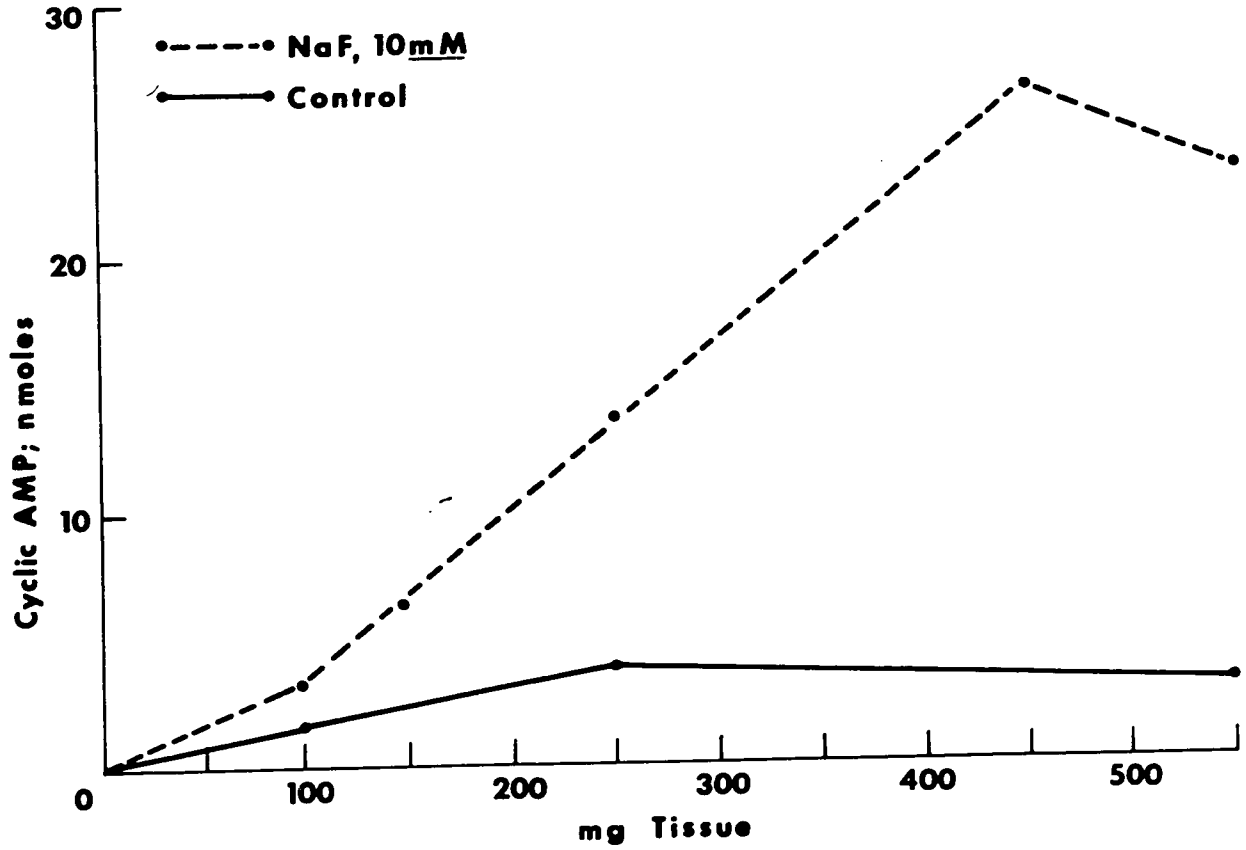


FIGURE 6: Cyclic AMP Accumulation as a Function of Tissue Concentration in the Rat Cerebral Cortex Preparation of Adenyl Cyclase

Increasing amounts of a 2,000 x g particulate preparation from rat cerebral cortex were incubated at 37°C for 10 minutes in the presence and absence of 10 mM NaF. The incubation medium of 2 ml volume contained: 40 mM Tris, pH 7.4; 3.5 mM $MgSO_4$; 6.67 mM theophylline and 2.1 mM ^{14}C-ATP (0.250 μCi/μmole). Cyclic AMP accumulation was determined as described in text.

TABLE 7

The Effect of Varying Concentrations of NaF on the Metabolism of ATP in the Rat Cerebral Cortex Preparation of Adenyl Cyclase

	AMP (μmoles)	Cyclic AMP (nmoles)	ADP (μmoles)	ATP (μmoles)
Control (7)	3.93 ± 0.08	4.70 ± 0.80	0.17 ± 0.05	0.08 ± 0.05
NaF 5.0 mM (4)	1.96 ± 0.36[a]	11.8 ± 0.56[a]	1.62 ± 0.40[a]	0.61 ± 0.24[b]
NaF 10.0 mM (9)	2.10 ± 0.09[a]	32.0 ± 5.40[c,d,a]	1.18 ± 0.08[a,c]	0.83 ± 0.14[a]
NaF 50.0 mM (4)	0.75 ± 0.18[a]	6.50 ± 2.50	2.06 ± 0.14[a]	1.38 ± 0.25[a]

[a] $P<0.001$ compared to control; [b] $P<0.05$ compared to control; [c] $P<0.02$ compared to 50 mM NaF; [d] $P<0.05$ compared to 5 mM NaF.

2,000 x g particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37°C for 10 minutes in the presence and absence of varying concentrations of NaF (5, 10 and 50 mM). The incubation medium of 2 ml volume contained: 40 mM Tris, pH 7.4; 3.5 mM $MgSO_4$; 6.67 mM theophylline and 2.1 mM ^{14}C-ATP (0.250 μCi/μmole). The adenine nucleotides were determined as described in the text. Numbers in brackets indicate the number of experiments per group. Results are expressed as mean ± S.E. AMP, ADP and ATP recovered expressed in μmoles and cyclic AMP recovered expressed in nmoles.

that in the controls ($P<0.001$), accumulation in the presence of 50 mM NaF was not ($P<0.50$).

In Table 8, the effect of the ATP regenerating system on the metabolism of ATP in the presence and absence of NaF (10 mM) is summarized. In the control experiments over 98% of the ATP was hydrolyzed during the 10 minute incubation period. The addition of the ATP regenerating system did not maintain the ATP concentration. Following the 10 minute incubation period there was no significant difference in the amount of ATP, ADP and AMP present in the control vessels and in those containing the ATP regenerating system. The amount of cyclic AMP accumulated in the presence of the ATP regenerating system was slightly greater than that accumulated in the controls, but this difference was not statistically significant.

In the presence of 10 mM NaF the amount of ATP hydrolyzed and the amount of AMP accumulated was significantly less than that produced in the controls or in the presence of the ATP regenerating system ($P<0.001$ in all cases). ADP accumulation in the presence of 10 mM NaF was significantly increased ($P<0.01$ compared to controls and ATP regenerating system). Approximately 80% of the ATP was hydrolyzed under these conditions: 50% was recovered as AMP and 30% as ADP. The amount of cyclic AMP accumulated in the presence of 10 mM NaF was significantly greater than that accumulated in the presence or absence of an ATP regenerating system ($P<0.001$). The combination of 10 mM NaF and the ATP regenerating system further reduced the rate of disappearance of ATP and appearance of AMP ($P<0.001$ in all cases). The rate of accumulation of ADP was the same as in the presence of NaF alone. Under these conditions approximately 50% of the ATP was hydrolyzed; 20% was

TABLE 8

The Effect of the ATP Regenerating System on the Metabolism of ATP in the Presence and Absence of NaF in the Rat Cerebral Cortex Preparation of Adenyl Cyclase

	AMP (μmoles)	Cyclic AMP (nmoles)	ADP (μmoles)	ATP (μmoles)
Control (7)	3.93 ± 0.08	4.70 ± 0.80	0.17 ± 0.05	0.08 ± 0.05
Control + ATP regenerating system (8)	3.94 ± 0.05	5.80 ± 0.86	0.16 ± 0.03	0.08 ± 0.01
NaF (9)	2.10 ± 0.09[a,b]	32.0 ± 5.40[a,b]	1.18 ± 0.08[a,b]	0.83 ± 0.14[a,b]
NaF + ATP regenerating system (8)	0.88 ± 0.21[a,b,c]	57.30 ± 18.30[a,b,d]	1.17 ± 0.13[a,b]	2.0 ± 0.21[a,b,c]

[a] $P<0.001$ compared to control ; [b] $P<0.001$ compared to control + ATP regenerating system; [c] $P<0.001$ compared to NaF; [d] $P<0.01$ compared to NaF.

2,000 x g particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37°C for 10 minutes in the presence and absence of NaF, 10 mM and an ATP regenerating system consisting of PEP, 10 mM and PK 25 μg/ml. The incubation medium of 2 ml volume contained: 40 mM Tris, pH 7.4; 3.5 mM $MgSO_4$; 6.67 mM theophylline and 2.1 mM ^{14}C-ATP (0.250 μCi/μmole). The adenine nucleotides were determined as described in the text. Numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E. AMP, ADP and ATP recovered expressed in μmoles and cyclic AMP recovered expressed in nmoles.

recovered as AMP and 28% as ADP. The amount of cyclic AMP accumulation in the presence of 10 mM NaF and the ATP regenerating system was significantly greater than that accumulated in the presence of either 10 mM NaF or the ATP regenerating system alone ($P<0.01$ compared to NaF and $P<0.001$ compared to ATP regenerating system).

Figures 7-9 show the metabolic fate of ATP incubated with the rat cerebral cortex preparation of adenyl cyclase in the absence of either an ATP regenerating system or NaF, in the presence of NaF and in the presence of the ATP regenerating system.

Figure 7 shows the metabolic fate of ATP incubated with the rat cerebral cortex preparation of adenyl cyclase. The ATP rapidly hydrolyzed; 95% was destroyed in the first minute of incubation and more than 98% in the first 4 minutes. ADP accumulated during the first minute of incubation, then declined rapidly to a concentration of 0.45 μmoles after 7 minutes of incubation. AMP accumulated at a rapid rate; after 2.5 minutes of incubation more than 70% of the added ATP could be accounted for as AMP. The AMP concentration after 7 minutes accounted for 90% of the total adenine nucleotides. Cyclic AMP accumulation reached a maximum after 2.5 minutes (2.86 nmoles) and fell slightly through the rest of the incubation period.

Figure 8 summarizes the metabolic fate of ATP incubated with this preparation of adenyl cyclase in the presence of 10 mM NaF. In the first minute of incubation the ATP concentration decreased by 77% to 0.94 μmoles. Thereafter the concentration of this nucleotide declined slowly, to 0.72 μmoles at 4 minutes and 0.61 μmoles after 7 minutes of incubation. ADP accumulated rapidly in the first minute of incubation and accounted for more

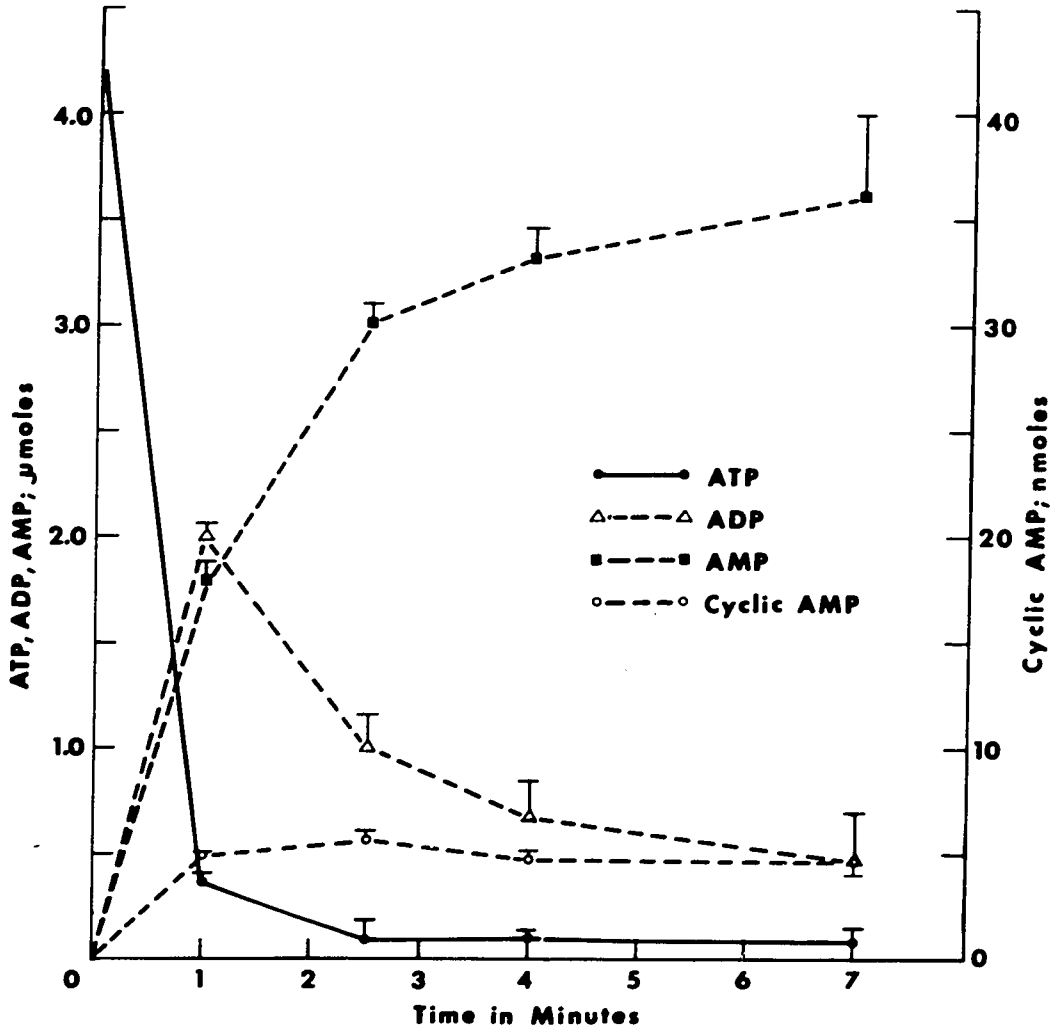


FIGURE 7: The Metabolic Fate of ATP Incubated with the Rat Cerebral Cortex Preparation of Adenyl Cyclase

2,000 x g particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37°C in a total volume of 2 ml, in a medium containing: 40 mM Tris, pH 7.4; 3.5 mM $MgSO_4$; 6.67 mM theophylline and 2.1 mM ^{14}C-ATP (0.250 µCi/µmole). The incubation was stopped at the times indicated and the adenine nucleotides determined as described in the text. Results are a mean of 3 observations and vertical bars represent ± S.E. AMP, ADP and ATP recovered expressed in µmoles and cyclic AMP recovered expressed in nmoles.

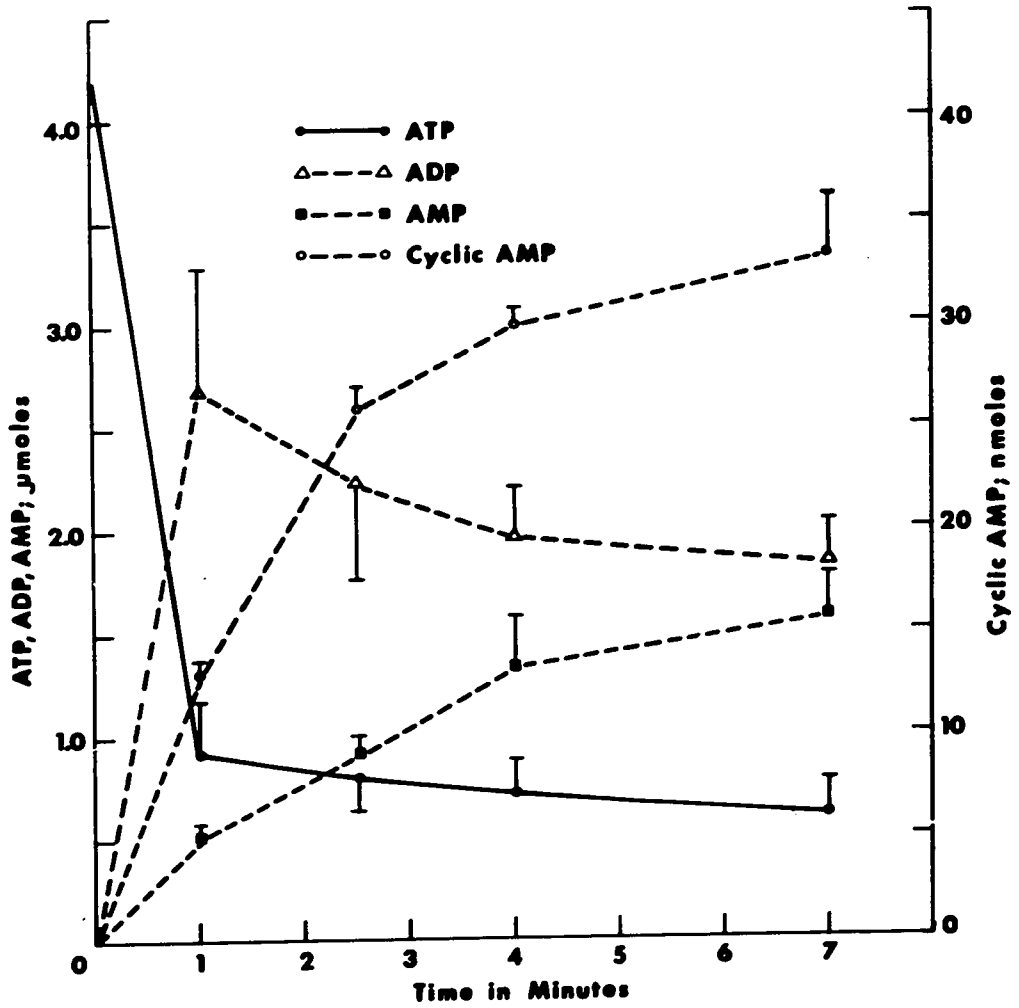


FIGURE 8: The Metabolic Fate of ATP Incubated with the Rat Cerebral Cortex Preparation of Adenyl Cyclase in the Presence of NaF

2,000 x g particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37°C in a total volume of 2 ml, in a medium containing: 40 mM Tris, pH 7.4; 3.5 mM $MgSO_4$; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 µCi/µmole) and 10 mM NaF. The incubation was stopped at the times indicated and the adenine nucleotides determined as described in the text. Results are a mean of 3 observations and vertical bars represent ± S.E. AMP, ADP and ATP recovered expressed in µmoles and cyclic AMP recovered expressed in nmoles.

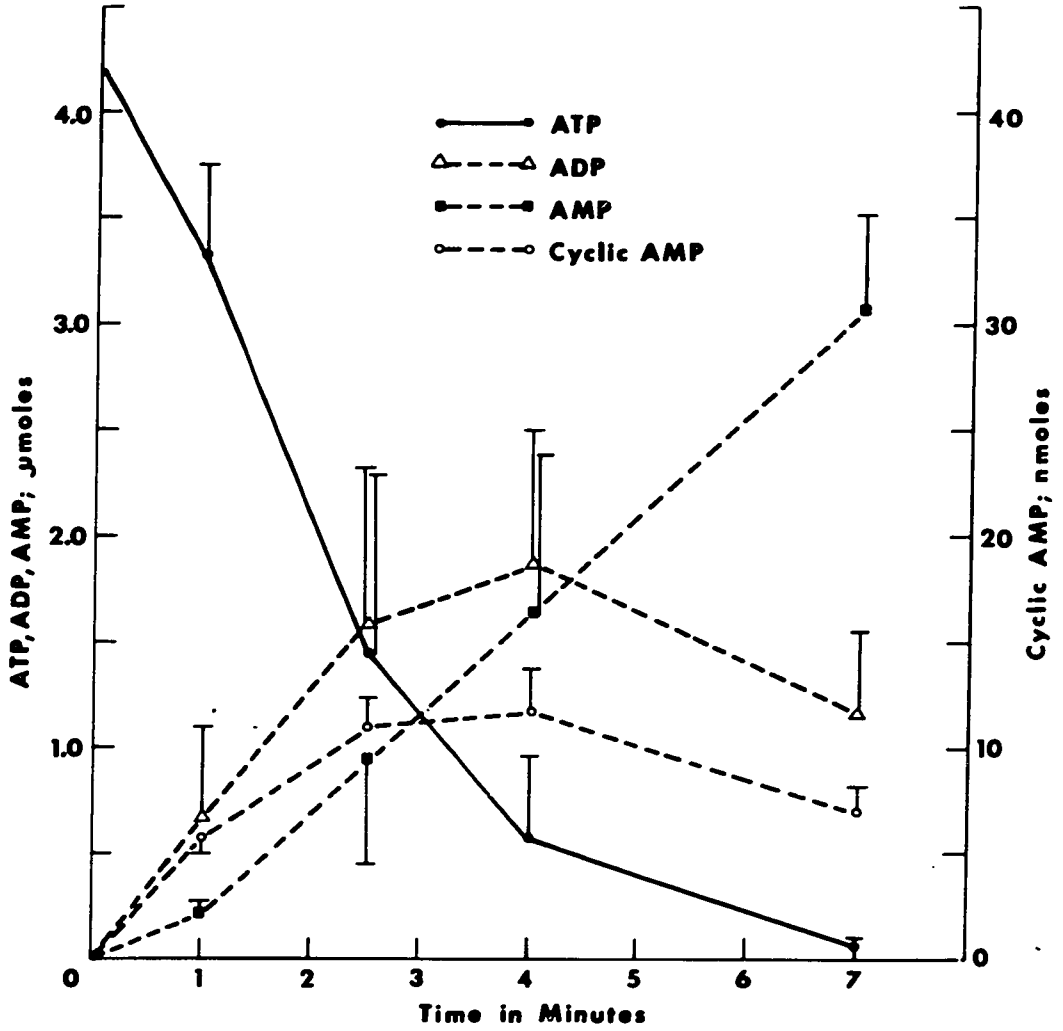


FIGURE 9: The Metabolic Fate of ATP Incubated with the Rat Cerebral Cortex Preparation of Adenyl Cyclase in the Presence of the ATP Regenerating System

2,000 x g particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37°C in a total volume of 2 ml, in a medium containing: 40 mM Tris, pH 7.4; 3.5 mM $MgSO_4$; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 µCi/µmole) and the ATP regenerating system consisting of 10 mM PEP and 25 µg/ml PK. The incubation was stopped at the times indicated and the adenine nucleotides determined as described in the text. Results are a mean of 3 observations and vertical bars represent ± S.E. AMP, ADP and ATP recovered expressed in µmoles and cyclic AMP recovered expressed in nmoles.

than 80% of the ATP that disappeared during this time. The ADP concentration fell slowly for the rest of the incubation period to a concentration of 1.83 μmoles at 7 minutes. AMP accumulated slowly over the entire incubation period to reach a concentration of 1.57 μmoles at 7 minutes. The accumulation of cyclic AMP was linear for the first 2.5 minutes of incubation and lower from there on reaching a value of 16.7 nmoles after 7 minutes of incubation.

Figure 9 shows the metabolism of ATP incubated with this preparation of adenyl cyclase in the presence of the ATP regenerating system. The rate of disappearance of ATP was clearly slower than it was in the absence of the regenerating system or when NaF alone was present. The concentration of ATP fell by only 25% in the first minute of incubation. From this time, the ATP concentration fell rapidly so that by 4 minutes it was at a lower concentration than it was in the presence of NaF (0.59 μmoles compared to 0.72 μmoles). After 7 minutes the ATP concentration was similar to that present in the controls (0.07 μmoles compared to 0.09 μmoles). ADP concentration increased during the first 2.5 minutes of incubation at a rate that approximately paralleled the rate of disappearance of ATP. The ADP concentration remained constant for the next 1.5 minutes and then fell rapidly over the final 3 minutes of incubation to 1.16 μmoles. The rate of accumulation of AMP was slow for the initial 4 minutes of incubation. Its appearance in the next 3 minutes paralleled the rate of disappearance of ADP. There was a net accumulation of 11.0 nmoles cyclic AMP during the first 2.5 minutes of incubation. The rate of accumulation of cyclic AMP fell significantly between 2.5 and 4 minutes and this occurred when the ATP concentration fell from 1.45 μmoles to 0.59 μmoles. During the last

3 minutes of incubation there was a net destruction of cyclic AMP so that its final concentration was 7.0 nmoles as compared to 11.7 nmoles at the peak of its accumulation. The destruction of this nucleotide coincided in time with the disappearance of ADP.

Figure 10 summarizes the rate of accumulation of cyclic AMP under the three conditions tested. In the controls, the amount of cyclic AMP accumulated during the first minute was 5.0 nmoles. The rate of accumulation was positive but lower during the next 1.5 minutes (0.37 nmoles/minute) and negative from thereon. In the presence of 10 mM NaF the rate of cyclic AMP accumulation was always positive. The largest net accumulation occurred at the early time periods; 13.0 nmoles/minute from 0-1 minute and 8.6 nmoles/minute from 1-2.5 minutes. Cyclic AMP accumulation was reduced to 2.7 nmoles/minute during the next 1.5 minutes and 1.3 nmoles/minute in the final 3 minutes of incubation. Thus, although the rate of accumulation of cyclic AMP in the presence of NaF was falling during the final stages of the incubation, it remained greater than the rate of cyclic AMP destruction. The rate of accumulation of cyclic AMP in the presence of the ATP regenerating system was rapid for the first 2.5 minutes of incubation: 5.7 nmoles/minute from 0-1 minute and 4.5 nmoles for the next 1.5 minutes. During the final 3 minutes, however, there was a net destruction of cyclic AMP (1.5 nmoles/minute). Due to this net destruction of cyclic AMP, the amount of cyclic AMP accumulated after 7 minutes incubation in the presence of the ATP regenerating system was significantly less than that accumulated in the presence of NaF ($P<0.05$). In fact, whereas after 4 minutes of incubation the regenerating system increased cyclic AMP accumulation 110%, compared to

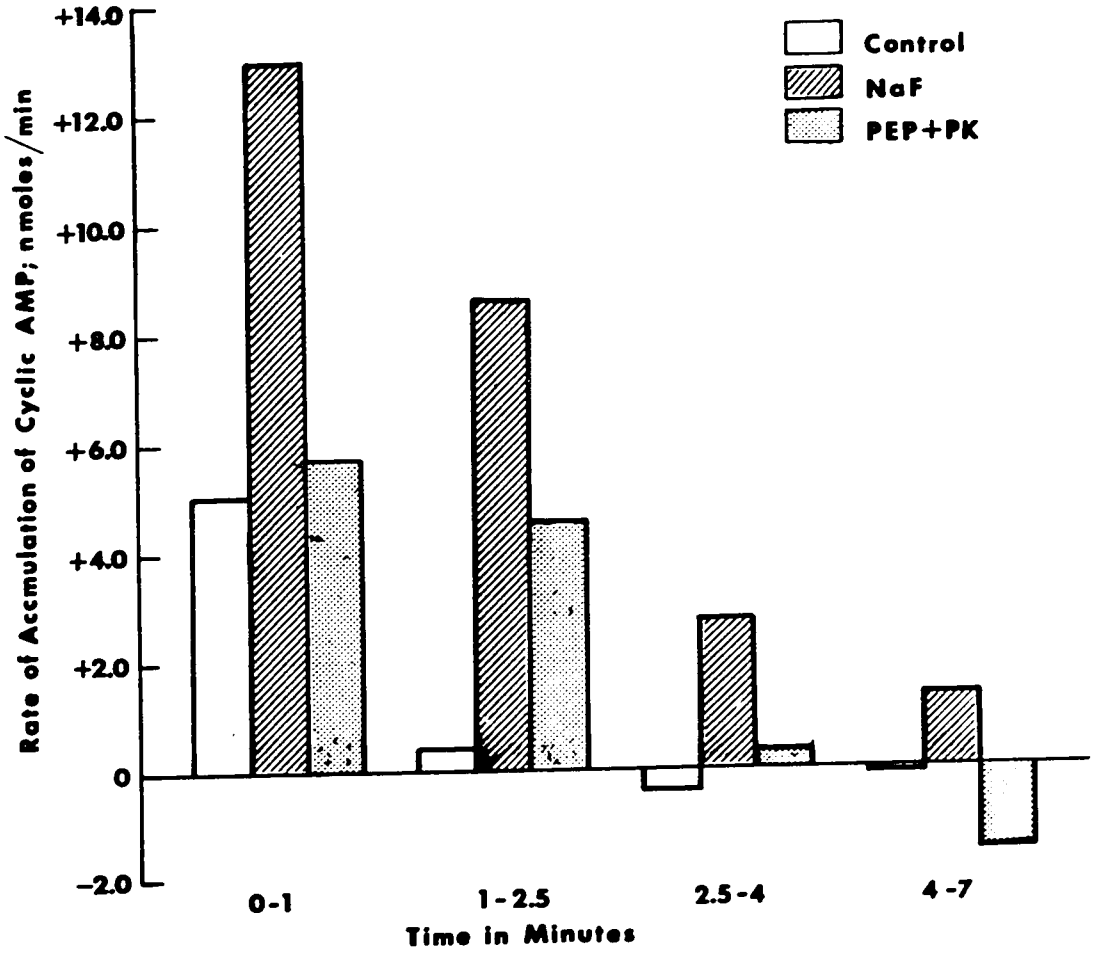


FIGURE 10: The Effect of NaF and the ATP Regenerating System on the Rate of Accumulation of Cyclic AMP in the Standard Cerebral Cortex Adenyl Cyclase Assay

The accumulation of cyclic AMP under the incubation conditions described in the controls (FIG. 8), in the presence of NaF (FIG. 9) and in the presence of the ATP regenerating system (FIG. 10) expressed in nmoles/minute.

the controls, after 7 minutes there was no difference between the amount accumulated in the presence and absence of the regenerating system.

Table 9 summarizes the effects of adrenaline (0.1 mM) or ouabain (0.1 mM) on ATP metabolism and cyclic AMP accumulation in the rat cerebral cortex preparation of adenyl cyclase. There was little difference in the amount of AMP, ADP and ATP recovered in the presence and absence of adrenaline or of ouabain after 10 minutes of incubation. There was a significant increase, however, in the amount of cyclic AMP accumulated in the presence of adrenaline or ouabain compared to the controls ($P<0.02$ in both cases).

The effect of α- and β-adrenergic blocking agents on cyclic AMP accumulation in this preparation of adenyl cyclase is summarized in Table 10. Phenoxybenzamine (POB) was used as the α-adrenergic blocker and pronethalol as the β-adrenergic blocking drug. Both these agents were used at a concentration of 0.1 mM and were added to the incubation medium 10 minutes prior to the start of the reaction. Neither POB nor pronethalol had any effect on control levels of cyclic AMP accumulation. Both agents, however, inhibited adrenaline-stimulated cyclic AMP accumulation.

Table 11 summarizes the effect of Mg^{++} on ATP metabolism and cyclic AMP accumulation in this preparation of adenyl cyclase. When equimolar (3.5 mM) Mn^{++} was added to the incubation medium in the place of Mg^{++}, the amount of ATP hydrolyzed during the 10 minute incubation period was significantly reduced ($P<0.01$). There was also a significant reduction in AMP accumulation ($P<0.001$) and a significant increase in ADP accumulation ($P<0.001$). The amount of cyclic AMP accumulated in the presence of Mn^{++} was significantly increased ($P<0.001$). Incubation in the presence of both Mn^{++} and 10 mM NaF produced a slightly greater inhibition of ATP hydrolysis

TABLE 9

The Effect of Adrenaline and Ouabain on ATP Metabolism in the Rat Cerebral Cortex Preparation of Adenyl Cyclase

	AMP (μmoles)	Cyclic AMP (nmoles)	ADP (μmoles)	ATP (μmoles)
Control (9)	3.94 ± 0.06	5.80 ± 0.86	0.16 ± 0.03	0.08 ± 0.01
Ouabain, 0.1 mM (7)	3.73 ± 0.26	12.30 ± 2.20[a]	0.29 ± 0.08	0.08 ± 0.02
Adrenaline, 0.1 mM (6)	3.81 ± 0.33	10.90 ± 1.76[a]	0.19 ± 0.05	0.10 ± 0.03

[a] $P<0.02$ compared to control

2,000 x g particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37°C for 10 minutes in the presence or the absence of adrenaline (0.1 mM) or ouabain (0.1 mM). The incubation medium of 2 ml volume also contained: 40 mM Tris, pH 7.4; 3.5 mM $MgSO_4$; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 μCi/μmole), and the ATP regenerating system consisting of 10 mM PEP and 25 μg/ml PK. The adenine nucleotides were determined as described in the text. Numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E. AMP, ADP and ATP recovered expressed in μmoles and cyclic AMP recovered expressed in nmoles.

TABLE 10

The Effect of α- and β-Adrenergic Blocking Drugs on Cyclic AMP Accumulation in Rat Cerebral Cortex Preparation of Adenyl Cyclase

Additions		Cyclic AMP (nmoles)
None	(9)	5.80 ± 0.86[a]
Adrenaline	(6)	10.90 ± 1.76
POB	(3)	5.50 ± 2.10
Pronethalol	(3)	5.40 ± 2.27
Adrenaline + POB	(3)	5.50 ± 2.10
Adrenaline + Pronethalol	(3)	5.60 ± 1.95

[a] $P<0.02$ compared to adrenaline

2,000 x g particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37°C for 20 minutes in the presence or the absence of 0.1 mM POB or 0.1 mM pronethalol. The incubation medium of 2 ml volume also contained: 40 mM Tris, pH 7.4; 3.5 mM $MgSO_4$; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 μCi/μmole); 10 mM PEP and 25 μg/ml PK and where indicated 0.10 mM adrenaline. The reaction was begun with the addition of ^{14}C-ATP after the blocking agents had been in contact with the preparation for 10 minutes. The adenine nucleotides were determined as described in the text. Numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E. Cyclic AMP recovered expressed in nmoles.

TABLE 11

The Effect of Mn^{++} on ATP Metabolism in the Rat Cerebral Cortex Preparation of Adenyl Cyclase

		AMP (μmoles)	Cyclic AMP (nmoles)	ADP (μmoles)	ATP (μmoles)
Mg^{++}	(8)	3.88 ± 0.06	5.80 ± 0.86	0.16 ± 0.03	0.05 ± 0.01
Mg^{++} + NaF	(8)	0.88 ± 0.21	57.30 ± 6.20	1.17 ± 0.13	2.00 ± 0.21
Mn^{++}	(4)	1.67 ± 0.22[a]	30.10 ± 4.50[a]	0.98 ± 0.17[a]	1.42 ± 0.51[b]
Mn^{++} + NaF	(3)	0.84 ± 0.45[a]	114.00 ± 18.30[c,d,a]	0.57 ± 0.28	2.58 ± 0.61[a]

[a] $P<0.001$ compared to Mg^{++}; [b] $P<0.01$ compared to Mg^{++}; [c] $P<0.001$ compared to Mn^{++}; [d] $P<0.01$ compared to Mg^{++} + NaF

2,000 x g particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37°C for 10 minutes in the presence of either 3.5 mM $MgSO_4$ or 3.5 mM $MnCl_2$. In addition, the incubation medium of 2 ml volume contained: 40 mM Tris, pH 7.4; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 μCi/μmole); 10 mM PEP and 25 μg/ml PK and where indicated, 10 mM NaF. The adenine nucleotides were determined as described in the text. Numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E. AMP, ADP and ATP recovered expressed in μmoles and cyclic AMP recovered expressed in nmoles.

and AMP accumulation but these changes were not significantly different from the values obtained in the presence of Mn^{++} alone. Under these conditions, however, there was a significant increase in cyclic AMP accumulation ($P<0.001$). The amount of cyclic AMP accumulated was also significantly greater than that seen in the presence of Mg^{++} and NaF ($P<0.01$). The amount of ATP hydrolyzed and AMP accumulated in the presence of Mn^{++} and NaF was not significantly different from that observed in the presence of Mg^{++} and NaF.

Table 12 summarizes the effect of 0.1 mM adrenaline and 0.1 mM ouabain on cyclic AMP accumulation in the rat cerebral cortex preparation of adenyl cyclase incubated in the presence of Mn^{++} (3.5 mM). Adrenaline had no effect on the rate of cyclic AMP accumulation. There was a slight increase in the amount of cyclic AMP accumulated in the presence of ouabain, but this difference was not statistically significant. Thus, whereas adrenaline and ouabain (0.1 mM) caused a significant increase in the amount of cyclic AMP accumulated in this preparation in the presence of Mg^{++} (Table 9), these agents were ineffective when Mg^{++} was replaced by an equimolar concentration of Mn^{++}.

B. GUINEA-PIG PANCREAS PREPARATION.

Figure 11 shows the results of a typical experiment that tested the relationship of cyclic AMP accumulation to tissue concentration of the guinea-pig pancreas preparation of adenyl cyclase. From 50 mg wet weight tissue to 650 mg wet weight tissue was tested. The ATP regenerating system was present throughout these experiments. In the absence of NaF the amount of cyclic AMP accumulated was very small and varied little over the range of

TABLE 12

The Effect of Adrenaline and Ouabain on Cyclic AMP Accumulation in the Rat Cerebral Cortex Preparation of Adenyl Cyclase Incubated in Mn^{++}

		Cyclic AMP (nmoles)
Control	(4)	30.10 ± 4.50
Adrenaline	(5)	26.60 ± 3.60
Ouabain	(5)	35.80 ± 3.70

2,000 x g particulate preparation of rat cerebral cortex (450 mg wet weight tissue) was incubated at 37°C for 10 minutes in $MnCl_2$, 3.5 mM in the presence and absence of 0.1 mM adrenaline or 0.1 mM ouabain. The incubation medium also contained: 40 mM Tris, pH 7.4; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 µCi/µmole) and PEP 10 mM and PK 25 µg/ml. Cyclic AMP accumulation was determined as described in the text. Numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E. Cyclic AMP recovered expressed in nmoles.

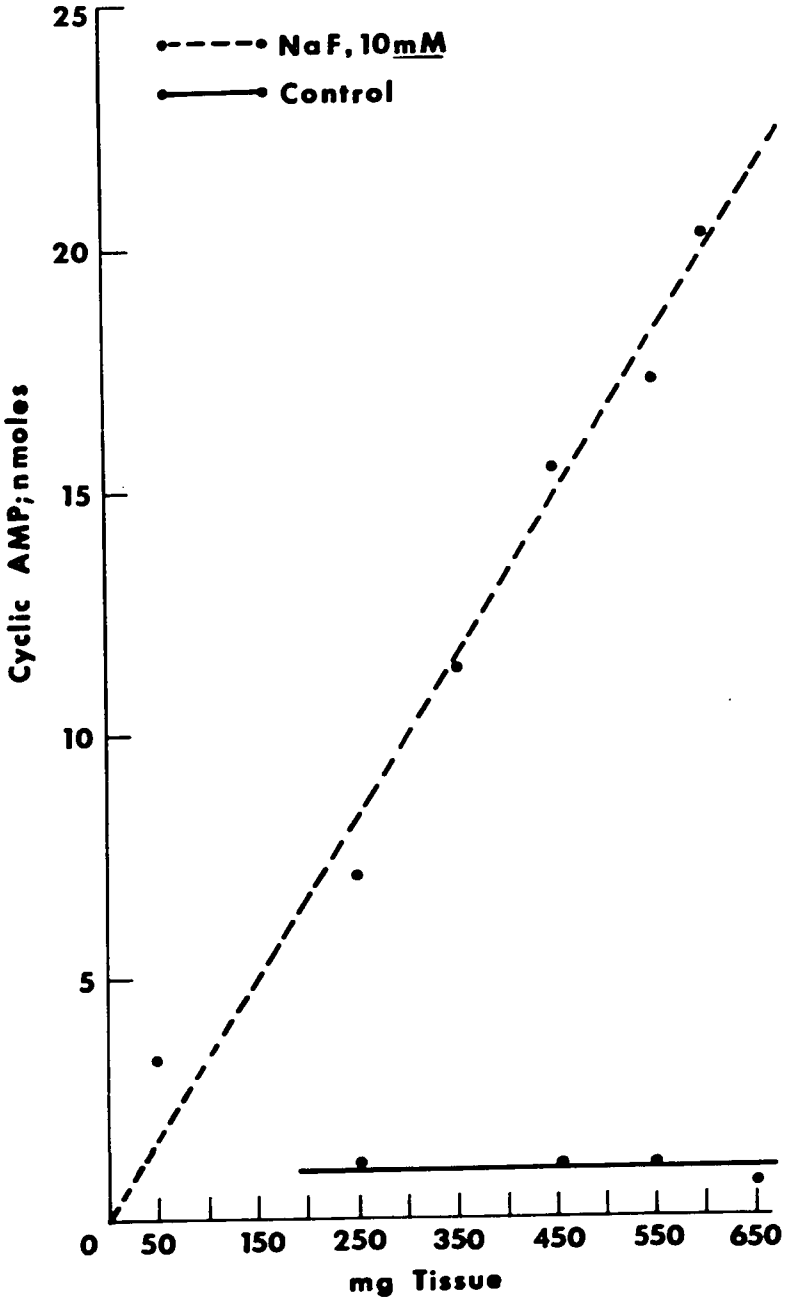


FIGURE 11: Cyclic AMP Accumulation as a Function of Tissue Concentration in the Guinea-Pig Pancreas Preparation of Adenyl Cyclase

Increasing amounts of a 3,000 x g particulate preparation from guinea-pig pancreas were incubated at 37°C for 10 minutes in the presence and absence of 10 mM NaF. Incubation medium of 2 ml volume contained: 40 mM Tris; pH 7.4; 3.5 mM $MgSO_4$; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 μCi/μmole) and the ATP regenerating system consisting of 10 mM PEP and 25 μg/ml PK. Cyclic AMP accumulation was determined as described in text.

tissue concentrations tested. In the presence of 10 mM NaF cyclic AMP accumulation was greatly increased and was linear through the entire range of tissue concentrations tested.

A tissue concentration of 450 mg wet weight tissue was used in all further experiments with the guinea-pig pancreas adenyl cyclase preparation.

Table 13 summarizes the metabolic fate of ATP in the presence and absence of 10 mM NaF in this preparation of adenyl cyclase. In the control experiments approximately 40% of the ATP was hydrolyzed during the 10 minute incubation period; 25% was recovered as ADP and 14% as AMP. In the presence of 10 mM NaF the amount of ATP hydrolyzed was slightly but not significantly reduced; 25% of the ATP was hydrolyzed, 18% recovered as ADP and 5% as AMP. There was a significant increase in the accumulation of cyclic AMP in the presence of 10 mM NaF ($P<0.001$).

The effects of a wide range of agents on cyclic AMP accumulation in this preparation of adenyl cyclase is summarized in Figure 12. Carbachol (0.1 mM and 1 mM), pancreozymin (0.9 and 1.8 international units (I.U.)), adrenaline (0.1 mM and 1.0 mM), and secretin (1.0 and 3.0 I.U.) were tested. None of these agents significantly increased the accumulation of cyclic AMP. Other experiments showed that preparations of adenyl cyclase from rat and cat pancreas were also stimulated by NaF and were not affected by the agents listed above.

DISCUSSION: STUDIES ON CYCLIC AMP ACCUMULATION IN ADENYL CYCLASE PREPARATIONS OF RAT CEREBRAL CORTEX AND GUINEA-PIG PANCREAS.

In these experiments the relationship of adenyl cyclase to the activity of other membrane-bound ATP utilizing enzymes was studied in low-speed sediment

TABLE 13

The Metabolic Fate of ATP in the Presence and Absence of NaF in the Guinea-Pig Pancreas Preparation of Adenyl Cyclase

		AMP (μmoles)	Cyclic AMP (nmoles)	ADP (μmoles)	ATP (μmoles)
Control	(11)	0.54 ± 0.21	2.90 ± 0.40	1.15 ± 0.25	2.47 ± 0.37
NaF	(11)	0.21 ± 0.07	8.90 ± 1.30[a]	0.74 ± 0.18	3.21 ± 0.24

[a] $P<0.001$ compared to control

3,000 x g particulate preparation of guinea-pig pancreas (450 mg wet weight tissue), was incubated at 37°C for 10 minutes in the presence and absence of 10 mM NaF. The incubation medium of 2 ml volume contained: 40 mM Tris, pH 7.4; 3.5 mM $MgSO_4$; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 μCi/μmole) and 10 mM PEP and 25 μg/ml PK. The adenine nucleotides were determined as described in the text. Numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E. AMP, ADP and ATP recovered expressed in μmoles and cyclic AMP recovered expressed in nmoles.

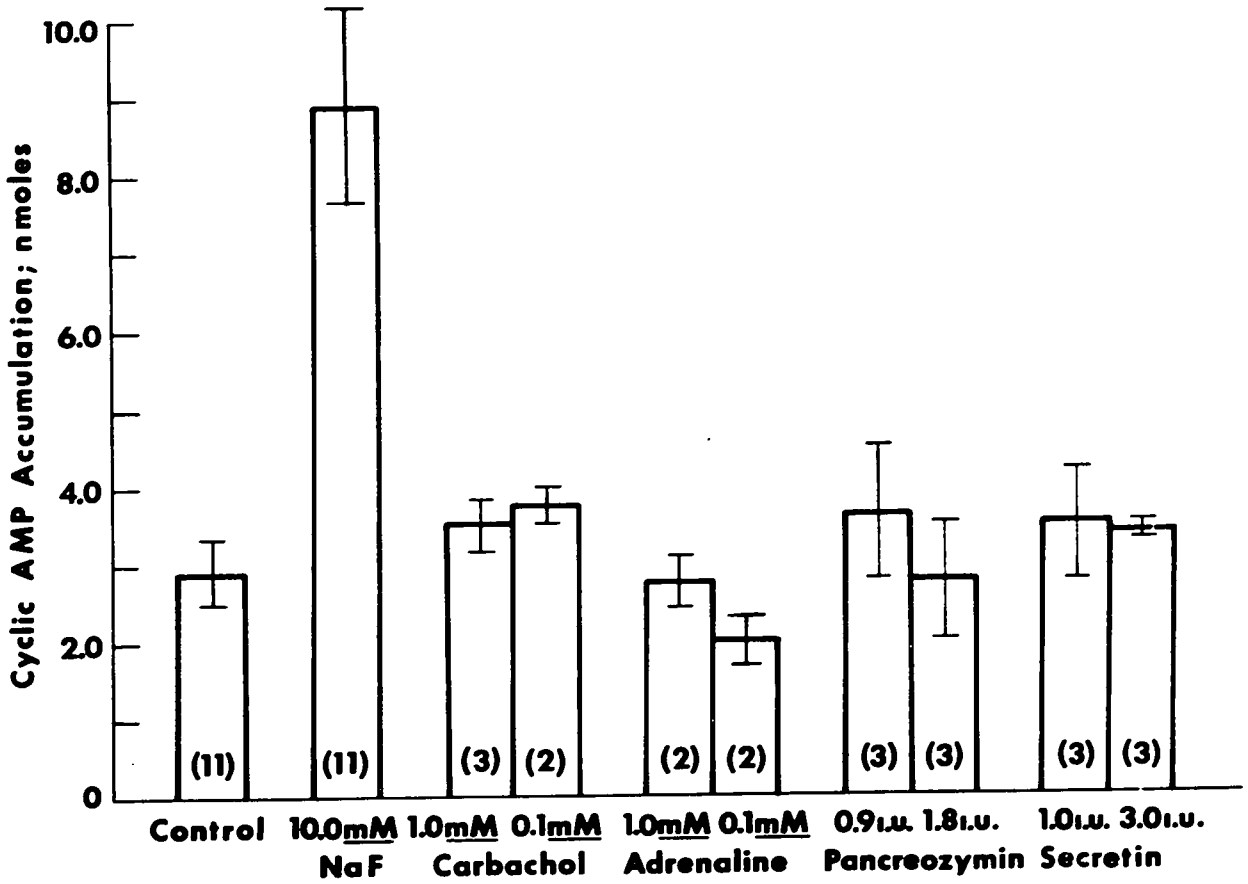


FIGURE 12: The Effect of Various Agents on the Accumulation of Cyclic AMP in the Guinea-Pig Pancreas Preparation of Adenyl Cyclase

3,000 x g particulate preparation of guinea-pig pancreas (450 mg wet weight tissue) was incubated at 37°C for 10 minutes in the presence of the various agents illustrated in the graph. The incubation medium of 2 ml volume also contained: 40 mM Tris, pH 7.4; 3.5 mM $MgSO_4$; 6.67 mM theophylline; 2.1 mM ATP (0.250 μCi/μmole) and the ATP regenerating system consisting of 10 mM PEP and 25 μg/ml PK. Cyclic AMP accumulation was determined as described in the text. Numbers in brackets indicate number of experiments per group. Vertical lines represent ± S.E.

preparations from rat brain cortex and guinea-pig pancreas. Preparations of adenyl cyclase from brain cortices had previously been shown to accumulate the largest amount of cyclic AMP of any tissue investigated (Sutherland et al., 1962), and were thus ideal preparations in which to study cyclic AMP metabolism.

The present studies confirm and extend previous demonstrations that cyclic AMP accumulation in this preparation is stimulated by NaF, Mn^{++}, adrenaline and ouabain. They show, in addition, that there was a rapid hydrolysis of ATP in this preparation, greater than could be accounted for by the production of cyclic AMP. ADP hydrolysis, as measured by the rate of AMP accumulation was also observed. It was also found that the ATP regenerating system used did not maintain the ATP concentration.

Agents that stimulated cyclic AMP accumulation in this preparation may have doneso by affecting the other enzyme systems present. This is suggested by the observation that in the presence of 10 mM NaF there was a significant inhibition of ATP and ADP hydrolysis as well as a significant increase in cyclic AMP accumulation. This suggestion is also supported by the finding that Mn^{++} significantly inhibited ATP hydrolysis and AMP accumulation while significantly increasing cyclic AMP accumulation. On the other hand, ouabain and adrenaline stimulated cyclic AMP accumulation in this preparation without affecting the rate of ATP hydrolysis or ADP and AMP accumulation.

Preparations of adenyl cyclase from guinea-pig pancreas exhibited lower amounts of ATP and ADP hydrolysis than the rat cerebral cortex preparation. Cyclic AMP accumulation, however, could not be stimulated by agents other than NaF in this preparation.

On the basis of these findings it appeared desirable to prepare an adenyl cyclase fraction free of contaminating enzymes in order to show conclusively that the various agents that stimulate cyclic AMP accumulation are affecting adenyl cyclase directly.

It was shown (De Robertis et al., 1967) that synaptic membrane preparations of brain adenyl cyclase accumulated large quantities of cyclic AMP and that these preparations were relatively free of mitochondrial contamination; they should therefore have reduced levels of "non-specific" ATP hydrolysis. A synaptic membrane preparation of rat cerebral cortex adenyl cyclase was used in the next group of experiments to further elucidate the relationship of adenyl cyclase to other membrane-bound ATP utilizing enzymes.

C. SYNAPTIC MEMBRANE PREPARATION OF ADENYL CYCLASE FROM RAT CEREBRAL CORTEX.

Figure 13 shows the results of a typical experiment that tested the relationship of cyclic AMP accumulation to tissue concentration of the synaptic membrane preparation of adenyl cyclase from rat cerebral cortex. From 1.1-4.4 mg protein, the equivalent of 250-1160 mg wet weight tissue, was used. The amount of cyclic AMP accumulated during the 10 minute incubation period increased with tissue concentration both in the absence and presence of NaF (10 mM).

In all subsequent experiments with this preparation, 2.0 mg protein per vessel was used. The results were expressed as μmoles nucleotide per mg protein and that of cyclic AMP accumulation as nmoles per mg protein.

Table 14 summarizes the effect of the ATP regenerating system on the metabolism of ATP in the presence and absence of NaF in this preparation of adenyl cyclase. In the control experiments, approximately 43% of the ATP

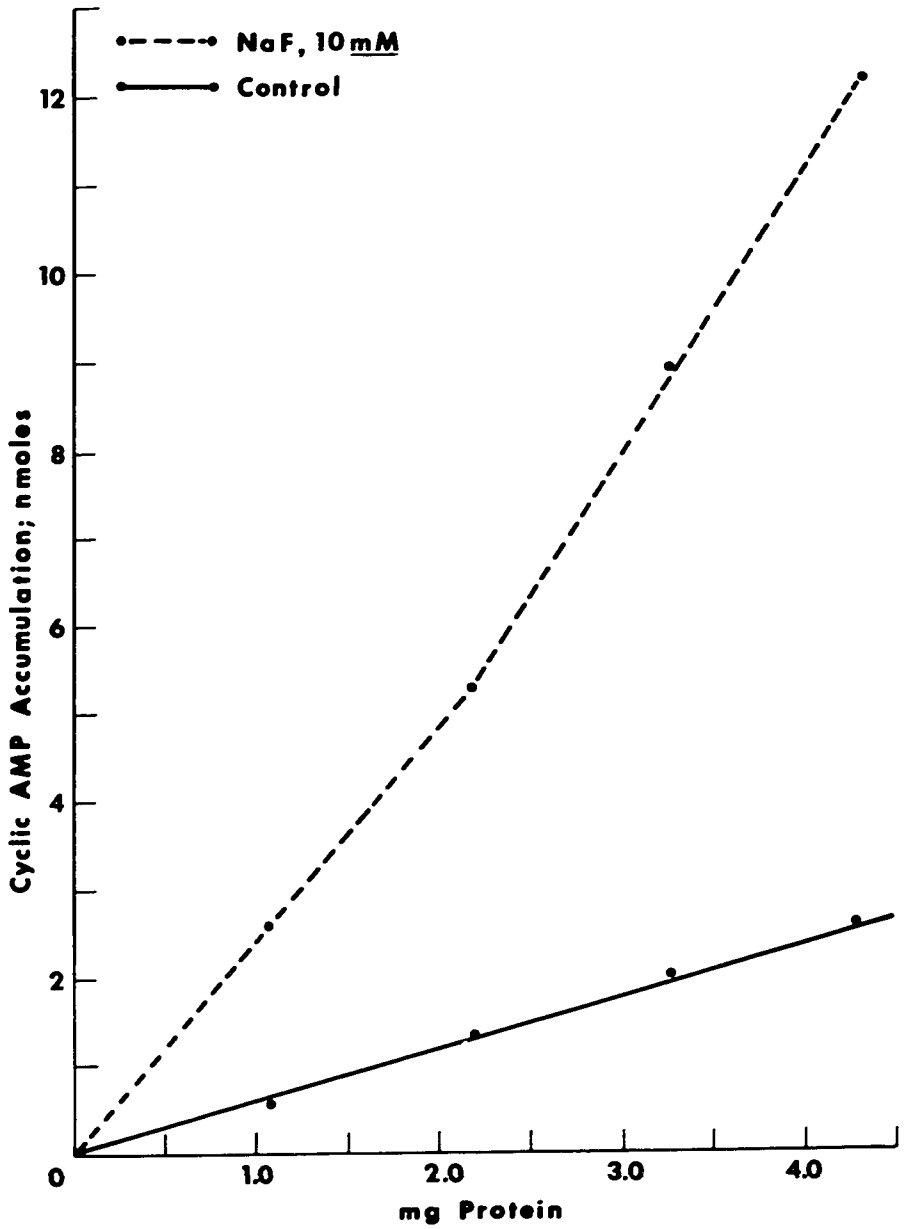


FIGURE 13: Cyclic AMP Accumulation as a Function of Tissue Concentration in the Synaptic Membrane Preparation of Adenyl Cyclase

Increasing amounts of a 1.2 M_1 fraction of rat cerebral cortex homogenate prepared as described in methods was incubated for 10 minutes at 37°C in the presence and absence of 10 mM NaF. The incubation medium of 2 ml volume contained: 40 mM Tris, pH 7.4; 5.0 mM $MgSO_4$; 6.67 mM theophylline; and 2.1 mM ^{14}C-ATP (0.250 µCi/µmole). Cyclic AMP accumulation was determined as described in text.

TABLE 14

The Effect of the ATP Regenerating System on the Metabolism of ATP in the Presence and Absence of NaF in the Synaptic Membrane Preparation of Adenyl Cyclase

	AMP (μmoles/mg protein)	Cyclic AMP (nmoles/mg protein)	ADP (μmoles/mg protein)	ATP (μmoles/mg protein)
Control (5)	0.45 ± 0.04	1.60 ± 0.26	0.69 ± 0.11	0.90 ± 0.17
Control + ATP regenerating system (7)	0.21 ± 0.06[b]	2.10 ± 0.34	0.61 ± 0.14	1.16 ± 0.19
NaF (5)	0.14 ± 0.01[a]	5.60 ± 1.30[c,g]	0.49 ± 0.10	1.40 ± 0.16
NaF + ATP regenerating system (5)	0.04 ± 0.01[a,d,g]	6.60 ± 0.84[c,f]	0.01 ± 0.00[a,e,f]	1.97 ± 0.02[a,e,f]

[a] $P<0.001$ compared to control; [b] $P<0.01$ compared to control; [c] $P<0.02$ compared to control; [d] $P<0.001$ compared to NaF; [e] $P<0.01$ compared to NaF; [f] $P<0.01$ compared to control + ATP regenerating system; [g] $P<0.02$ compared to control + ATP regenerating system

1.2 M_1 fraction of rat cerebral cortex homogenate prepared as described in methods (2.0 mg protein) was incubated for 10 minutes at 37°C in the presence and absence of NaF, 10 mM and an ATP regenerating system consisting of PEP, 10 mM and PK, 25 μg/ml. The incubation medium of 2 ml volume also contained: 40 mM Tris, pH 7.4; 5.0 mM $MgSO_4$; 6.67 mM theophylline; and 2.1 mM ^{14}C-ATP (0.250 μCi/μmole). Numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E. AMP, ADP and ATP recovered expressed in μmoles/mg protein and cyclic AMP recovered expressed in nmoles/mg protein.

concentration was present following the 10 minute incubation period, with 33% of total nucleotide recovered as ADP and 23% as AMP. The amount of cyclic AMP accumulated on a mg protein basis was 11 times higher than that observed in the controls of the rat cerebral cortex preparation of adenyl cyclase (Part 2, section A). When 10 mM NaF was added to the incubation mixture the amount of ATP hydrolyzed was somewhat reduced, but this reduction was not statistically significant ($P<0.10$). The amount of AMP accumulated in the presence of NaF, however, was significantly reduced from that accumulated in the controls ($P<0.001$). NaF produced a significant increase in the amount of cyclic AMP accumulated ($P<0.02$). The amount of cyclic AMP accumulated on a mg protein basis was 5.6 times higher than that observed in the presence of 10 mM NaF in the rat cerebral cortex preparation of adenyl cyclase. When the ATP regenerating system was present, the concentration of ATP following the 10 minute incubation period was not significantly different from the controls but the amount of AMP accumulated was significantly decreased ($P<0.01$). The amount of cyclic AMP accumulated under these conditions was slightly but not significantly higher than that accumulated in the controls ($P<0.30$) but significantly lower than that accumulated in the presence of 10 mM NaF ($P<0.02$). In the presence of 10 mM NaF and the ATP regenerating system the concentration of ATP had fallen only 2%. Under these conditions the concentration of ATP, following the 10 minute incubation period, was significantly higher than the other three conditions ($P<0.01$ compared to NaF alone and the ATP regenerating system alone, and $P<0.001$ compared to the controls), and the amount of AMP recovered was significantly reduced compared to the amount recovered in the controls or with NaF alone ($P<0.001$) and that recovered in the presence of the ATP regenerating

system alone ($P<0.02$). The amount of ADP produced under these conditions was also significantly reduced as compared to the three other conditions ($P<0.001$ compared to the controls, $P<0.01$ compared to the ATP regenerating system alone and NaF alone). The amount of cyclic AMP accumulated in the presence of 10 mM NaF and the ATP regenerating system was slightly greater than that accumulated in the presence of NaF alone, but this difference was not statistically significant ($P<0.60$).

Figures 14, 15, 16 and 17 show the metabolic fate of ATP incubated with the synaptic membrane preparation of adenyl cyclase of rat cerebral cortex under the following conditions: in the absence of NaF or the ATP regenerating system, in the presence of 10 mM NaF, in the presence of the ATP regenerating system and in the presence of both NaF and the ATP regenerating system.

In the absence of NaF or the ATP regenerating system (Fig. 14), 33% of the ATP was hydrolyzed in the first minute of incubation. Following 3 minutes of incubation 48% of the ATP had been hydrolyzed, and 57% had been hydrolyzed after 6 minutes of incubation. From this time to the end of the incubation period little further change in the level of ATP occurred. ADP accumulated rapidly during the first minute of incubation, reached a plateau at 3 minutes, then declined to a concentration of 0.59 μmoles/mg protein after 10 minutes of incubation. The AMP concentration increased steadily with time to a value of 0.45 μmoles/mg protein after 10 minutes. Cyclic AMP accumulation under these conditions was linear for the first 3 minutes of incubation reaching a maximum of 2.0 nmoles/mg protein; the concentration of cyclic AMP then fell slightly during the next 3 minutes and remained constant throughout the rest of the incubation period.

Figure 15 shows the metabolic fate of ATP incubated with this

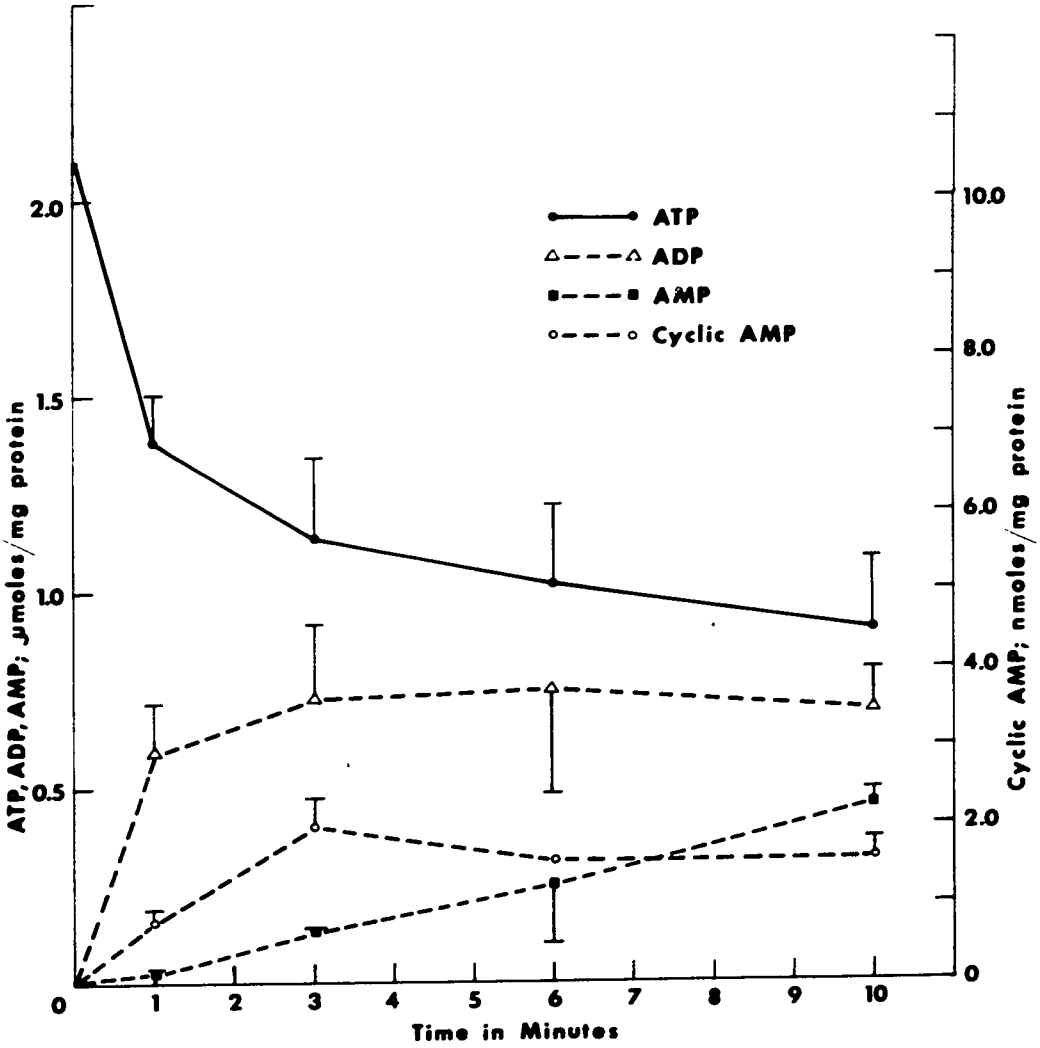


FIGURE 14: The Metabolic Fate of ATP Incubated with the Synaptic Membrane Preparation of Adenyl Cyclase

1.2 M_1 fraction of rat cerebral cortex homogenate prepared as described in methods (2.0 mg protein) was incubated at 37°C in a total volume of 2 ml, in a medium containing: 40 mM Tris, pH 7.4; 5.0 mM $MgSO_4$; 6.67 mM theophylline and 2.1 mM ^{14}C-ATP (0.250 μCi/μmole). The incubation was stopped at the times indicated and the adenine nucleotides determined as described in text. Results are a mean of 2 observations at 1, 3 and 6 minutes and 5 observations at 10 minutes. Vertical bars represent ± S.E. AMP, ADP and ATP recovered expressed in μmoles/mg protein and cyclic AMP expressed in nmoles/mg protein.

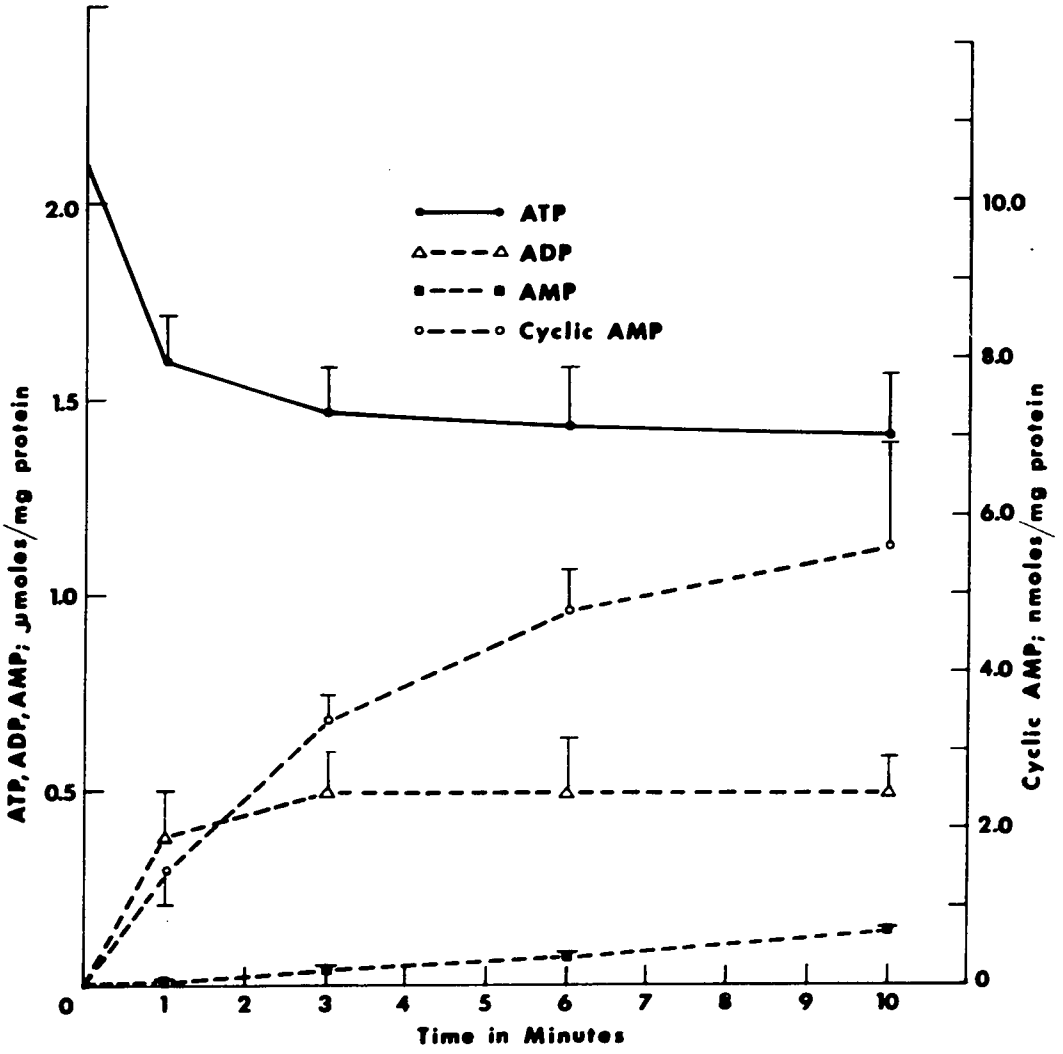


FIGURE 15: The Metabolic Fate of ATP Incubated with the Synaptic Membrane Preparation of Adenyl Cyclase in the Presence of NaF

1.2 M_1 fraction of rat cerebral cortex homogenate prepared as described in methods (2.0 mg protein) was incubated at 37°C in a total volume of 2 ml, in a medium containing: 40 mM Tris, pH 7.4; 5.0 mM $MgSO_4$; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 µCi/µmole) and 10 mM NaF. The incubation was stopped at the times indicated and the adenine nucleotides determined as described in the text. Results are a mean of 2 observations at 1, 3 and 6 minutes and 5 observations at 10 minutes. Vertical bars represent ± S.E. AMP, ADP and ATP recovered expressed in µmoles/mg protein and cyclic AMP recovered expressed in nmoles/mg protein.

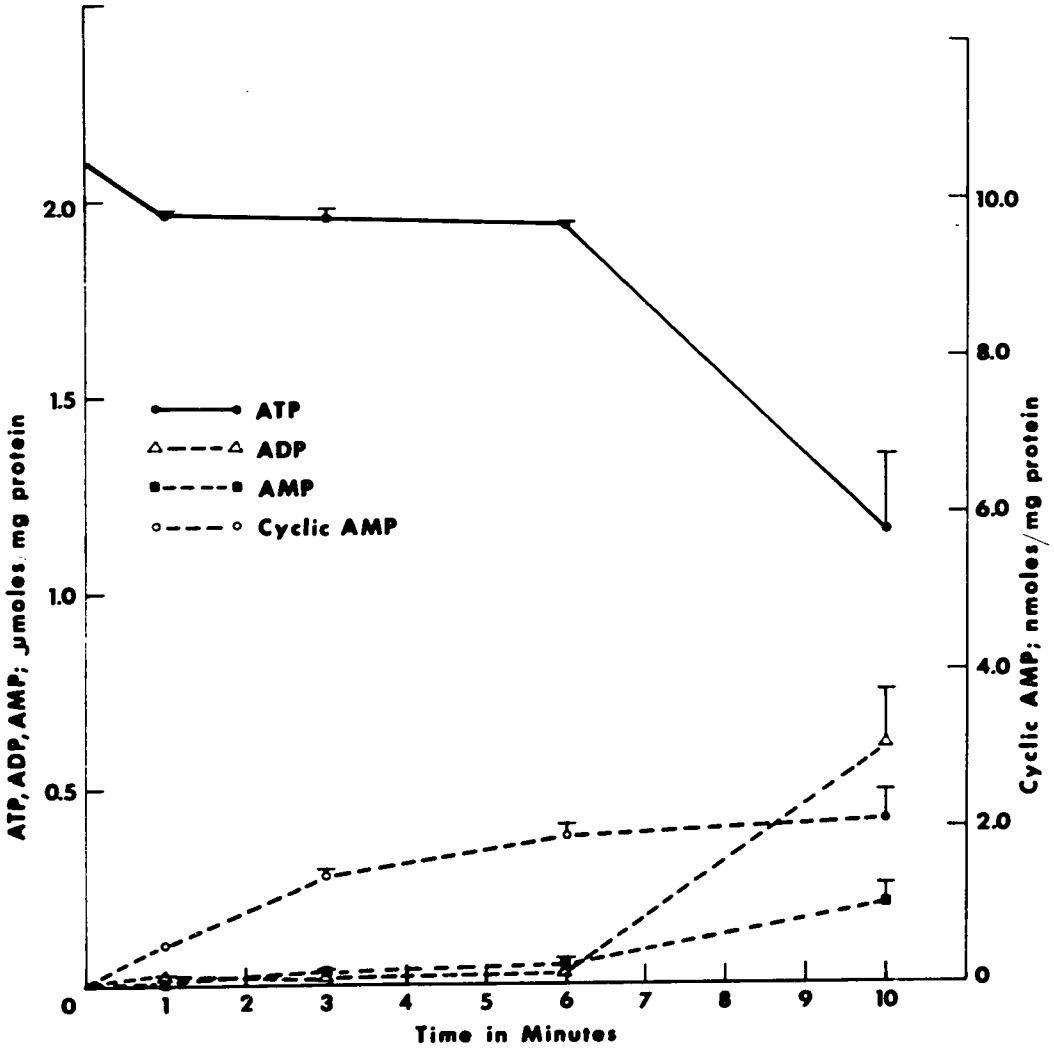


FIGURE 16: The Metabolic Fate of ATP Incubated with the Synaptic Membrane Preparation of Adenyl Cyclase in the Presence of the ATP Regenerating System

1.2 M_1 fraction of rat cerebral cortex homogenate prepared as described in methods (2.0 mg protein) was incubated at 37°C in a total volume of 2 ml, in a medium containing: 40 mM Tris, pH 7.4; 5.0 mM $MgSO_4$; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 µCi/µmole) and the ATP regenerating system consisting of 10 mM PEP and 25 µg/ml PK. The incubation was stopped at the times indicated and the adenine nucleotides determined as described in text. Results are a mean of 2 observations at 1, 3 and 6 minutes and 5 observations at 10 minutes. Vertical bars represent ± S.E. AMP, ADP and ATP recovered expressed in µmoles/mg protein and cyclic AMP recovered expressed in nmoles/mg protein.

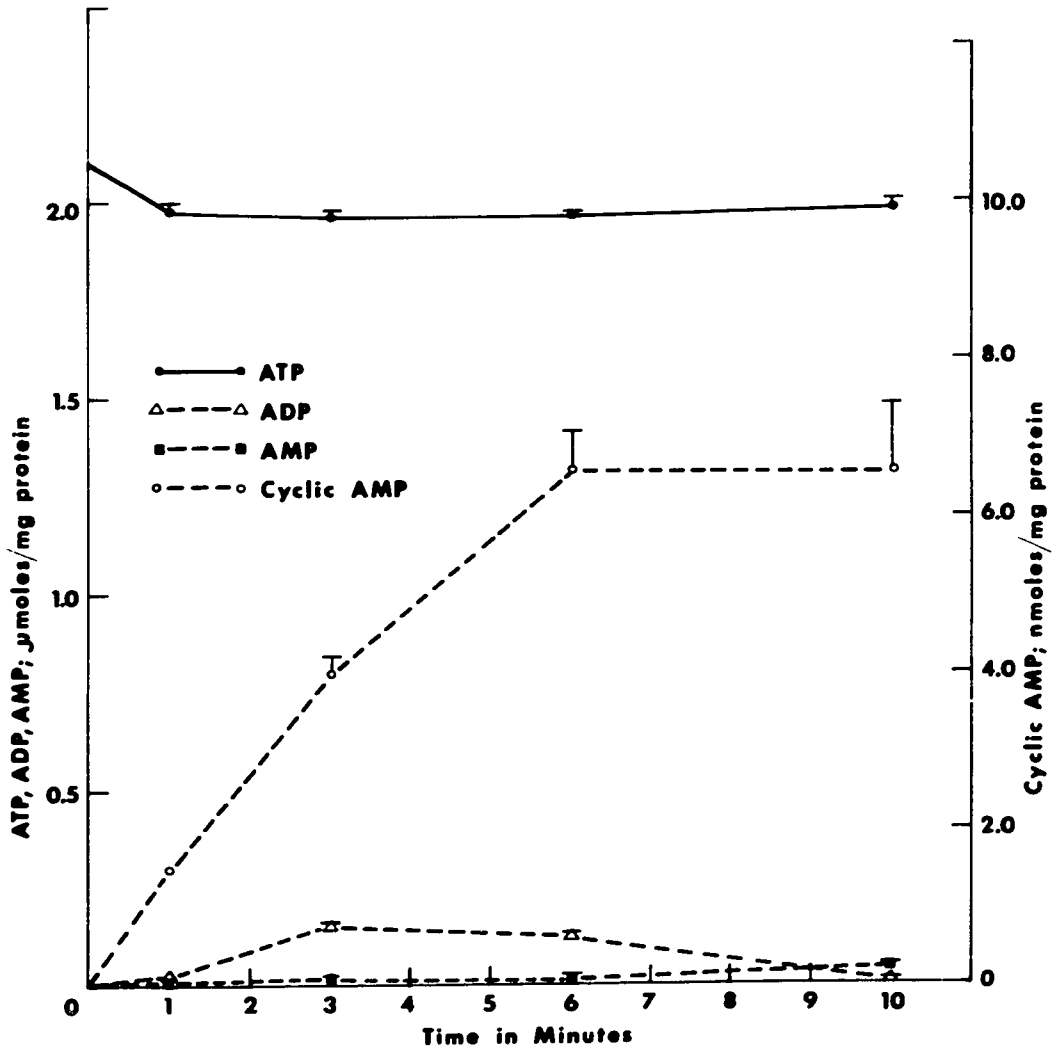


FIGURE 17: The Metabolic Fate of ATP Incubated with the Synaptic Membrane Preparation of Adenyl Cyclase in the Presence of the ATP Regenerating System and NaF

1.2 M_1 fraction of rat cerebral cortex homogenate prepared as described in methods (2.0 mg protein) was incubated at 37°C in a total volume of 2 ml, in a medium containing: 40 mM Tris, pH 7.4; 5.0 mM $MgSO_4$; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 μCi/μmole) and the ATP regenerating system consisting of 10 mM PEP and 25 μg/ml PK and 10 mM NaF. The incubation was stopped at the times indicated and the adenine nucleotides determined as described in text. Results are a mean of 2 observations at 1, 3 and 6 minutes and 5 observations at 10 minutes. Vertical bars represent ± S.E. AMP, ADP and ATP recovered expressed in μmoles/mg protein and cyclic AMP recovered expressed in nmoles/mg protein.

preparation of adenyl cyclase in the presence of 10 mM NaF. The concentration of ATP fell 24% during the first minute of incubation from an initial value of 2.1 μmoles/mg protein to 1.6 μmoles/mg protein, and then to 1.47 μmoles/mg protein during the next 3 minutes. The concentration of ATP stayed essentially the same for the last 7 minutes of incubation. ADP accumulated rapidly during the first 3 minutes of incubation and accounted for more than 77% of the ATP that disappeared during this time. The concentration of ADP then remained unchanged until the end of the 10 minute incubation period. The concentration of AMP increased slowly and accounted for only 20% of the ATP hydrolyzed during the incubation period. The accumulation of cyclic AMP rose linearly for the first 3 minutes to 3.4 nmoles/mg protein, then more slowly to 4.8 nmoles/mg protein after 6 minutes and to 5.6 nmoles/mg protein after 10 minutes of incubation.

Figure 16 shows the metabolic fate of ATP incubated with this preparation of adenyl cyclase in the presence of the ATP regenerating system. The concentration of ATP fell 8% during the first 6 minutes of incubation. From 6 minutes to the end of the incubation period, the concentration of ATP fell very rapidly from 1.93 μmoles/mg protein to 1.16 μmoles/mg protein. During this period ADP accumulation rose rapidly, accounting for 67% of the ATP that disappeared during this time, with AMP accumulation accounting for the rest. Cyclic AMP accumulated rapidly during the first 6 minutes of incubation to 1.9 nmoles/mg protein and then more slowly during the next 4 minutes to 2.1 nmoles/mg protein.

Figure 17 shows the metabolic fate of ATP in the synaptic membrane preparation of adenyl cyclase incubated in the presence of the ATP regenerating system and NaF (10 mM). The amount of ATP hydrolyzed during

the 10 minute incubation period was essentially nil and the amount of AMP and ADP accumulated was negligible. Cyclic AMP accumulated rapidly during the first 6 minutes of incubation to 6.6 nmoles/mg protein, but did not increase during the final 4 minutes of incubation.

Figure 18 summarizes the rate of accumulation of cyclic AMP under the various conditions tested. In the control experiments, the amount of cyclic AMP accumulated during the first minute was 0.86 nmoles/mg protein, the rate of accumulation was positive but lower during the next 2 minutes, 0.57 nmoles/mg protein/minute, and negative from thereon. The rate of accumulation of cyclic AMP in the presence of NaF (10 mM) was positive throughout the incubation period (1.50 nmoles/mg protein/minute from 0-1 minute, 0.95 nmoles/mg protein/minute from 103 minutes, 0.50 nmoles/mg protein/minute from 3-6 minutes and 0.22 nmoles/mg protein/minute from 6-10 minutes). The rate of accumulation of cyclic AMP in the presence of the ATP regenerating system was constant for the first 3 minutes of incubation (0.50 nmoles/mg protein/minute from 0-1 minute and 0.47 nmoles/mg protein/minute from 1-3 minutes), but during the final 7 minutes of incubation the accumulation rate of cyclic AMP decreased sharply though it remained positive (0.15 nmoles/mg protein/minute from 3-6 minutes and 0.05 nmoles/mg protein/minute from 6-10 minutes). Cyclic AMP accumulation in the presence of the ATP regenerating system and NaF was high and relatively constant for the first 3 minutes of incubation (1.50 nmoles/mg protein/minute from 0-1 minute and 1.25 nmoles/mg protein from 1-3 minutes). During the 3-6 minute period cyclic AMP accumulation was reduced (0.83 nmoles/mg protein/minute) and there was no net accumulation during the 6-10 minute incubation period.

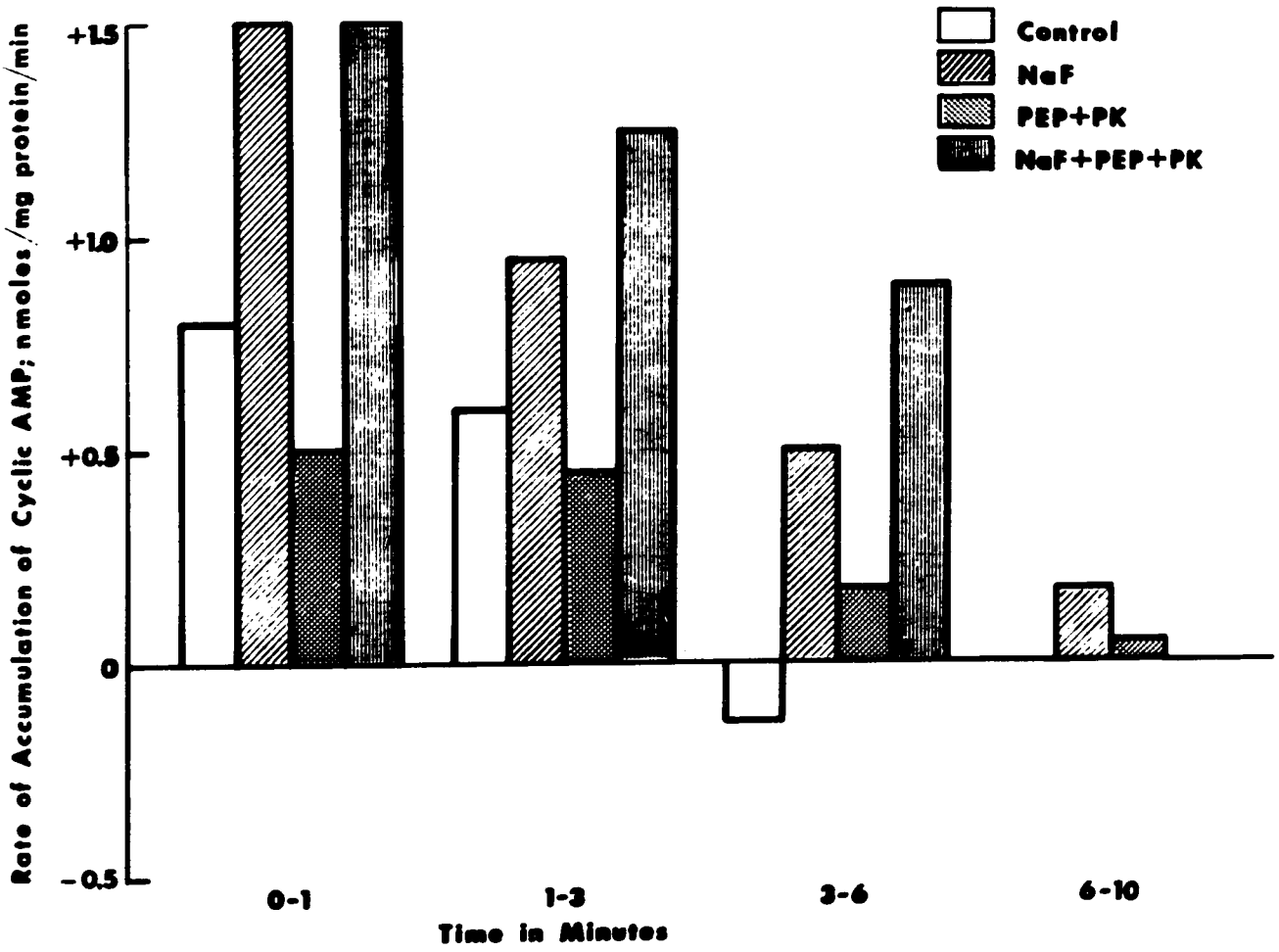


FIGURE 18: The Effect of NaF and the ATP Regenerating System on the Rate of Accumulation of Cyclic AMP in the Standard Synaptic Membrane Adenyl Cyclase Assay

The accumulation of cyclic AMP under the incubation conditions described, in the controls (FIG. 14), in the presence of NaF, (FIG. 15) in the presence of the ATP regenerating system (FIG. 16) and in the presence of the ATP regenerating system and NaF (FIG. 17) expressed in nmoles/mg protein/minute.

Table 15 shows the effects of adrenaline (0.1 mM) or ouabain (0.1 mM) on ATP metabolism and cyclic AMP accumulation in the synaptic membrane preparation of adenyl cyclase from rat cerebral cortex; the incubation time was 6 minutes. There was no increase in cyclic AMP accumulation with these agents when the synaptic membranes were prepared in the usual manner ($MgSO_4$, 1 mM and Tris, 2 mM, pH 7.2) and little difference in the concentrations of ATP, ADP and AMP was found. When the synaptic membranes were prepared with 0.01 mM Ca^{++} present throughout the preparation procedure however, there was a significant increase in the amount of cyclic AMP accumulated in the presence of adrenaline ($P<0.02$), but the amount of cyclic AMP accumulated in the presence of ouabain was not significantly increased by this procedure ($P<0.50$). The addition of 0.01 mM Ca^{++} to the preparation media significantly increased ADP accumulation in the controls ($P<0.05$). The amount of ATP hydrolyzed and of ADP and AMP accumulated in the presence of adrenaline in tissue prepared in the presence of Ca^{++} was significantly higher than adrenaline-induced levels in tissue prepared in the absence of Ca^{++} ($P<0.05$ for ATP and ADP and $P<0.01$ for AMP). The addition of 0.01 mM Ca^{++} to the preparation media did not affect the amount of ATP hydrolyzed in the presence of ouabain. In this case, ATP levels were slightly but not significantly higher than in the presence of adrenaline ($P<0.10$) while ADP levels were significantly higher than in the presence of adrenaline ($P<0.005$).

Thus with the addition of Ca^{++} to the preparation media, cyclic AMP accumulation in the presence of adrenaline was significantly increased even though this procedure increased ATP hydrolysis as well.

The effect of POB (0.1 mM) or pronethalol (0.1 mM) on cyclic AMP accumulation in this preparation of adenyl cyclase is summarized in Table 16;

TABLE 15

The Effects of Adrenaline and Ouabain on ATP Metabolism in the Synaptic Membrane Preparation of Adenyl Cyclase

	Preparation in Mg^{++}			Preparation in Mg^{++} + Ca^{++}		
	Control (5)	Adrenaline (3)	Ouabain (3)	Control (7)	Adrenaline (7)	Ouabain (3)
AMP (μmoles/mg protein)	0.05 ± 0.02	0.04 ± 0.01	0.08 ± 0.04	0.07 ± 0.02	0.09 ± 0.01[a]	0.05 ± 0.02
Cyclic AMP (nmoles/mg protein)	2.90 ± 0.30	3.20 ± 0.60	2.70 ± 0.20	2.60 ± 0.30	3.90 ± 0.30[b]	3.00 ± 0.50
ADP (μmoles/mg protein)	0.04 ± 0.01	0.04 ± 0.00	0.02 ± 0.01	0.41 ± 0.14[d]	0.65 ± 0.09[c,e]	0.07 ± 0.05
ATP (μmoles/mg protein)	2.01 ± 0.24	2.01 ± 0.12	1.98 ± 0.18	1.52 ± 0.15	1.26 ± 0.21[c]	1.95 ± 0.27

[a] $P<0.01$ compared to adrenaline prepared in Mg^{++}; [b] $P<0.02$ compared to control prepared in Mg^{++} + Ca^{++};
[c] $P<0.05$ compared to adrenaline prepared in Mg^{++}; [d] $P<0.05$ compared to control prepared in Mg^{++};
[e] $P<0.005$ compared to ouabain prepared in Mg^{++} + Ca^{++}

1.2 M_1 fraction of rat cerebral cortex homogenate prepared as described in methods with and without the addition of 0.01 mM calcium ion to the preparation media (2.0 mg protein) was incubated at 37°C for 6 minutes in the presence or the absence of adrenaline (0.1 mM) or ouabain (0.1 mM). The incubation medium of 2 ml volume also contained: 40 mM Tris, pH 7.4; 5.0 mM $MgSO_4$; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 μCi/μmole), and the ATP regenerating system consisting of 10 mM PEP and 25 μg/ml PK. The adenine nucleotides were determined as described in the text. Numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E. AMP, ADP and ATP recovered expressed in μmoles/mg protein and cyclic AMP recovered expressed in nmoles/mg protein.

TABLE 16

The Effect of α- and β-Adrenergic Blocking Drugs on Cyclic AMP Accumulation in the Synaptic Membrane Preparation of Adenyl Cyclase

Additions		Cyclic AMP (nmoles/mg protein)
None	(7)	2.60 ± 0.30[(a)]
Adrenaline	(7)	3.90 ± 0.30
POB	(2)	2.50 ± 0.50
Pronethalol	(2)	2.55 ± 0.65
Adrenaline + POB	(2)	2.40 ± 0.60
Adrenaline + Pronethalol	(2)	2.55 ± 0.65

(a) $P<0.02$ compared to adrenaline

1.2 M_1 fraction of rat cerebral cortex homogenate prepared as described in methods, with the addition of 0.01 mM calcium ion to the preparation media, (2.0 mg protein) was incubated for 16 minutes in the presence and absence of 0.1 mM POB or 0.1 mM pronethalol. The incubation medium of 2 ml volume also contained: 40 mM Tris, pH 7.4; 5.0 mM $MgSO_4$; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 μCi/μmole); the ATP regenerating system consisting of 10 mM PEP and 25 μg/ml PK and where indicated, 0.10 mM adrenaline. The reaction was begun with the addition of ^{14}C-ATP after the blocking agents had been in contact with the preparation for 10 minutes. The adenine nucleotides were determined as described in the text. Numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E. Cyclic AMP recovered expressed in nmoles/mg protein.

both drugs were added to the incubation media 10 minutes before the start of the reaction and Ca^{++} (0.01 mM) was present throughout the preparation procedure. Neither POB nor pronethalol effected the control levels of cyclic AMP accumulation but both these agents inhibited adrenaline-stimulated cyclic AMP accumulation.

Figure 19 summarizes the effect of varying the Mg^{++} concentration in the incubation medium on ATP hydrolysis and cyclic AMP accumulation in the presence and absence of adrenaline in this preparation of adenyl cyclase; a concentration of 0.01 mM Ca^{++} was present throughout the preparation procedure and the time of incubation was 6 minutes. When the concentration of Mg^{++} was reduced from the standard 5.0 mM to 3.5 mM or 2.0 mM the amount of ATP hydrolyzed in the controls decreased only slightly. In the presence of adrenaline, decreasing the Mg^{++} concentration from 5.0 mM to 2.0 mM significantly increased ATP hydrolysis ($P<0.02$ compared to the controls at 2.0 mM Mg^{++}). When the concentration of Mg^{++} was increased from 5.0 mM to 10.0 mM, the amount of ATP hydrolyzed in the controls changed little, whereas in the presence of adrenaline it decreased by 22%. There was little change in the amount of ADP accumulated in the controls when the Mg^{++} concentration was reduced from 5.0 mM to 1.0 mM, and a slight, but insignificant, increase ($P<0.60$) when the Mg^{++} concentration was increased from 5.0 mM to 10.0 mM. The amount of ADP accumulated in the presence of adrenaline was highest at 2.0 mM Mg^{++} and lowest at 10 mM Mg^{++}. AMP accumulation was highest at low Mg^{++} concentrations (from 1.0 mM to 5.0 mM Mg^{++}) in both the controls and in the presence of adrenaline. Cyclic AMP accumulation increased steadily in the controls with increasing Mg^{++} concentration. The accumulation of cyclic AMP in the presence of adrenaline

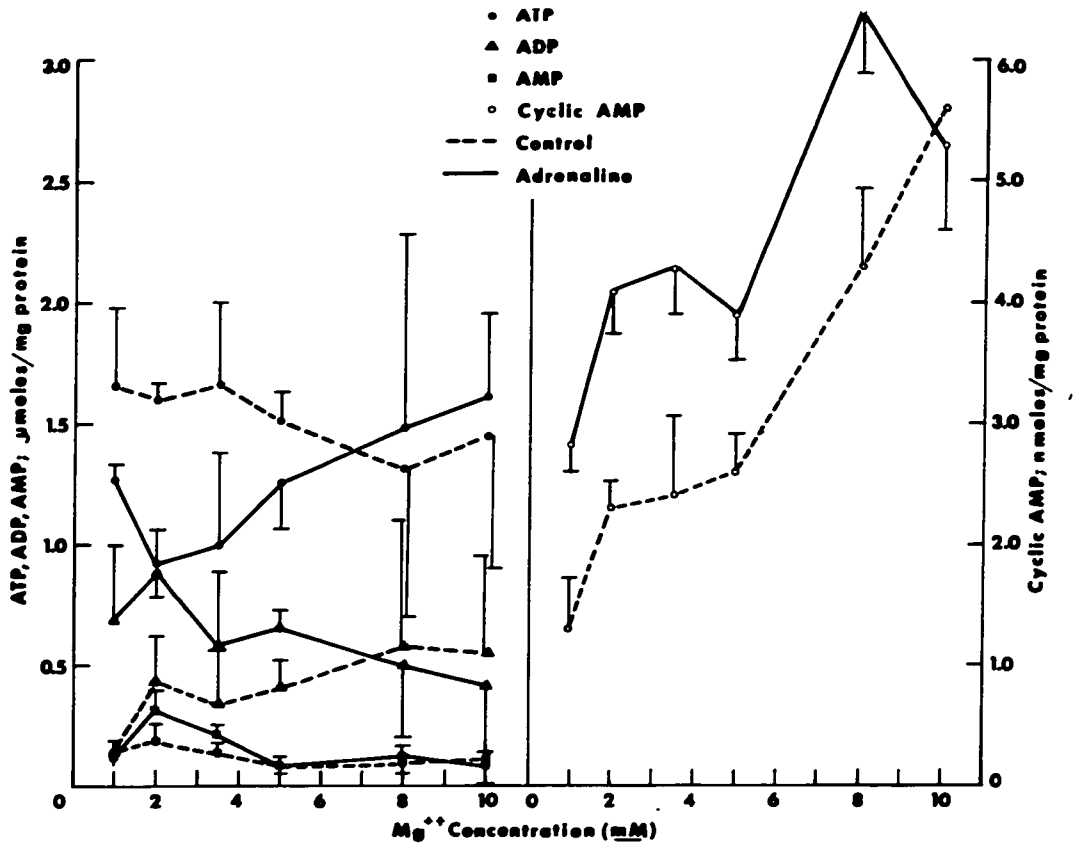


FIGURE 19: The Effect of the Mg^{++} Concentration of the Incubation Medium on ATP Metabolism in the Presence and Absence of Adrenaline in the Synaptic Membrane Preparation of Adenyl Cyclase

1.2 M_1 fraction of rat cerebral cortex homogenate prepared as described in methods (2.0 mg protein) with 0.01 mM Ca^{++} present throughout the preparation procedure was incubated for 6 minutes in the presence and absence of 0.1 mM adrenaline at varying Mg^{++} concentrations (from 1.0 mM - 10.0 mM). The incubation medium of 2 ml volume also contained: 40 mM Tris, pH 7.4; 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 μCi/μmole) and the ATP regenerating system consisting of 10 mM PEP and 25 μg/ml PK. The adenine nucleotides were determined as described in text. Results are a mean of at least 3 observations per group. Vertical bars represent ± S.E. AMP, ADP and ATP recovered expressed in μmoles/mg protein and cyclic AMP recovered expressed in nmoles/mg protein.

increased to a peak value at 8.0 mM Mg^{++}. At all concentrations of Mg^{++} the amount of cyclic AMP accumulated in the presence of adrenaline was higher than that accumulated in the controls ($P<0.01$ at 2.0 mM Mg^{++}, $P<0.02$ at 5.0 mM Mg^{++}) except at the highest Mg^{++} concentration when the amount of cyclic AMP accumulated in both conditions was the same.

Thus at low Mg^{++} concentrations in the presence of adrenaline, although ATP hydrolysis was significantly greater than in the controls, cyclic AMP accumulation was significantly increased. In the control experiments, variation in the Mg^{++} concentration did not affect ATP hydrolysis but cyclic AMP accumulation was significantly increased at high Mg^{++} concentrations ($P<0.001$ at 10 mM Mg^{++} compared to cyclic AMP accumulation at 2.0 mM Mg^{++}, $P<0.01$ compared to 5.0 mM Mg^{++}, $P<0.05$ compared to 3.5 mM Mg^{++}).

Table 17 illustrates the effect of substituting equimolar Mn^{++} for Mg^{++} in the preparation and incubation of the synaptic membrane fraction of adenyl cyclase from rat cerebral cortex; no ATP regenerating system was used in this experiment. When tissue prepared in Mg^{++} was incubated in the presence of Mn^{++} (Part A), the amount of ATP hydrolyzed was significantly reduced ($P<0.05$ compared to incubation in Mg^{++}). The amount of AMP accumulated was also significantly reduced ($P<0.001$) and the accumulation of cyclic AMP was significantly increased ($P<0.01$). The addition of 10 mM NaF to the Mn^{++} incubated preparation further decreased the amount of ATP hydrolyzed ($P<0.50$) and increased the amount of cyclic AMP accumulated ($P<0.10$). ATP hydrolysis and cyclic AMP accumulation in the presence of Mn^{++} and NaF (10 mM) were essentially the same as in the presence of Mg^{++} and NaF (10 mM).

TABLE 17

The Effect of Mn^{++} on ATP Metabolism in the Synaptic Membrane Preparation of Adenyl Cyclase

	(A) Tissue Prepared in Mg^{++}				(B) Tissue Prepared in Mn^{++}			
	Incubated in Mg^{++}		Incubated in Mn^{++}		Incubated in Mg^{++}		Incubated in Mn^{++}	
	C	NaF	C	NaF	C	NaF	C	NaF
AMP (μmoles/mg protein)	± 0.45 ± 0.03	± 0.14 ± 0.01	± 0.04 ± 0.01(b)	± 0.04 ± 0.01	± 0.57 ± 0.07(e)	± 0.18 ± 0.03(e)	± 0.13 ± 0.00	± 0.10 ± 0.00
Cyclic AMP (nmoles/mg protein)	± 1.60 ± 0.26	± 5.60 ± 1.30	± 4.40 ± 0.60(c)	± 6.10 ± 0.80	± 4.80 ± 1.20(a)	± 12.00 ± 2.10	± 4.80 ± 1.00	± 8.80 ± 1.10
ADP (μmoles/mg protein)	± 0.69 ± 0.11	± 0.49 ± 0.10	± 0.53 ± 0.07	± 0.35 ± 0.05	± 0.88 ± 0.15	± 0.58 ± 0.06	± 0.75 ± 0.06	± 0.61 ± 0.05
ATP (μmoles/mg protein)	± 0.90 ± 0.17	± 1.40 ± 0.16	± 1.47 ± 0.14(a)	± 1.65 ± 0.19	± 0.60 ± 0.11(d,g)	± 1.27 ± 0.02(f)	± 1.14 ± 0.07	± 1.31 ± 0.06

(a) $P<0.05$ compared to Mg^{++} prepared-Mg^{++} incubated control; (b) $P<0.001$ compared to Mg^{++} prepared-Mg^{++} incubated control; (c) $P<0.01$ compared to Mg^{++} prepared-Mg^{++} incubated control; (d) $P<0.01$ compared to Mn^{++} prepared-Mn^{++} incubated control; (e) $P<0.001$ compared to Mn^{++} prepared-Mn^{++} incubated control; (f) $P<0.01$ compared to Mn^{++} prepared-Mg incubated control; (g) $P<0.01$ compared to Mg^{++} prepared-Mn^{++} incubated control

1.2 M_1 fraction of rat cerebral cortex homogenates (2.0 mg protein) prepared as described in methods in either 1 mM Mg^{++} (A), or 1 mM Mn^{++} (B), was incubated at 37°C for 10 minutes in either 5.0 mM $MgSO_4$ or 5.0 mM $MnCl_2$. The incubation medium of 2 ml volume also contained: 6.67 mM theophylline; 40 mM Tris, pH 7.4; 2.1 mM ^{14}C-ATP (0.250 μCi/μmole) and where indicated, 10 mM NaF. The adenine nucleotides were determined as described in the text. Results are expressed as the mean of 5 experiments ± S.E. AMP, ADP and ATP recovered expressed in μmoles/mg protein and cyclic AMP recovered expressed in nmoles/mg protein.

Part B of Table 17 shows the effect on ATP metabolism produced when the synaptic membrane preparation of adenyl cyclase was prepared in media in which 1 mM Mn^{++} was substituted for the normally present 1.0 mM Mg^{++}; except for this substitution the preparation procedure was exactly the same as that previously used. When tissue prepared in Mn^{++} was incubated in Mg^{++} the amount of ATP hydrolyzed during the 10 minute incubation period was significantly increased compared to that measured when the Mn^{++} prepared tissue was incubated in Mn^{++} ($P<0.01$). Similarly, AMP accumulation was significantly higher in the Mg^{++} incubated vessels ($P<0.001$). The cyclic AMP accumulation was the same in both conditions. When tissue prepared in Mn^{++} was incubated in the presence of 10 mM NaF, ATP hydrolysis in the Mg^{++} incubated vessels was significantly inhibited ($P<0.01$) but in the Mn^{++} incubated vessels was virtually unchanged. The concentrations of ADP, ATP and AMP were similar under these conditions whether Mg^{++} or Mn^{++} was present in the incubation media. The amount of cyclic AMP accumulated in the presence of Mg^{++} and NaF was slightly greater than that accumulated in the presence of Mn^{++} and NaF but this difference was not statistically significant ($P<0.60$).

Comparing part A and B of Table 17 shows that the highest amount of ATP hydrolysis in the absence of NaF was obtained when the synaptic membranes preparation of adenyl cyclase was prepared in Mn^{++} and incubated in the presence of Mg^{++} ($P<0.01$ compared to tissue prepared in Mg^{++} and incubated in Mn^{++}). Conversely, the level of cyclic AMP accumulated under these conditions was equal to that produced under any other conditions and significantly higher than that seen in Mg^{++} prepared and Mg^{++} incubated

tissue ($P<0.05$). In the presence of NaF, the amount of ATP hydrolyzed was the same regardless of whether the tissue was prepared or incubated with Mg^{++} or Mn^{++}, but the amount of cyclic AMP accumulated in tissue prepared in Mn^{++} was considerably higher.

It was shown previously that adrenaline stimulated cyclic AMP accumulation when the synaptic membrane preparation of adenyl cyclase was prepared in 1.0 mM Mg^{++} and 0.01 mM Ca^{++}. In the study summarized in Figure 20, tissue was prepared in either Mg^{++} or Mn^{++} and incubated in either Mg^{++} (5.0 mM) or Mn^{++} (5.0 mM) and the amount of cyclic AMP accumulated in the presence of adrenaline (0.1 mM) or ouabain (0.1 mM) measured; Ca^{++} (0.01 mM) was present throughout the preparation procedure. When the synaptic membrane preparation of adenyl cyclase was prepared in the normal manner (1.0 mM Mg^{++}) and then incubated in Mn^{++}, adrenaline did not stimulate cyclic AMP production. Adrenaline did not stimulate cyclic AMP accumulation in the synaptic membranes prepared in Mn^{++} regardless of whether they were incubated in the presence of Mn^{++} or Mg^{++}. Ouabain did not stimulate cyclic AMP accumulation in any of these four conditions.

DISCUSSION: STUDIES ON THE SYNAPTIC MEMBRANE PREPARATION OF ADENYL CYCLASE.

The relationship of adenyl cyclase to other membrane-bound ATP utilizing enzymes was further studied in a synaptic membrane preparation of adenyl cyclase from rat cerebral cortex. It was shown that this preparation of adenyl cyclase had a specific activity 11 times higher than that observed in the rat cerebral cortex preparation (Part 2, section A) in the controls and a specific activity 5.6 times higher in the presence of NaF. It was also shown that cyclic AMP accumulation in this synaptic membrane preparation of

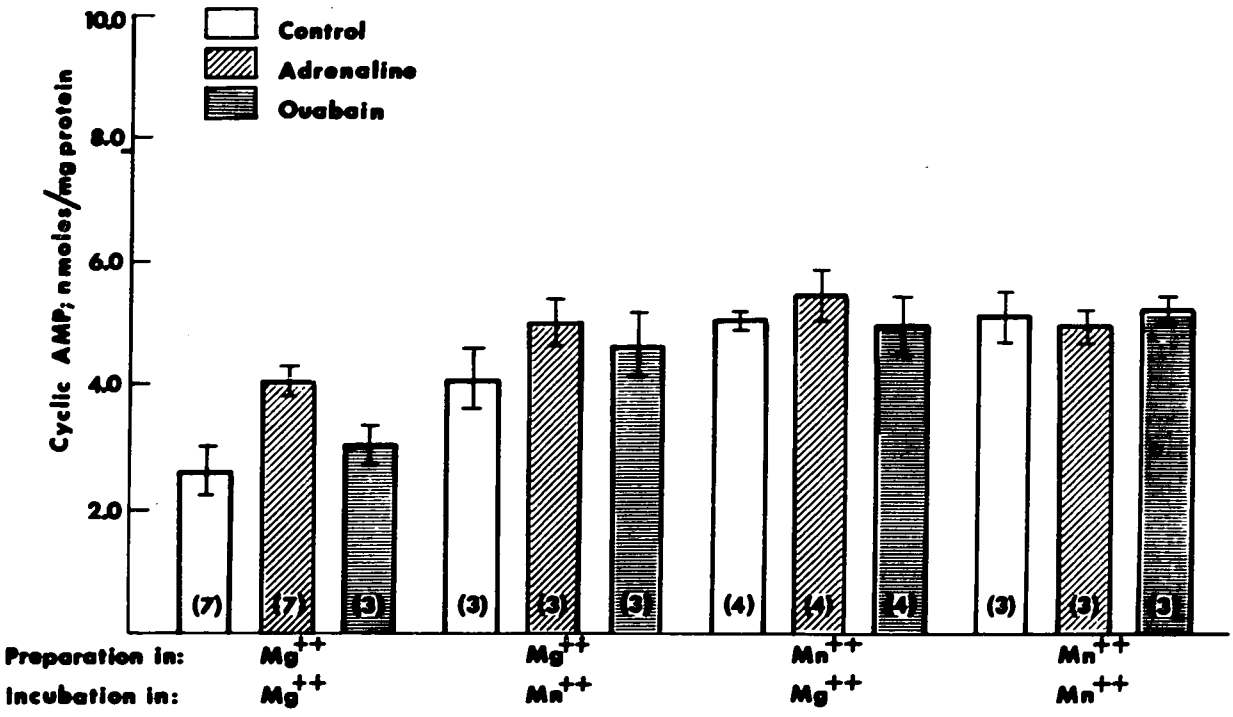


FIGURE 20: The Effect of Adrenaline and Ouabain on Cyclic AMP Accumulation in the Synaptic Membrane Preparation of Adenyl Cyclase Prepared and/or Incubated in Mn^{++}

1.2 M_1 fraction of rat cerebral cortex homogenate prepared as described in methods (2.0 mg protein) in either 1 mM $MgSO_4$ or 1 mM $MnCl_2$ with 0.01 mM Ca^{++} present throughout the preparation procedure and incubated at 37°C for 6 minutes in the presence of either 5.0 mM $MgSO_4$ or 5.0 mM $MnCl_2$. The incubation media also contained 6.67 mM theophylline; 2.1 mM ^{14}C-ATP (0.250 μCi/μmole); and an ATP regenerating system consisting of 10 mM PEP and 25 μg/ml PK and, where indicated, 0.1 mM adrenaline or 0.1 mM ouabain in a final volume of 2 ml. The adenine nucleotides were determined as described in methods. Numbers in brackets indicate number of experiments per group. Vertical bars represent ± S.E. Cyclic AMP recovered expressed in nmoles/mg protein.

adenyl cyclase was significantly stimulated by NaF, Mn^{++} and adrenaline.

Although other membrane-bound ATP utilizing enzymes appeared to be present in this preparation, the amount of ATP hydrolysis and ADP and AMP accumulation were much reduced compared to that found under the same assay conditions in the rat cerebral cortex preparation of adenyl cyclase. In this preparation the ATP regenerating system was still unable to maintain high substrate levels throughout the incubation period. In the presence of the ATP regenerating system and NaF, however, the concentration of ATP was maintained throughout the 10 minute incubation period.

Experiments with the synaptic membrane preparation of adenyl cyclase indicated that substances that stimulated cyclic AMP accumulation were not doing so merely by inhibiting the other ATP utilizing enzymes and thus providing substrate for adenyl cyclase. This conclusion is supported by the following observations: 1) In the presence of an ATP regenerating system and NaF, when ATP levels were maintained at significantly higher concentrations than in the presence of NaF alone, cyclic AMP accumulation was not significantly increased. 2) In tissue prepared in the presence of 0.01 mM Ca^{++}, adrenaline significantly stimulated cyclic AMP accumulation even though preparation in Ca^{++} significantly increased ATP hydrolysis. Furthermore, at low Mg^{++} concentrations in the presence of adrenaline, although ATP hydrolysis was significantly greater than in the controls, cyclic AMP accumulation was significantly increased. In the absence of adrenaline, variation of the Mg^{++} concentration did not affect ATP hydrolysis but cyclic AMP accumulation was significantly greater at high Mg^{++} concentrations. 3) When synaptic membrane adenyl cyclase was prepared in Mn^{++} and incubated

in the presence of Mg^{++}, the amount of ATP hydrolyzed was significantly increased compared to that noted in Mn^{++} prepared and Mn^{++} incubated tissue. The amount of cyclic AMP accumulated in these two conditions was essentially the same. In the presence of NaF, the amount of ATP hydrolyzed was the same whether the tissue was prepared or incubated with Mg^{++} or Mn^{++}, but the amount of cyclic AMP accumulated in tissue prepared in Mn^{++} was considerably higher.

It has been shown that particulate preparations of adenyl cyclase contained a high amount of PDE activity (De Robertis et al., 1967). Theophylline was used throughout the present experiments, but it was shown by other workers in particulate preparations of adenyl cyclase to inhibit PDE activity only 50% (Streeto and Reddy, 1967). PDE activity must therefore be considered when estimating cyclic AMP accumulation in these preparations. It seemed possible that agents which stimulated cyclic AMP accumulation in these preparations could be doing so by directly or indirectly inhibiting cyclic AMP destruction. The effects of NaF, Mn^{++}, epinephrine, ouabain and adenine nucleotides on PDE activity were therefore studied. Three PDE systems were utilized: 1) a partially purified preparation of soluble PDE, 2) the rat cerebral cortex preparation of adenyl cyclase and 3) the synaptic membrane preparation of adenyl cyclase.

PART 3. ADENOSINE 3',5'-MONOPHOSPHATE DESTRUCTION IN PREPARATIONS OF RAT BRAIN PHOSPHODIESTERASE.

A. RESULTS OF STUDIES WITH A PARTIALLY PURIFIED PREPARATION OF PDE FROM RAT CEREBRAL CORTEX

Figure 21 shows the results of a typical experiment that tested the activity of a partially purified preparation of PDE from rat cerebral cortex. The amount of cyclic AMP hydrolyzed increased linearly with the amount of PDE preparation present up to 2.4 mg protein. The specific activity of the PDE preparation was 0.5 μmoles cyclic AMP hydrolyzed/10 minute incubation/ mg protein. In all subsequent experiments with this preparation 3 mg protein was used.

Figure 22 summarizes the effect of ATP, ADP and AMP in the presence or absence of NaF on the activity of partially purified PDE from rat cerebral cortex. Both ATP and ADP were potent inhibitors of the enzyme. One mM ATP inhibited enzyme activity 65% and 2 mM ATP inhibited the enzyme activity almost completely (90%). One mM ADP inhibited the preparation 46% and 2 mM, 70%. In contrast AMP was only a weak inhibitor of the enzyme, as 2 mM AMP inhibited the enzyme activity only 25%. NaF alone at a concentration of 10 mM had no effect on enzyme activity nor did it alter the inhibitory effects of ADP or ATP. NaF, however, increased the inhibitory effect of 1.0 mM AMP from 4% to 18% ($P<0.02$) and that of 2.0 mM from 25% to 35% ($P<0.20$).

Table 18 shows the effect of increasing Mg^{++} concentration on the ATP inhibition of PDE. The Mg^{++} concentration used in the incubation media throughout these studies, 1.8 mM, was increased to 3.5 mM, 7.0 mM or 10.0 mM, and the effect of ATP on the activity of this enzyme was again measured.

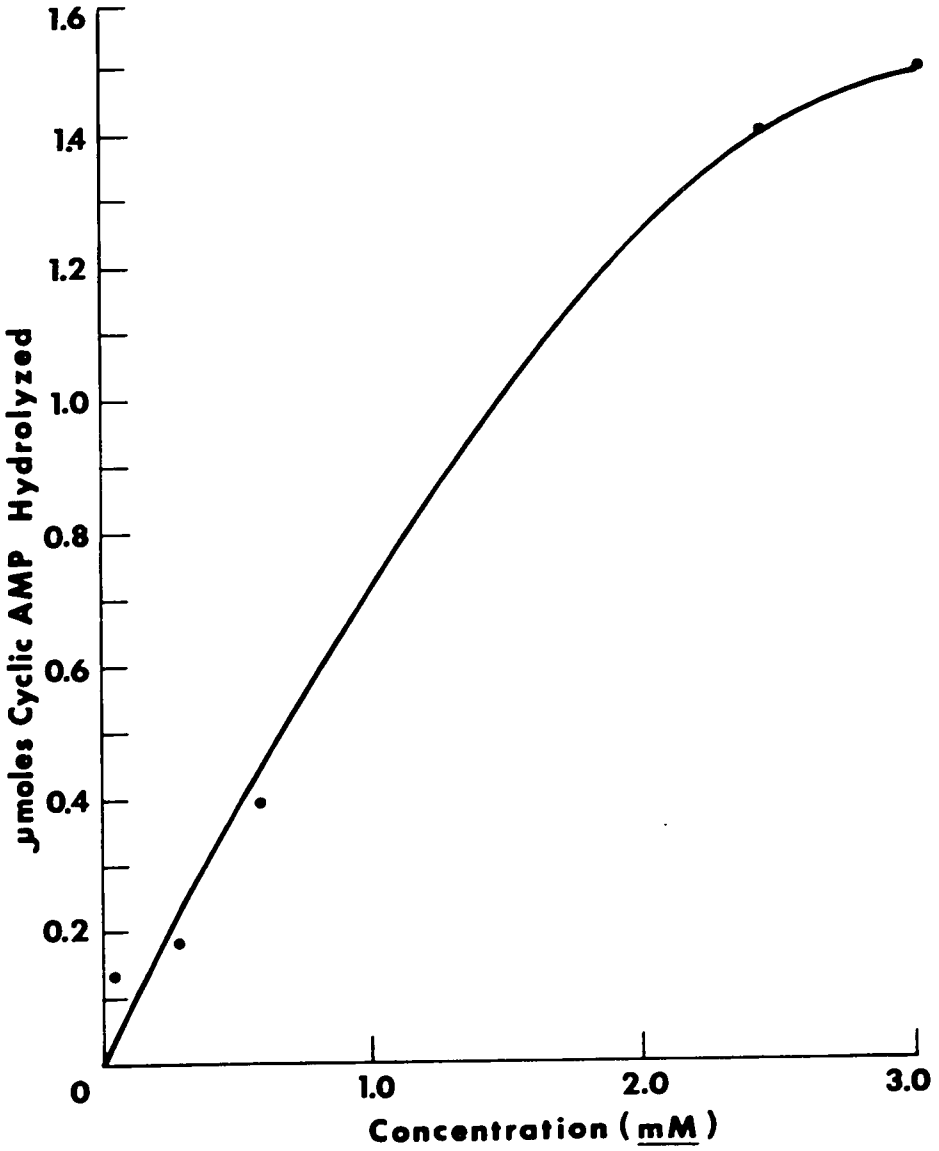


FIGURE 21: Cyclic AMP Hydrolysis as a Function of Tissue Concentration in the Partially Purified Preparation of PDE from Rat Cerebral Cortex

Increasing concentrations of the soluble PDE preparation from rat cerebral cortex prepared as described in methods was incubated with 1.8 mM $MgSO_4$; 40 mM Tris, pH 7.5 and 2 mM ^{14}C-cyclic AMP (0.012 μCi/μmole) in a final volume of 1 ml. Incubation was for 10 minutes at 37°C and cyclic AMP remaining was determined as described in text.

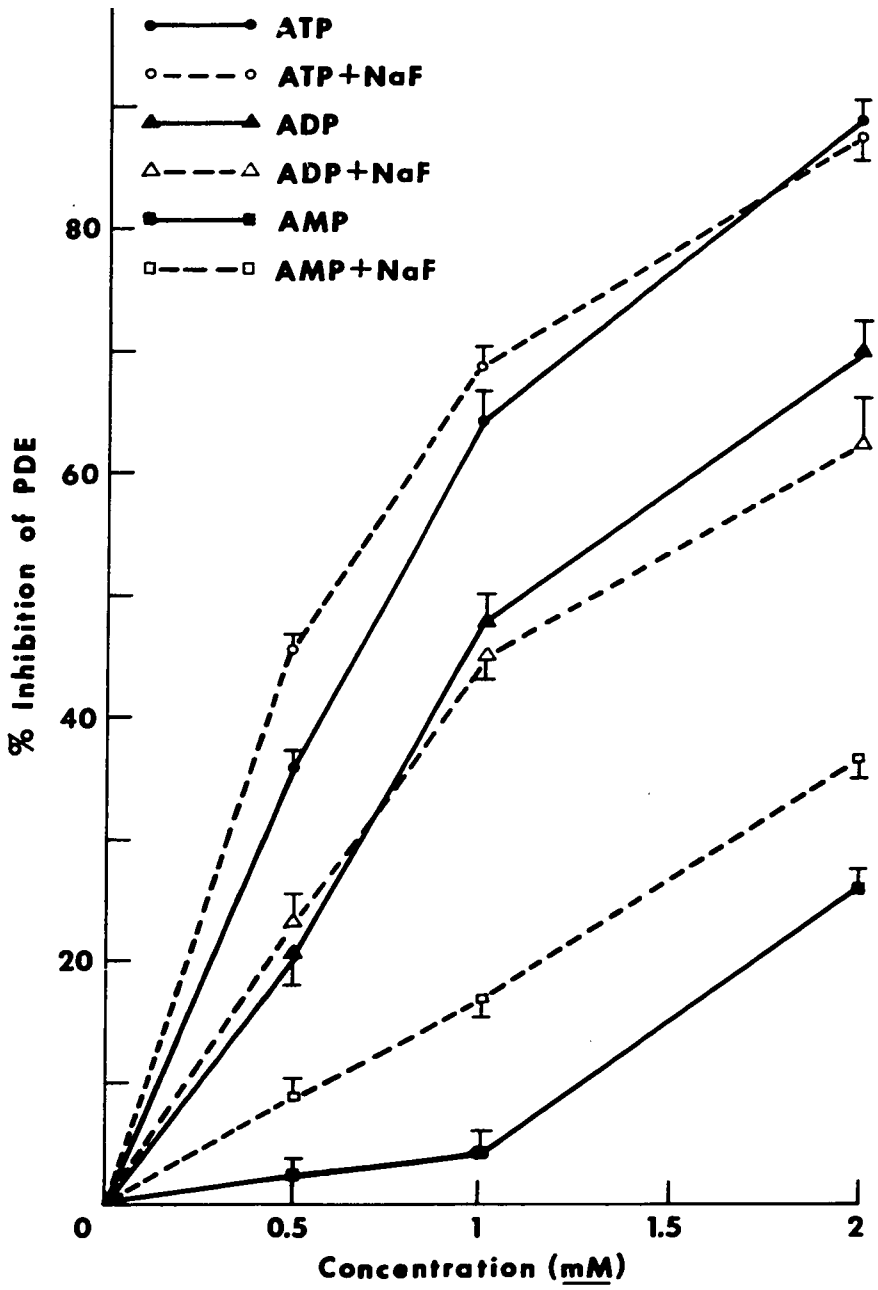


FIGURE 22: The Effect of ATP, ADP, AMP and NaF on the Activity of the Partially Purified PDE Preparation from Rat Cerebral Cortex

Soluble PDE preparation from rat cerebral cortex prepared as described in methods (3 mg protein) was incubated for 10 minutes at 37°C in a total volume of 1 ml, containing: 40 mM Tris, pH 7.5; 1.8 mM $MgSO_4$; 2 mM ^{14}C-cyclic AMP (0.012 μCi/μmole) and ATP, ADP, AMP (0.5 mM-2.0 mM) and NaF (10 mM) where indicated. Cyclic AMP remaining was determined as described in text. Results are a mean of at least 3 observations per group and vertical bars represent ± S.E.

TABLE 18

The Effect of Mg^{++} Concentration on the Inhibition of the Partially Purified Preparation of PDE by ATP

Mg^{++} Concentration	% Cyclic AMP Remaining	
	Control	2 mM ATP
1.8 mM	20.4 ± 2.7 (8)	91.2 ± 2.10 (8)
3.5 mM	20.6 ± 2.6 (2)	91.0 ± 4.6 (2)
7.0 mM	20.2 ± 2.4 (2)	90.7 ± 4.4 (2)
10.0 mM	20.4 ± 2.5 (2)	90.7 ± 4.5 (2)

Soluble PDE preparation from rat cerebral cortex, prepared as described in methods (3.0 mg protein) was incubated at 37°C for 10 minutes in varying concentrations of $MgSO_4$ (1.8 mM to 10 mM) in the presence and absence of 2 mM ATP. The incubation medium of 1 ml volume also contained: 40 mM Tris, pH 7.5 and 2 mM ^{14}C-cyclic AMP (0.012 μCi/μmole). Cyclic AMP remaining was determined as described in text. Numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E.

Increasing the Mg^{++} concentration to 5 times the amount normally present in the incubation medium did not reverse the inhibitory effect of ATP on the hydrolysis of cyclic AMP, nor did it have any effect on the amount of cyclic AMP hydrolysis in the control experiments.

Figure 23 shows the effect of PP_i on the activity of the partially purified preparation of PDE. Five mM and 8 mM PP_i have little inhibitory effect on the activity of this enzyme preparation but 10 mM PP_i inhibited enzyme activity by 34%; 10 mM NaF did not enhance the weak inhibition by PP_i.

Table 19 shows the effects of α-β- and β-γ-methylene adenosine triphosphate (α-β mATP and β-γ mATP respectively) on the activity of this preparation of PDE. These ATP analogs differ from ATP in that one of the bridging pyrophosphate oxygens, either between the α and β phosphate or β and γ phosphate of ATP has been replaced by a methylene group. Whereas 1 mM ATP inhibited enzyme activity 65%, 1.0 mM α-β mATP inhibited only very slightly (12%). A concentration of 2.0 mM α-β mATP inhibited cyclic AMP hydrolysis only 15.7% and 4.0 mM only 40%. At higher concentrations of this analog the activity of PDE was inhibited to a greater extent, with 8.0 mM inhibiting cyclic AMP hydrolysis by 70% and 16.0 mM by 83%. Thus this analog has approximately 1/8th the inhibitory activity of ATP. The β-γ mATP was an even weaker inhibitor of PDE activity with 1.0, 2.0 and 4.0 mM of this compound not inhibiting the enzyme to a measurable extent; a concentration of 8.0 mM and 16.0 mM inhibited cyclic AMP hydrolysis 13.5% and 29.7% respectively.

Table 20 shows the effect on the PDE activity of this preparation produced by substituting Mg^{++} for Mn^{++} in the incubation media. Equimolar

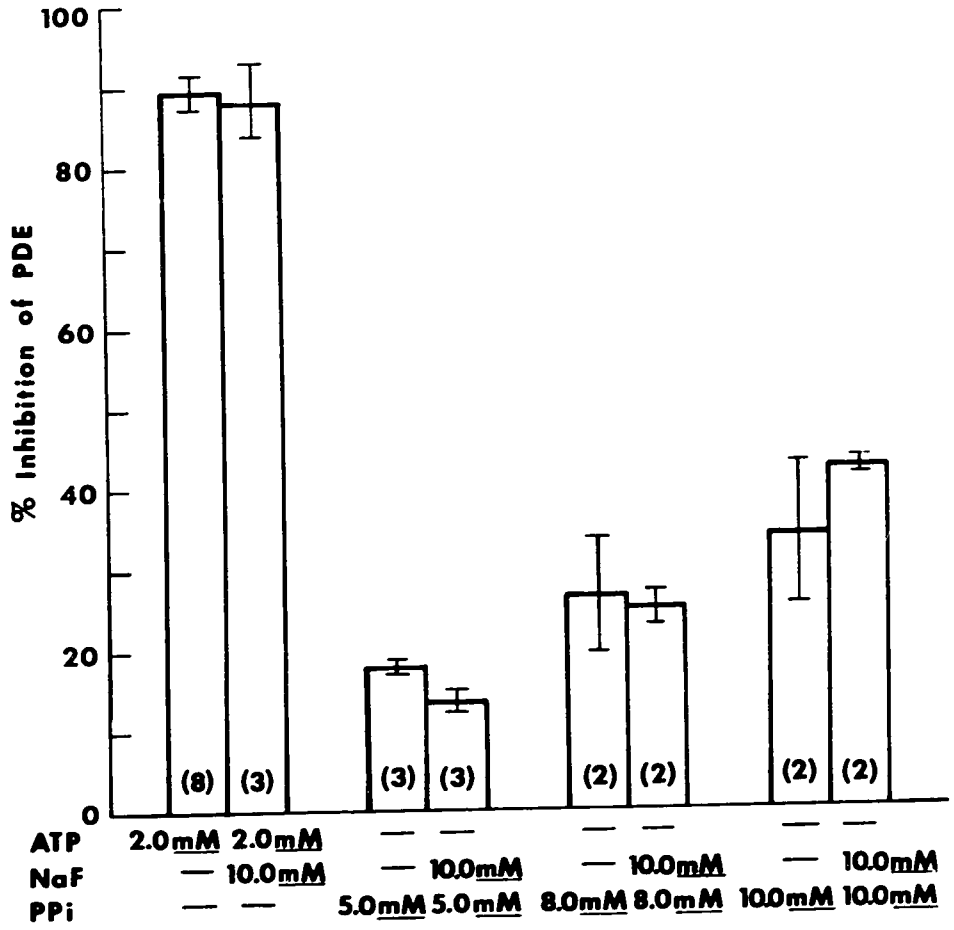


FIGURE 23: The Effect of PP_i on the Activity of the Partially Purified PDE Preparation from Rat Cerebral Cortex

Soluble PDE preparation from rat cerebral cortex prepared as described in methods (3 mg protein) was incubated for 10 minutes in a total volume of 1 ml, containing: 40 mM Tris, pH 7.5; 1.8 mM $MgSO_4$; 2 mM ^{14}C-cyclic AMP (0.012 µCi/µmole) in the presence and absence of PP_i (5 mM, 8 mM and 10.0 mM) and where indicated, 10 mM NaF. Cyclic AMP remaining was determined as described in text. Numbers in brackets indicate number of experiments per group. Vertical lines indicate ± S.E.

TABLE 19

The Effect of α-β and β-γ Methylene ATP on the Activity of the Partially Purified Preparation of PDE from Rat Cerebral Cortex

	ATP		α-β Methylene Triphosphate					β-γ Methylene Triphosphate				
	1.0 mM	2.0 mM	1.0 mM	2.0 mM	4.0 mM	8.0 mM	16.0 mM	1.0 mM	2.0 mM	4.0 mM	8.0 mM	16.0 mM
% Inhibition of cyclic AMP destruction	65.1 ± 5.1	89.7 ± 1.5	12.0 ± 6.0	15.7 ± 10.1	40.2 ± 11.7	70.4 ± 4.4	83.0 ± 2.0	0	0	0	13.5 ± 9.7	29.7 ± 3.0
	(6)	(8)	(3)	(4)	(4)	(4)	(3)	(3)	(3)	(3)	(3)	(2)

Soluble PDE preparation from rat cerebral cortex prepared as described in methods (3 mg protein) was incubated at 37°C for 10 minutes in the presence and absence of α-β methylene ATP or β-γ methylene ATP (1.0 mM to 16.0 mM). The incubation medium of 1 ml volume also contained: 40 mM Tris, pH 7.5; 1.8 mM $MgSO_4$ and 2 mM ^{14}C-cyclic AMP (0.012 μCi/μmole). Cyclic AMP remaining was determined as described in text. Numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E.

TABLE 20

The Effect of Mn^{++} on the Activity of the Partially Purified Preparation of PDE

		% Cyclic AMP Remaining
Mg^{++}	(8)	21.4 ± 2.7
Mn^{++}	(3)	14.7 ± 3.7

Soluble PDE preparation from rat cerebral cortex, prepared as described in methods (3.0 mg protein) was incubated at 37°C for 10 minutes in either 1.8 mM $MgSO_4$ or $MnCl_2$. The incubation medium of 1 ml volume also contained: 40 mM Tris, pH 7.5 and 2 mM ^{14}C-cyclic AMP (0.012 μCi/μmole). Cyclic AMP was determined as described in text. Numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E.

Mn^{++} was substituted for Mg^{++} and had no effect on the hydrolysis of cyclic AMP by this PDE preparation.

B. PDE ACTIVITY OF THE RAT CEREBRAL CORTEX PREPARATION OF ADENYL CYCLASE

Table 21 shows the PDE activity of the rat cerebral cortex preparation of adenyl cyclase. In these experiments the effect of NaF, ADP with ATP, and ADP with ATP and NaF was studied in the presence and absence of theophylline. The concentration of NaF was 10 mM, the concentration that was shown to maximally stimulate cyclic AMP accumulation in this same preparation. The concentrations of ADP, ATP and cyclic AMP were chosen to approximate those measured in the adenyl cyclase assay after 30 seconds incubation in the presence of NaF (in μmoles/ml incubation media). The cyclic AMP concentration was 1.0 μM, the ADP concentration 0.5 mM and the ATP concentration 1.0 mM. A short incubation time was used in order to limit any effect that the synthesis of unlabelled cyclic AMP might have on the rate of destruction of ^{14}C-cyclic AMP. In the absence of any added ATP, ADP or NaF, 85% of the cyclic AMP was destroyed in the first minute of incubation. NaF did not augment this effect. The addition of 0.5 mM ADP and 1.0 mM ATP significantly inhibited this destruction of cyclic AMP ($P<0.01$) and the addition of 0.5 mM ADP and 1.0 mM ATP in the presence of 10 mM NaF inhibited cyclic AMP destruction even further, so that only 55% of the cyclic AMP was now destroyed during the one minute incubation period ($P<0.01$ compared to the controls). The standard concentration of theophylline used in the studies on cyclic AMP accumulation in this preparation, 6.7 mM, was shown to inhibit PDE activity approximately 59%. ADP and ATP added together with theophylline inhibited the enzyme by 71% and the addition of

TABLE 21

PDE Activity of Rat Cerebral Cortex Preparation of Adenyl Cyclase

Additions	% Cyclic AMP Remaining	% Inhibition of PDE	
None	14.6 ± 2.3	--	
NaF	14.3 ± 2.2	--	
ADP + ATP	34.4 ± 3.7[a]	19.4 ± 4.4	
ADP + ATP + NaF	44.2 ± 6.7[a]	28.5 ± 5.0	
Theophylline	58.7 ± 5.9	52.1 ± 7.2	(--)
Theophylline + ADP + ATP	71.2 ± 5.9	67.8 ± 7.2	(30.1)
Theophylline + ADP + ATP + NaF	81.6 ± 3.4[b]	77.7 ± 2.2[c]	(48.9)

[a] $P<0.01$ compared to controls; [b] $P<0.05$ compared to theophylline; [c] $P<0.05$ compared to theophylline

2,000 x g particulate preparation of rat cerebral cortex, prepared as described in methods (225 mg wet weight tissue) was incubated at 37°C for 1 minute in 1 ml medium containing 40 mM Tris, pH 7.5; 1.0 µM ^{14}C-cyclic AMP (0.250 mCi/µmole) and 3.5 mM $MgSO_4$. The concentrations of the various substances added were: theophylline, 6.7 mM; ADP, 0.5 mM; ATP, 1.0 mM and NaF, 10 mM. Cyclic AMP remaining was determined as described in the text. The numbers in brackets represent the percent inhibition of PDE relative to the activity of the enzyme in the presence of theophylline. Results are a mean of 3 experiments ± S.E.

these nucleotides with NaF increased the inhibition to 82%. If the rate of destruction of cyclic AMP by the adenyl cyclase preparation in the presence of 6.7 mM theophylline is taken as the basal PDE activity, then the addition of ADP (0.5 mM) and ATP (1.0 mM) inhibited this activity by 30%. The presence of NaF (10 mM) increased this inhibition to 49%. This increase in inhibition is significant to $P<0.05$ compared to the inhibition obtained when theophylline alone was present.

Figure 24 shows the effect on cyclic AMP destruction of ADP with ATP, and ADP with ATP and NaF during a more prolonged incubation with this preparation of adenyl cyclase. The same concentrations of nucleotides and NaF used in the previous study (Table 21) were used here and theophylline, 6.7 mM, was present throughout. After 3 minutes of incubation ADP with ATP and ADP with ATP and NaF continued to inhibit the PDE activity of this preparation. The destruction of cyclic AMP in the presence of theophylline at this time was 67% and ADP and ATP reduced this destruction to 50% ($P<0.02$ compared to theophylline alone); the addition of NaF further reduced this destruction to 43% ($P<0.01$ compared to theophylline alone). If the incubation was allowed to proceed to 5 minutes, 86% of the cyclic AMP added was hydrolyzed in the presence of theophylline. In the presence of ADP and ATP only 66% of the nucleotide was hydrolyzed ($P<0.02$ compared to theophylline alone), and the addition of NaF with ADP and ATP further inhibited cyclic AMP destruction so that 42% of the nucleotide still remained at this time ($P<0.01$ compared to theophylline alone). The difference between the amount of cyclic AMP remaining in the presence of theophylline alone and that remaining in the presence of theophylline and the nucleotides became more marked with time.

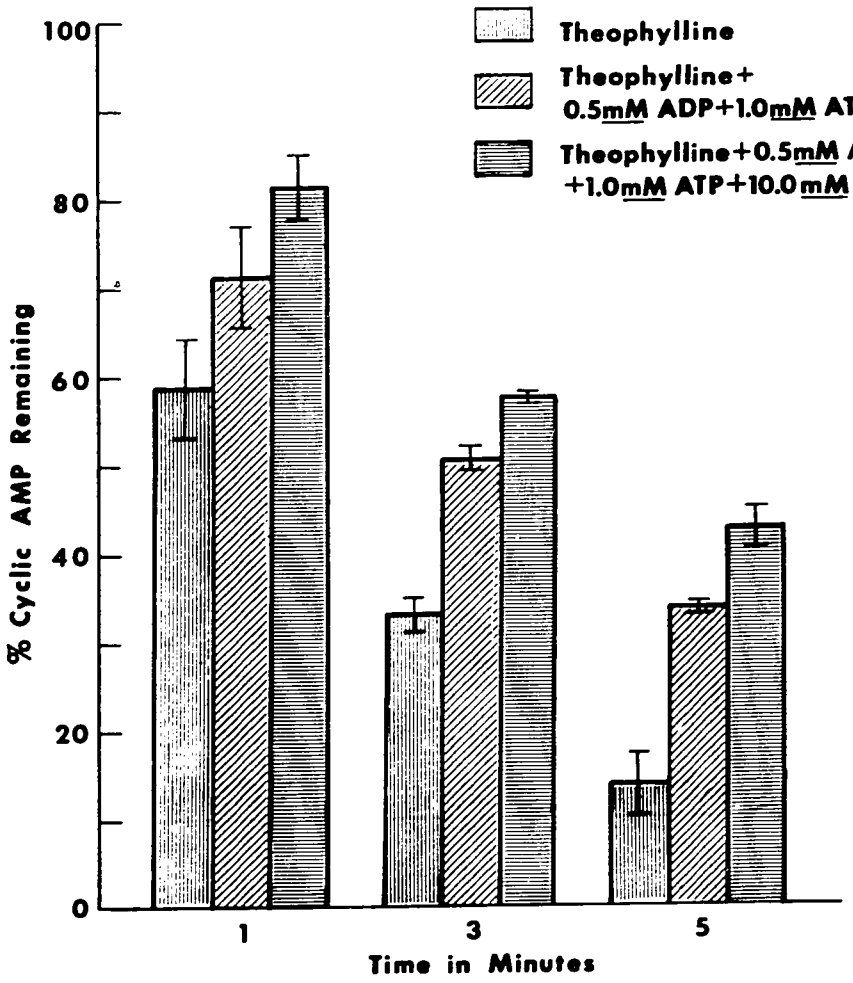


FIGURE 24: The Effect of ADP and ATP in the Presence and Absence of NaF on the PDE Activity of the Rat Cerebral Cortex Preparation of Adenyl Cyclase

2,000 x g particulate preparation of rat cerebral cortex prepared as described in methods (225 mg wet weight tissue) was incubated in 1 ml medium containing 40 mM Tris, pH 7.5; 1.0 M ^{14}C-cyclic AMP (0.250 mCi/μmole); 3.5 mM $MgSO_4$ and theophylline, 6.67 mM and 0.5 mM ADP and 1.0 mM ATP and 10 mM NaF where indicated. Incubation at 37°C was stopped at the times indicated and the cyclic AMP remaining determined by the method described in text. Results are a mean of 3 experiments with vertical lines representing ± S.E.

As shown previously (Part 2, section A) the substitution of Mn^{++} for Mg^{++} in the incubation medium greatly stimulated cyclic AMP accumulation in this preparation of rat cerebral cortex adenyl cyclase. The effect of Mn^{++} on the destruction of cyclic AMP in this preparation was therefore studied; the results are shown in Table 22. Theophylline, 6.67 mM, was present in the incubation medium. Following the 5 minute incubation period, in the presence of Mg^{++}, only 13.5% of the cyclic AMP added remained unhydrolyzed. If equimolar Mn^{++} (3.5 mM) was substituted for Mg^{++} the amount of cyclic AMP remaining following the incubation period was significantly increased ($P<0.05$). Thus, Mn^{++} was shown to be a potent inhibitor of cyclic AMP destruction in this preparation. The effect of Mn^{++} on the activity of PDE was further studied using the synaptic membrane preparation of adenyl cyclase.

C. PDE ACTIVITY OF THE SYNAPTIC MEMBRANE PREPARATION OF ADENYL CYCLASE OF RAT CEREBRAL CORTEX.

Table 23 shows the PDE activity of the synaptic membrane preparation of adenyl cyclase prepared normally, in 1 mM Mg^{++}, or prepared in 1 mM Mn^{++}. A concentration of 6.67 mM theophylline was used throughout this study. When this tissue was prepared in Mg^{++} and incubated in Mg^{++}, 51.3% of the cyclic AMP added was hydrolyzed. This destruction of cyclic AMP was not influenced by the addition of 10 mM NaF. If, however, 5 mM Mn^{++} was substituted for Mg^{++} in the incubation medium, only 40% of the cyclic AMP was hydrolyzed in the 10 minute incubation period. This inhibition of cyclic AMP destruction was significant when compared to the destruction of cyclic AMP produced in the presence of Mg^{++} ($P<0.01$). If the adenyl cyclase was

TABLE 22

Effect of Mn^{++} on the PDE Activity of Rat Cerebral Cortex Preparation of Adenyl Cyclase

		% Cyclic AMP Remaining
Mg^{++}	(3)	13.5 ± 3.2
Mn^{++}	(3)	26.2 ± 0.5[a]

[a] $P<0.05$ compared to Mg^{++}

2,000 x g particulate preparation of rat cerebral cortex, prepared as described in methods (225 mg wet weight tissue) was incubated for 5 minutes at 37°C in either 3.5 mM $MgSO_4$ or 3.5 mM $MnCl_2$. The incubation medium of 1 ml volume also contained: 40 mM Tris, pH 7.5; 1.0 µM ^{14}C-cyclic AMP (0.250 mCi/µmole) and 6.67 mM theophylline. Cyclic AMP remaining was determined as described in the text. Numbers in brackets indicate number of experiments per group. Results are expressed as mean ± S.E.

TABLE 23

Effect of Mn^{++} on PDE Activity of the Synaptic Membrane Preparation of Adenyl Cyclase from Rat Cerebral Cortex

Incubation Media	% Cyclic AMP Remaining			
	Tissue Prepared in Mg^{++}		Tissue Prepared in Mn^{++}	
Mg^{++}	48.7 ± 0.8	(6)	55.7 ± 2.3[b]	(3)
Mg^{++} + NaF	52.9 ± 2.1	(5)	57.0 ± 1.7	(3)
Mn^{++}	59.3 ± 3.1[a]	(4)	70.8 ± 2.1[c,d,e]	(5)
Mn^{++} + NaF	56.7 ± 4.7	(3)	74.1 ± 2.4[c,e,d]	(5)

[a] $P<0.01$ compared to Mg^{++} prepared-Mg^{++} incubated; [b] $P<0.02$ compared to Mg^{++} prepared-Mg^{++} incubated; [c] $P<0.001$ compared to Mg^{++} prepared-Mg^{++} incubated; [d] $P<0.02$ compared to Mg^{++} prepared-Mn^{++} incubated; [e] $P<0.01$ compared to Mn^{++} prepared-Mg^{++} incubated

1.2 M_1 fraction of rat cerebral cortex homogenates prepared as described in methods in either 1 mM $MnCl_2$ or 1 mM $MgSO_4$ (3.0 mg protein) was incubated at 37°C for 10 minutes in either 5.0 mM $MgSO_4$ or 5.0 mM $MnCl_2$. The incubation medium of 2 ml volume also contained: 40 mM Tris, pH 7.5; 6.67 mM theophylline; 0.1 µM ^{14}C-cyclic AMP (2.50 mCi/µmole) and where indicated, 10 mM NaF. Cyclic AMP remaining was determined as described in the text. Numbers in brackets indicate number of experiments in each group. Results are expressed as mean ± S.E.

prepared using Mn^{++} instead of Mg^{++} the inhibition of PDE was even more pronounced. Preparation in Mn^{++} and incubation in Mg^{++} caused a significant inhibition of cyclic AMP destruction compared to the hydrolysis of cyclic AMP produced in Mg^{++} prepared and Mg^{++} incubated adenyl cyclase ($P<0.02$). If, however, the Mn^{++} prepared tissue was incubated in Mn^{++} only 29% of the cyclic AMP was hydrolyzed in the 10 minute incubation period compared to 45% cyclic AMP hydrolyzed in the presence of Mn^{++} prepared and Mg^{++} incubated tissue ($P<0.01$) and over 50% cyclic AMP hydrolyzed in the presence of Mg^{++} prepared and Mg^{++} incubated tissue ($P<0.001$). NaF did not augment the effect of Mn^{++} on cyclic AMP destruction.

Table 24 summarizes the effects of various agents on the PDE activity of the synaptic membrane preparation of adenyl cyclase of rat cerebral cortex. This table includes only data obtained in Mg^{++} prepared tissue. As previously shown, 10 mM NaF had no significant effect on cyclic AMP destruction in this preparation. Five mM Ca^{++} also had no effect on cyclic AMP destruction, nor did Ca^{++} (5.0 mM) plus NaF (10 mM). Adrenaline at a concentration (0.1 mM) that stimulated cyclic AMP accumulation in this preparation did not inhibit the PDE activity of this fraction, nor did ouabain at a concentration of 0.1 mM.

DISCUSSION: STUDIES ON CYCLIC AMP DESTRUCTION IN PREPARATIONS OF RAT BRAIN PDE.

In experiments using a partially purified preparation of soluble PDE from rat brain, ATP and ADP were shown to be potent inhibitors of cyclic AMP destruction. In contrast, AMP, PP_i , α-β mATP and β-γ mATP were shown to be poor inhibitors of cyclic AMP destruction. This finding raised the possibility that substances that inhibited the ATP utilizing enzymes were

TABLE 24

Effect of Various Agents on PDE Activity of Synaptic Membrane Preparation of Adenyl Cyclase from Rat Cerebral Cortex

Addition		% Cyclic AMP Remaining
None	(6)	48.7 ± 0.8
NaF	(5)	52.9 ± 2.2
Ca^{++}	(3)	53.3 ± 0.4
NaF + Ca^{++}	(3)	53.1 ± 0.6
Ouabain	(4)	48.4 ± 2.2
Adrenaline	(4)	45.3 ± 4.8

1.2 M_1 fraction of rat cerebral cortex homogenates prepared as described in methods in 1 mM $MgSO_4$ (3.0 mg protein) was incubated at 37°C for 10 minutes in 2 ml medium containing: 40 mM Tris, pH 7.5; 6.67 mM theophylline; 0.1 mM ^{14}C-cyclic AMP (2.50 mCi/μmole); 5.0 mM $MgSO_4$. The concentrations of the various substances added were: NaF, 10 mM; $CaCl_2$, 5 mM; ouabain 0.1 mM and adrenaline 0.1 mM. Cyclic AMP remaining was determined as described in the text. Number in brackets indicate number of experiments in each group. Results are expressed as mean ± S.E.

not only acting to preserve substrate for adenyl cyclase but also inhibiting cyclic AMP destruction. In the presence of 10 mM NaF, ATP hydrolysis was greatly inhibited in both the cerebral cortex and synaptic membrane preparations of adenyl cyclase (Part 2, sections A and C). A large percentage of the ATP hydrolyzed in the presence of NaF in these preparations was recovered as ADP. The amount of ATP and ADP present under these conditions was sufficient to produce a significant inhibition of cyclic AMP destruction. It was postulated that NaF might be stimulating cyclic AMP accumulation by inhibiting ATP and ADP hydrolysis, and thus reducing PDE activity.

This hypothesis was tested under standard experimental conditions in the cerebral cortex preparation of adenyl cyclase. It was shown that the addition of ADP and ATP in concentrations approximating those occurring in the presence of NaF produced a significant inhibition of cyclic AMP destruction. This inhibition was synergistic with that produced by theophylline alone; cyclic AMP destruction in the presence of both nucleotides and theophylline was significantly increased over that produced in the presence of theophylline alone. The nucleotide inhibition of cyclic AMP destruction persisted during longer incubation periods, with 3 times more cyclic AMP remaining after 5 minutes of incubation in the presence of added nucleotides than in the presence of theophylline alone. Thus the stimulatory effect of NaF on cyclic AMP accumulation can be explained in part by this indirect inhibition of PDE.

The experiments in sections A and C of Part 2 showed that in both rat cerebral cortex and synaptic membrane preparations of adenyl cyclase Mn^{++} was a potent stimulator of cyclic AMP accumulation. In the cerebral cortex

preparation (section A) Mn^{++} significantly inhibited ATP hydrolysis and ADP hydrolysis. Thus Mn^{++} could have been stimulating cyclic AMP accumulation in the same way as described for the action of NaF. In the synaptic membrane preparation of adenyl cyclase a clear dissociation was found between the stimulatory effect of Mn^{++} on cyclic AMP accumulation and ATP hydrolysis (section C). Mn^{++} in this preparation appeared to be stimulating cyclic AMP accumulation in a way not related to inhibition of other membrane-bound ATP utilizing enzymes. The effect of Mn^{++} on PDE activity was therefore studied.

It was shown that Mn^{++} was a potent inhibitor of PDE activity in both the cerebral cortex and synaptic membrane preparations of adenyl cyclase. In the cerebral cortex preparation twice as much cyclic AMP remained following the incubation period in the presence of Mn^{++} than in the presence of equimolar Mg^{++}. In the synaptic membrane preparation, incubation of Mg^{++} prepared tissue in Mn^{++} significantly inhibited PDE activity. Preparation in Mn^{++} and incubation in Mn^{++} further inhibited cyclic AMP destruction with 50% more cyclic AMP remaining in this condition than in the presence of Mg^{++} prepared and Mg^{++} incubated tissue. The finding that Mn^{++} is a potent inhibitor of the PDE activity in adenyl cyclase preparations explains, at least in part, the significant amount of cyclic AMP accumulation in the presence of Mn^{++}.

It was found (Part 2, section A) that adrenaline and ouabain stimulated cyclic AMP accumulation in the rat cerebral cortex preparation of adenyl cyclase. This stimulation of cyclic AMP accumulation was not due to inhibition of the other membrane-bound ATP utilizing enzymes since no difference in the ATP and ADP levels in these groups, as compared to the

controls, was found. In the synaptic membrane preparation of adenyl cyclase (Part 2, section C) adrenaline caused a significant increase in cyclic AMP accumulation while stimulating ATP and ADP hydrolysis. It appeared that here as well another mechanism of stimulation of cyclic AMP accumulation was necessary to explain the effects of these agents. In studies on the synaptic membrane preparation of adenyl cyclase it was shown that these agents did not have a significant effect on cyclic AMP destruction and were thus stimulating cyclic AMP accumulation in another manner.

DISCUSSION

The method of adenine nucleotide determination developed in this study was found to be accurate to 0.5 nmole at the specific activity of ^{14}C-labelled nucleotides used. The separation of AMP, ADP, ATP and cyclic AMP in pure form by this one-step technique provides a useful method for studying the relationship of adenyl cyclase to other membrane-bound ATP utilizing enzymes and PDE. The use of ^{3}H-labelled nucleotides as markers allows an easy method for quantitation of the ^{14}C-nucleotides recovered, and the ratio of ^{3}H to ^{14}C radioactivity provides a measure of the purity of the nucleotides eluted from the columns. The elution pattern of the nucleotides from the columns was very consistent and the time of appearance of each peak could be predicted with great accuracy. Thus, continuous monitoring was not necessary and samples were taken only at the expected peak times of each nucleotide. In this way only a small number of samples from each column was necessary and these could be eluted directly into scintillation vials and counted.

The method for adenine nucleotide determination developed in this study was also found suitable for assaying PDE activity using ^{14}C-cyclic AMP as substrate and measuring ^{14}C-AMP produced and/or the ^{14}C-cyclic AMP remaining in the sample. This method of PDE assay was quick and sensitive and appeared more suitable than inorganic phosphate determination (Butcher and Sutherland, 1962) due to the inherent difficulties in measuring small quantities of inorganic phosphate.

Despite the accuracy and simplicity of this method of adenine nucleotide determination there are a number of limitations to its use. This method does not measure the endogenous levels of cyclic AMP; isotopically labelled substrate must be used. This fact, as well as the use of ^{3}H-labelled adenine

nucleotides as markers, introduces the problem of the radiopurity of these compounds. Analysis of these compounds by either thin layer chromatography or anion-exchange column chromatography is necessary. If only cyclic AMP is to be studied this method of adenine nucleotide determination is not worthwhile because: 1) the sensitivity of this method is much lower than many of the methods available for quantitation of cyclic AMP alone; and 2) the time involved in collecting to the end of the cyclic AMP peak is greater than the time involved in other methods used for the exclusive determination of cyclic AMP.

NaF has been shown to increase cyclic AMP accumulation in particulate preparations of adenyl cyclase (Sutherland et al., 1962; Streeto and Reddy, 1967; Weiss, 1969). NaF has also been shown to be a potent inhibitor of ATP hydrolysis in these preparations (Sutherland et al., 1962; Weiss, 1969). These results suggested a possible mechanism for the NaF-induced increase in cyclic AMP accumulation, that is, the maintenance of substrate for adenyl cyclase by inhibition of ATP hydrolysis. This hypothesis was tested in the rat cerebral cortex preparation of adenyl cyclase. The simultaneous measurement of cyclic AMP accumulation and ATP disappearance clearly indicated that NaF did not increase the accumulation of cyclic AMP solely by maintaining substrate for adenyl cyclase. When varying concentrations of NaF was tested, the highest concentration, 50 mM, produced the greatest inhibition of ATP hydrolysis and AMP accumulation, but this concentration of NaF inhibited cyclic AMP accumulation. The mechanism of NaF stimulation of cyclic AMP accumulation was further studied by comparing its effects on adenine nucleotide metabolism to the effects of an ATP regenerating system. Under the conditions used the ATP regenerating system did not maintain the ATP concentration throughout the 10 minute incubation period. Approximately 98% of the ATP was hydrolyzed in

the presence or absence of the ATP regenerating system; cyclic AMP accumulation was similar under both conditions. In the presence of 10 mM NaF a significant inhibition of ATP hydrolysis was found, and cyclic AMP accumulation was significantly higher than that found in the controls or in the presence of the ATP regenerating system. In the presence of both NaF and the ATP regenerating system the concentration of ATP was higher and cyclic AMP accumulation was significantly increased compared to that accumulated in the presence of 10 mM NaF alone. A study of the time course of ATP metabolism in this preparation revealed that the initial rate of cyclic AMP accumulation in the presence of NaF was much higher than in the presence of the regenerating system but the concentration of ATP was much higher in the presence of the ATP regenerating system; at the end of 1 minute, 0.90 μmoles ATP remained in the presence of NaF and 3.05 μmoles remained in the presence of the ATP regenerating system. A large amount of the ATP metabolized in the presence of NaF was recovered as ADP and, when the concentration of ADP and ATP at this time were added together, the sum was found to be the same as the ADP plus ATP levels in the presence of the ATP regenerating system; 3.60 μmoles in the presence of NaF and 3.70 μmoles in the presence of the ATP regenerating system. For the next 1.5 minutes of incubation, cyclic AMP accumulation in the presence of NaF was twice as high as in the presence of the ATP regenerating system (9.0 nmoles/minute compared to 4.5 nmoles/minute), though at this time the ATP concentrations were not significantly different (1.40 μmoles in the presence of the ATP regenerating system and 0.80 μmoles in the presence of 10 mM NaF) and the ADP plus ATP concentrations were exactly the same (3.0 μmoles). At later times cyclic AMP accumulation in the presence of the ATP regenerating system was less than cyclic AMP destruction; this occurred when the ATP concentration fell from

0.60 μmoles to 0.06 μmoles and coincided with a fall in the ADP concentration from 1.80 to 1.10 μmoles. At this time the rate of cyclic AMP accumulation in the presence of NaF was lower than at earlier periods in the incubation, but was still positive (2.6 nmoles/minute from 2.5 to 4 minutes and 1.2 nmoles/minute from 4 to 7 minutes), while the ATP concentration remained constant at 0.60 μmoles and the ADP concentration varied from 2.0 μmoles to 1.8 μmoles. The sum of ADP plus ATP in the presence of NaF was thus twice as high as in the presence of the ATP regenerating system at this time (2.40 μmoles compared to 1.16 μmoles); the major difference was due to the high ADP concentration. It appeared from these studies that the maintenance of ADP, as well as ATP, concentration was necessary for maximum cyclic AMP accumulation. It was proposed that these nucleotides could be inhibiting PDE activity and thus maintaining a condition for positive cyclic AMP accumulation. In a soluble PDE preparation from rat cerebral cortex it was shown that ADP and ATP were both potent inhibitors of PDE activity. ADP, on a molar basis, was approximately 80% as effective as ATP in inhibiting cyclic AMP destruction in this preparation. When cyclic AMP destruction was studied in the rat cerebral cortex preparation of adenyl cyclase it was found that PDE activity was very high and in the presence of theophylline (6.7 mM) over half the cyclic AMP added was destroyed in the first minute of incubation. When ADP and ATP were added in concentrations chosen to approximate those seen in the adenyl cyclase assay after 30 seconds incubation in the presence of NaF, cyclic AMP destruction was significantly reduced. In the presence of the nucleotides and theophylline only 18% of the cyclic AMP added was hydrolyzed during this period and this represented a 49% inhibition of PDE activity relative to the activity of PDE in the presence of theophylline alone. This

inhibition of PDE by the nucleotides persisted for at least 5 minutes.

It was concluded from these studies that NaF increases cyclic AMP accumulation in this preparation by an indirect inhibition of PDE activity as a result of NaF inhibition of nucleotide hydrolysis, primarily ADP which is a potent inhibitor of PDE. This finding does not explain the complete mechanism of action of NaF in increasing cyclic AMP accumulation. This is evident from the following:

1) This does not explain the much higher initial rates of cyclic AMP accumulation observed in the presence of NaF compared to those in the presence of the ATP regenerating system, when the ADP plus ATP concentration was exactly the same in the two conditions.

2) This finding does not explain why the cyclic AMP accumulation in the guinea-pig pancreas adenyl cyclase preparation was significantly increased by NaF even though the ADP plus ATP concentration in the presence and absence of NaF was essentially the same (3.95 μmoles in the presence of NaF compared to 3.62 μmoles in the controls).

Although in the rat cerebral cortex preparation of adenyl cyclase inhibition of phosphatases and PDE could partially account for NaF stimulation of cyclic AMP accumulation, further purification of this preparation, indicated a more direct action of NaF. In the synaptic membrane preparation of adenyl cyclase the amount of ATP and ADP hydrolysis was much lower than in the cerebral cortex adenyl cyclase preparation. The concentration of these nucleotides found in the presence of 10 mM NaF was not significantly higher than that found in the presence of the controls or the ATP regenerating system, though cyclic AMP accumulation in the presence of NaF was significantly increased. In the presence of NaF and the ATP regenerating system the ATP

concentration following the 10 minute incubation period was significantly higher than that observed in the presence of NaF alone, but in these conditions cyclic AMP accumulation was not significantly increased over that observed in the presence of NaF alone. A time course study of the metabolism of ATP in the presence of the ATP regenerating system and NaF revealed that no cyclic AMP accumulation occurred from the 6 to 10 minute period of incubation even though ATP plus ADP levels were unchanged during this time. When ATP metabolism in the controls was studied, cyclic AMP destruction was seen to occur at a time when ADP plus ATP levels were still very high (1.87 μmoles/mg protein). In the presence of the ATP regenerating system the greatest decrease in ADP plus ATP concentrations occurred from the 6th to the 10th minute of incubation; despite this, cyclic AMP accumulation was positive at this time. Thus, changes in cyclic AMP accumulation in this preparation did not correlate with levels of ADP plus ATP. In addition, PDE activity was much lower in this preparation compared to the cerebral cortex preparation of adenyl cyclase; 50% of cyclic AMP added remained following the 10 minute incubation period in the presence of theophylline compared to less than 12% remaining in the presence of theophylline following 5 minutes of incubation in the cerebral cortex preparation of adenyl cyclase. Destruction of cyclic AMP, therefore, was a less important factor in this preparation.

Figure 25 summarizes the mechanisms by which NaF increases cyclic AMP accumulation in preparations of adenyl cyclase. These mechanisms are: 1) inhibition of ATP and ADP hydrolysis, which provides inhibitors of cyclic AMP destruction; 2) direct stimulation of adenyl cyclase; and 3) maintenance of substrate for adenyl cyclase by inhibition of ATP hydrolysis.

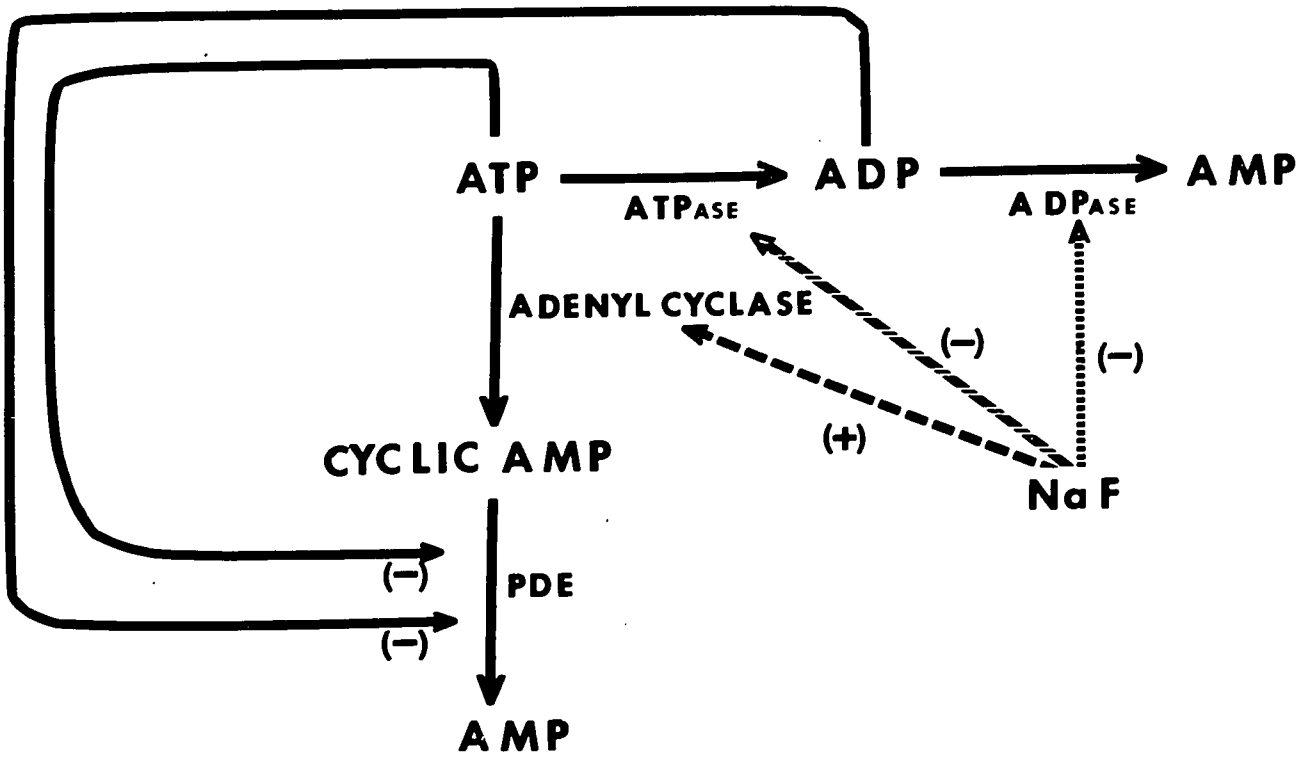


FIGURE 25: The Mechanism by which NaF Increases Cyclic AMP Accumulation in Preparations of Adenyl Cyclase

(—) indicates inhibition and (+) indicates stimulation.

Under the conditions used in this study, in the rat cerebral cortex preparation of adenyl cyclase NaF appears to be acting in two ways: by the maintenance of ADP and ATP concentrations which in turn inhibit PDE activity and by the direct stimulation of adenyl cyclase. In the rat synaptic membrane preparation of adenyl cyclase and the guinea-pig pancreas preparation of adenyl cyclase the more important mechanism of action of NaF is the direct stimulation of adenyl cyclase. The data shows that, when ATP concentration is high, NaF stimulates adenyl cyclase directly but as the ATP concentration falls and the rate of cyclic AMP synthesis falls, the mechanism by which NaF maintains the high concentration of cyclic AMP is an indirect inhibition of PDE, mainly due to the inhibition of ADP hydrolysis. The third mechanism of NaF action, the maintenance of substrate for adenyl cyclase, probably becomes an important factor when concentrations of ATP are very low. This hypothesis is supported by the studies of Dousa and Rychlik (1970b) in the rat kidney homogenate and by Weiss (1969) in the pineal gland homogenate. The suggested direct stimulation of adenyl cyclase by NaF is supported by Birnbaumer et al. (1969) in the rat fat cell ghost preparation, where NaF produced an increase in cyclic AMP accumulation when conditions were such that ATP concentrations were being held constant. In this preparation, NaF appeared to be increasing the affinity of adenyl cyclase for Mg^{++}. Studies by Bär and Hechter (1969a) in rat fat cell ghosts and by Perkins (1970) in rat cerebral cortex particulate preparations showed that NaF did not alter the apparent Km of adenyl cyclase for ATP to a significant extent, indicating that the stimulatory effects of NaF could not be due to an alteration of the affinity of the enzyme for ATP at the catalytic site.

It is difficult to conclusively demonstrate a direct effect of any agent on adenyl cyclase or to completely eliminate an indirect effect on PDE activity as a mechanism of action of any agent since most preparations of adenyl cyclase contain considerable "non-specific" phosphatase activity as well as PDE activity. Resolution of this difficulty awaits the purification of adenyl cyclase. Isolation of adenyl cyclase free of PDE activity has been accomplished in Escherichia coli (Tao and Lipmann, 1969) and in this preparation, NaF inhibits cyclic AMP accumulation. In contrast, a frog erythrocyte adenyl cyclase preparation free of PDE activity was stimulated by NaF (Rosen and Rosen, 1969). Adenyl cyclase possessing little PDE activity has been prepared from rat liver by two groups of investigators. The preparation used by Pohl et al. (1969) was stimulated by NaF and that by Marinetti et al. (1969) was inhibited by NaF. Both groups used the adenyl cyclase contained in purified rat liver plasma membranes, but the methods used to prepare the membranes were somewhat different. These studies suggested that there were some species differences as well as differences determined by the method of preparation of the enzyme.

In studies with the cerebral cortex preparation of adenyl cyclase it was shown that, when equimolar Mn^{++} was added to the incubation medium in the place of Mg^{++}, there was a significant increase in cyclic AMP accumulation. There was also a significant reduction in the amount of ATP hydrolyzed and a significant increase in ADP accumulation. Mn^{++} thus increased cyclic AMP accumulation while inhibiting ATP and ADP hydrolysis. Incubation in the presence of both Mn^{++} and 10 mM NaF produced a slightly greater inhibition of ATP hydrolysis and AMP accumulation and a significant increase in cyclic AMP accumulation. It appeared that Mn^{++} could be increasing cyclic AMP

accumulation by preserving substrate for adenyl cyclase and inhibiting PDE activity by maintaining high concentrations of ATP and ADP. A number of findings suggested that these two mechanisms were not sufficient to fully explain the action of Mn^{++} in increasing cyclic AMP accumulation:

1) In the presence of Mn^{++} and 10 mM NaF, although the amount of ATP, ADP and AMP were not significantly different from that found in the presence of Mg^{++} and 10 mM NaF (2.58 μmoles, 0.57 μmoles and 0.84 μmoles respectively in the case of Mn^{++} and NaF and 2.00 μmoles, 1.17 μmoles and 0.88 μmoles in the case of Mg^{++} and NaF), cyclic AMP accumulation was significantly greater (114 nmoles compared to 57.3 nmoles).

2) When the effect of Mn^{++} was further studied in the synaptic membrane preparation of adenyl cyclase it was found that, when tissue was prepared in Mn^{++} and then incubated in Mg^{++}, ATP hydrolysis and AMP accumulation during the 10 minute incubation period was significantly increased compared to Mn^{++} prepared and Mn^{++} incubated tissue, but cyclic AMP accumulation in these two cases was the same (ATP levels: 0.60 μmoles/mg protein compared to 1.14 μmoles/mg protein; cyclic AMP levels: 4.80 nmoles/mg protein in both cases).

3) In the synaptic membrane preparation, in the presence of 10 mM NaF, the amount of ATP hydrolyzed and ADP accumulated was the same regardless of whether the tissue was prepared and/or incubated in Mn^{++} or Mg^{++}, but the amount of cyclic AMP accumulated in the presence of Mn^{++} prepared tissue was considerably higher (12.0 nmoles/mg protein in Mn^{++} prepared Mg^{++} incubated tissue; 8.80 nmoles/mg protein in Mn^{++} prepared Mn^{++} incubated tissue; 5.60 nmoles/mg protein in Mg^{++} prepared Mg^{++} incubated tissue and 6.10 nmoles/mg protein in Mg^{++} prepared Mn^{++} incubated tissue).

It was proposed that in both these preparations PDE activity might be altered in the presence of Mn^{++} in a way unrelated to nucleotide inhibition of PDE, thus resulting in the difference in the amount of cyclic AMP accumulated in the presence of Mn^{++} and Mg^{++}. In the rat cerebral cortex preparation it was indeed shown that PDE activity in the presence of Mn^{++} was 50% lower than in the presence of Mg^{++}. In the synaptic membrane preparation of adenyl cyclase, PDE activity in the presence of tissue prepared and/or incubated in Mn^{++} was significantly lower than in the presence of Mg^{++} prepared Mg^{++} incubated tissue. For example, over 70% of cyclic AMP added remained after 10 minutes incubation in the presence of Mn^{++} prepared, Mn^{++} incubated tissue and only 48% remained in the presence of Mg^{++} prepared, Mg^{++} incubated tissue.

Figure 26 summarizes the mechanisms by which Mn^{++} increases cyclic AMP accumulation in preparations of adenyl cyclase. These mechanisms are: 1) inhibition of ATP and ADP hydrolysis, which provides inhibitors of cyclic AMP destruction; 2) direct stimulation of adenyl cyclase; 3) inhibition of cyclic AMP destruction in a way unrelated to nucleotide inhibition of PDE; and 4) maintenance of substrate for adenyl cyclase by inhibition of ATP hydrolysis.

In the rat cerebral cortex preparation of adenyl cyclase, Mn^{++} appeared to increase cyclic AMP accumulation by inhibition of ATP and ADP hydrolysis and thus by indirect inhibition of PDE and also by inhibition of cyclic AMP destruction in a manner unrelated to nucleotide inhibition. In the rat synaptic membrane preparation of adenyl cyclase where PDE activity was much lower these factors appeared to be secondary to a direct stimulation of adenyl cyclase by Mn^{++}. As in the case of NaF a direct stimulation of adenyl cyclase

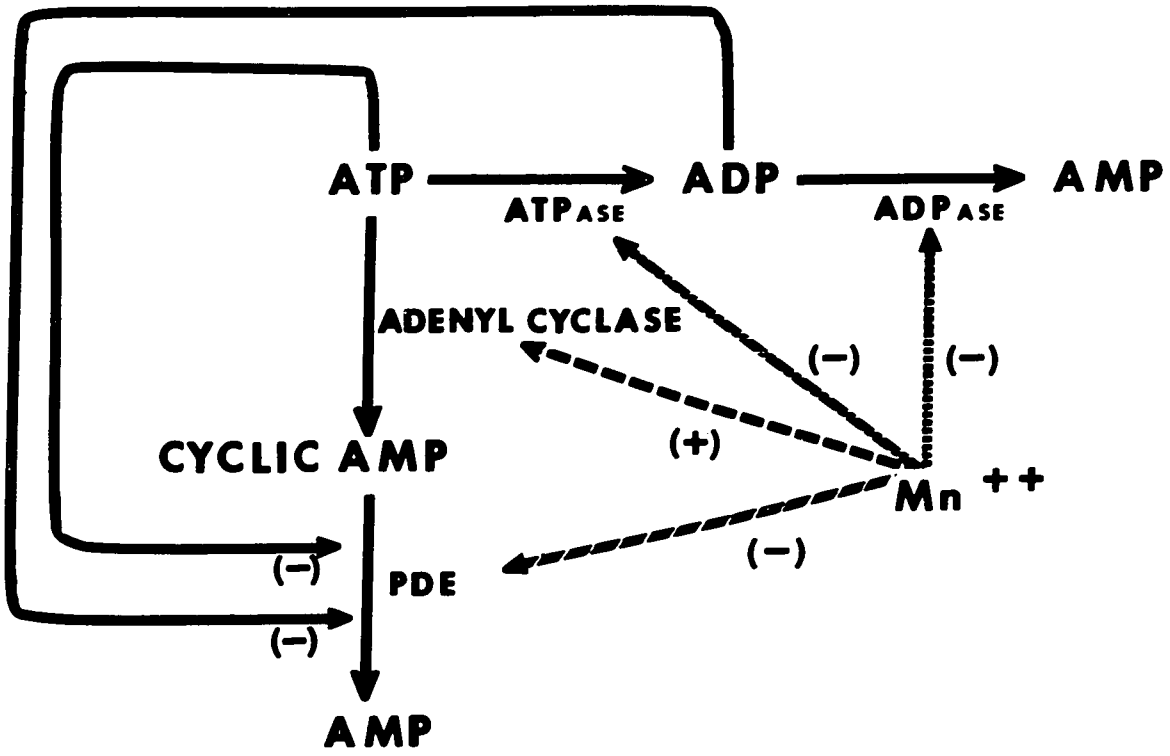


FIGURE 26: The Mechanisms by which Mn^{++} Increases Cyclic AMP Accumulation in Preparations of Adenyl Cyclase

(—) indicates inhibition and (+) indicates stimulation.

is difficult to prove conclusively in preparations of adenyl cyclase that are contaminated with so many other enzyme systems. The high accumulation of cyclic AMP, however, in the presence of Mn^{++} in the synaptic membrane preparation, a system where ATP and ADP hydrolysis was much reduced and PDE activity was less of a factor, strongly suggests a direct effect of Mn^{++} on adenyl cyclase. The maintenance of substrate for adenyl cyclase by Mn^{++} probably becomes important at very low concentrations of ATP and cannot be excluded as an important factor in promoting cyclic AMP accumulation in systems where ATP hydrolysis is very high. Sutherland et al. (1962) found that Mn^{++} could substitute for Mg^{++} as the metal ion requirement in particulate preparations of brain adenyl cyclase. Perkins (1970) found that cyclic AMP accumulation in cerebral cortex particulate preparations was stimulated by Mn^{++} as well as by Mg^{++}. The apparent Km value for Mn-ATP and Mg-ATP was the same, 0.16 mM; no indication of the relative effects of the two metal ions on cyclic AMP accumulation was given. The results reported in this thesis are supported by the work of Birnbaumer et al. (1969) in the rat fat cell ghost preparation where Mn^{++} was shown to stimulate basal and NaF stimulated cyclic AMP accumulation; no suggestion as to the mechanism of Mn^{++} stimulation of cyclic AMP accumulation was given in this study.

Experiments by Roberts and Simonsen (1970) on mouse brain soluble PDE preparations, showed that Mn^{++} was considerably more effective on a molar basis than Mg^{++} in activating PDE. In dog heart soluble PDE preparations (Nair, 1966) Mg^{++} was equally as effective as Mn^{++} in promoting PDE activity and in rabbit brain PDE preparations Mg^{++} was more effective than Mn^{++}. This indicates that PDE enzymes might differ from one species to another and possibly from one tissue to another. Again, no indication as to the relative

effects of Mg^{++} and Mn^{++} on cyclic AMP accumulation was given in these studies.

The present studies show that although Mn^{++} inhibited PDE activity in the rat cerebral cortex preparation and synaptic membrane preparations of adenyl cyclase, this ion did not inhibit the soluble PDE preparation of rat cerebral cortex. This was also shown by Cheung (1966) using a similar preparation of rat cerebral cortex PDE. The fact that the adenyl cyclase preparations used were shown to possess other enzyme activities, such as nucleotidases, and that the soluble PDE preparation was relatively free of other contaminating enzymes, suggests that Mn^{++} might have produced its inhibition on PDE activity in the adenyl cyclase preparations through another enzyme system not present in the soluble PDE preparation.

These studies in broken cell preparations demonstrate that an effect on other enzyme systems can result in a net accumulation of cyclic AMP. All of these enzyme systems exist in the intact cell and it is not improbable that some of the physiological stimulators of cyclic AMP act through these systems in the same way as described for Mn^{++} and NaF.

Adrenaline and ouabain had been previously shown to stimulate cyclic AMP accumulation in various preparations of rat brain (Klainer et al., 1962; Shimizu et al., 1970c), but the mechanism of action of these drugs was not known.

In the present studies, stimulation of cyclic AMP accumulation by adrenaline and ouabain was shown not to be due to an effect on other membrane-bound ATP utilizing enzymes or on PDE activity:

1) In the rat cerebral cortex preparation, there was little difference in the amount of AMP, ADP and ATP recovered in the presence and absence of

adrenaline or of ouabain after 10 minutes of incubation; 95% of total nucleotide, in all three cases was recovered as AMP. There was, however, a significant increase in the amount of cyclic AMP accumulated in the presence of either of these agents.

2) In the synaptic membrane preparation of adenyl cyclase prepared in the presence of Ca^{++}, adrenaline significantly increased cyclic AMP accumulation even though ATP hydrolysis and AMP accumulation were also significantly increased.

3) Neither ouabain nor adrenaline had any effect on the PDE activity of the synaptic membrane preparation of adenyl cyclase.

It was concluded that ouabain and adrenaline were acting in a more direct manner to stimulate cyclic AMP accumulation in these preparations of adenyl cyclase. The requirement of Ca^{++} in the preparation media of the synaptic membrane preparation of adenyl cyclase, in order for cyclic AMP accumulation to be stimulated by adrenaline, further demonstrated the difference in the mechanism of stimulation of cyclic AMP accumulation by NaF or Mn^{++} and by adrenaline.

The stimulation by adrenaline of cyclic AMP accumulation in the synaptic membrane preparation of adenyl cyclase is the first report of stimulation by a biogenic amine of cyclic AMP accumulation in a high speed brain adenyl cyclase preparation. Indeed, there have been very few reports of adrenaline stimulation of adenyl cyclase present in either low- or high-speed particulate preparations, of any tissue. A review of the few reports available in this area points out the difficulty in stimulating cyclic AMP accumulation in these preparations by adrenaline and the possible explanation.

1) Klainer et al. (1962) found that adrenaline produced a small increase in the formation of cyclic AMP by rat cerebral cortex homogenates, but there was considerable variation in the drug's effect.

2) Bitensky et al. (1968) noted that adrenaline stimulation of adenyl cyclase in liver homogenates was found to be reduced by heating or sonication, while the response to glucagon in the preparation was unchanged.

3) Pohl et al. (1969) using a high-speed plasma membrane preparation of rat parenchymal cells demonstrated stimulation of cyclic AMP accumulation by glucagon but not by adrenaline; they considered that adrenaline responsiveness may have been destroyed by the method of preparation of the adenyl cyclase.

4) Øye and Sutherland (1966) found that extensively fragmented membrane preparations from turkey erythrocytes did not respond to adrenaline but larger fragments exhibited adenyl cyclase activity in the presence of this hormone.

5) Rall and Kakiuchi (1966) also noted hormone sensitivity of adenyl cyclase preparations from nearly all tissues may be lost during vigorous fractionation procedures with relatively small losses in adenyl cyclase activity in response to NaF.

The results of these studies suggest the following possibilities:

1) The molecular configuration of adenyl cyclase was changed by the extensive fragmentation of the tissue and thus the response to hormones was lost during the process.

2) The hormone responsiveness of the adenyl cyclase required an entity distinct from the enzyme and this entity was lost or separated from the adenyl

cyclase during the fractionation of the tissue.

3) A requirement for hormone stimulation of adenyl cyclase that was present in whole cells, such as a cytoplasmic component or ion requirement, was lost during the fractionation procedures.

Marinetti et al. (1969) found that the addition of Ca^{++} (0.50 mM) to the homogenizing medium increased adenyl cyclase activity in high-speed plasma membrane preparations; a minimum concentration of Ca^{++} of 0.01 mM was necessary for stimulation of this preparation by adrenaline. Perkins (1970) found that Ca^{++} in low concentrations (0.05 mM) stimulated the accumulation of cyclic AMP by low-speed homogenates of rat cerebral cortex but higher concentrations of Ca^{++}, above 0.1 mM, inhibited the activity of adenyl cyclase. Bradham et al. (1970), in a similar preparation, found that the concentration of Ca^{++} appearing as a contaminant in the incubation medium (approximately 0.033 mM) was enough to allow cyclic AMP accumulation; inclusion of EGTA, 0.1 mM, reduced adenyl cyclase activity by 60% and an equal concentration of added Ca^{++} prevented this inhibition. A similar requirement for Ca^{++} was demonstrated for ACTH stimulation of adenyl cyclase in the rat fat cell ghost preparation and in the bovine adrenal membrane preparation (Birnbaumer et al., 1969; Bar and Hechter, 1969b).

Ray (1970) stated that Ca^{++} in the homogenizing medium was a critical factor in obtaining plasma membrane preparations in high yield and with high activity of the characteristic plasma membrane enzymes. Ca^{++} has been found to maintain the structural and functional integrity of the plasma membrane (Kavanau, 1965; Abood and Gabel, 1965; Cavallito, 1967). The stabilization of the membrane structure by calcium may be the result of coordination of Ca^{++} to the macromolecular components of the membrane. When

tissues are homogenized in the absence of Ca^{++}, Ca^{++} is dissociated from the membrane structures. The presence of Ca^{++} in the homogenizing medium may help to prevent dissociation of the membrane-bound Ca^{++} and thus maintain the integrity of the plasma membrane, resulting in greater activity of the enzymes present in the membrane. In the present studies it was shown that, in addition to the ability of adrenaline to stimulate cyclic AMP accumulation under these conditions, ATP hydrolysis was higher in membranes prepared in the presence of Ca^{++}.

It is concluded that the requirement of Ca^{++} for adrenaline stimulation of cyclic AMP accumulation in the synaptic membrane preparation of adenyl cyclase is probably related to the preservation of the integrity of the membrane preparation. The requirement of a stable membrane-enzyme complex for hormone stimulation of adenyl cyclase would explain the unresponsiveness of the soluble adenyl cyclase preparations to hormone stimulation (Sutherland et al., 1962; Levey, 1970). Ca^{++} does not appear to be an ion requirement per se for adenyl cyclase. In fact, at concentrations slightly greater than that required for membrane integrity, Ca^{++} is a potent inhibitor of adenyl cyclase (Perkins, 1970; Bär and Hechter, 1969b).

In the present studies, ouabain stimulated cyclic AMP accumulation in the low-speed cerebral cortex preparation but did not stimulate cyclic AMP accumulation in the high-speed synaptic membrane preparation of adenyl cyclase. The lack of stimulation of cyclic AMP accumulation by ouabain in the synaptic membrane preparation probably indicates a loss of some property in the fractionation of this homogenate that is necessary for ouabain stimulation of adenyl cyclase. In this case, the addition of Ca^{++} to the preparation media did not restore the effect of ouabain on cyclic AMP accumulation.

Ouabain had previously been shown to stimulate brain slice preparations of adenyl cyclase (Shimizu et al., 1970c) but this is the first report of its stimulation of cyclic AMP accumulation in any kind of broken-cell preparation. In the brain slice preparation, ouabain appeared to be stimulating cyclic AMP accumulation in a manner different from that of the biogenic amines, noradrenaline and histamine; it was postulated that ouabain stimulated cyclic AMP accumulation by a depolarizing effect requiring the presence of Ca^{++}. The present studies do not support this explanation as ouabain was shown to stimulate cyclic AMP accumulation in a particulate preparation of adenyl cyclase in which, presumably, membrane depolarization is not a factor. The present experiments, though, do not clarify the mechanism of stimulation of cyclic AMP accumulation by ouabain in the particulate rat cerebral cortex preparation nor do they indicate the reason for the lack of stimulation of cyclic AMP accumulation in the synaptic membrane preparation of adenyl cyclase.

The mechanism of stimulation of cyclic AMP accumulation by adrenaline was further investigated by an attempt to characterize the response as either an α- or β-adrenergic type of effect. Adrenaline-stimulated cyclic AMP accumulation in rat cerebral cortex and synaptic membrane preparations of adenyl cyclase was inhibited by both POB, and α-adrenergic blocking agent, and pronethalol, a β-adrenergic blocking agent. In previous studies by Weiss (1969) DCI and propranolol, both β-adrenergic blocking agents, significantly decreased noradrenaline-stimulated cyclic AMP accumulation in rat pineal gland homogenates while α-adrenergic blocking agents were without effect. In studies on rabbit cerebellar slices (Kakiuchi and Rall, 1968a) DCI inhibited noradrenaline stimulated cyclic AMP accumulation but POB did not. Thus the inhibition of adrenaline-stimulated cyclic AMP accumulation by α-adrenergic

blockade in these present studies appears at variance with other studies on brain preparations. It seemed possible that the concentration of POB (0.1 mM) used in this study produced a "non-specific" inhibition of these preparations, though this concentration did not affect the control levels of cyclic AMP accumulation. Furthermore, the concentration of POB used by Kakiuchi and Rall (1968a) was 5 times higher than the concentration used in the present studies and Weiss (1969) used 1 mM phentolamine. It is possible that the different membrane preparations in which these effects were studied could explain the differing results. It is thus difficult to conclude anything as to the nature of the adrenaline response from these studies with the blocking agents.

An attempt was made to elucidate further the nature of the adrenaline stimulation of cyclic AMP accumulation by varying the Mg^{++} concentration in the incubation medium of the synaptic membrane preparation of adenyl cyclase prepared in the presence of Ca^{++}. At concentrations of 1.0 mM Mg^{++} to 8.0 mM Mg^{++} cyclic AMP accumulation in the presence of adrenaline was higher than that observed in the absence of adrenaline. In the absence of adrenaline, cyclic AMP accumulation increased with increasing Mg^{++} concentration so that at 10 mM Mg^{++} cyclic AMP accumulation was higher in the controls than in the presence of adrenaline. These results are similar to those of Birnbaumer et al. (1969) in studies on the effect of ACTH on cyclic AMP accumulation in rat fat cell ghosts. As in their studies, in the absence of hormones Mg^{++} concentration in excess of that required for maximal Mg-ATP levels (Walaas, 1958; Burton, 1959) enhanced the basal activity of adenyl cyclase. If, as suggested by Birnbaumer et al. (1969), Mg-ATP is the true substrate of adenyl cyclase, free ATP an inhibitor and free Mg^{++} an allosteric

effector, it is possible that the observed accumulation of cyclic AMP in the presence of adrenaline is an action of the hormone in increasing the affinity of adenyl cyclase for Mg^{++} at a site that is distinct from the catalytic site.

Although adrenaline and ouabain both stimulated cyclic AMP accumulation in the rat cerebral cortex preparation of adenyl cyclase in the presence of Mg^{++}, these agents did not stimulate cyclic AMP accumulation in the presence of Mn^{++}. This was also true for adrenaline-stimulated cyclic AMP accumulation in the synaptic membrane preparation. The absence of an effect in the presence of Mn^{++} was not due to the large basal concentration of cyclic AMP accumulated, because the addition of NaF (10 mM), significantly increased cyclic AMP accumulation in both preparations in the presence of Mn^{++}. It was previously noted that adenyl cyclase has, in addition to the catalytic site, a site for Mg^{++} that influences the catalytic activity of this enzyme, and that this is the site that is altered in the presence of the activators. It is suggested that: 1) Mn^{++} binds to this allosteric site better than Mg^{++} and under basal conditions stimulates the catalytic activity of the enzyme, and 2) adrenaline and ouabain cannot further alter the binding of Mn^{++} at this site. The present experiments offer no support for this hypothesis as no binding studies were carried out. Only one report could be found in which a hormone effect on cyclic AMP accumulation was tested in the presence of Mn^{++}. In this case, in the rat fat cell ghost preparation (Birnbaumer et al., 1969) stimulation of cyclic AMP accumulation by ACTH was not observed in the presence of Mn^{++}.

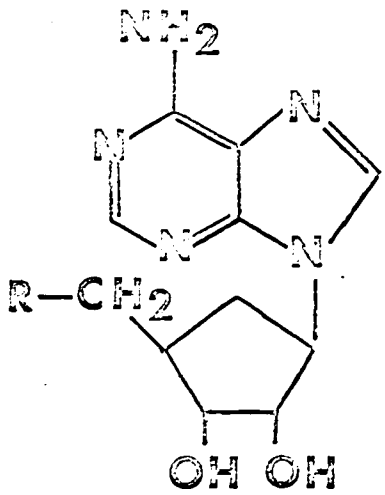
This study again suggests that the mode of action of adrenaline and ouabain and that of NaF in stimulating cyclic AMP accumulation are different;

stimulation of cyclic AMP accumulation by adrenaline and ouabain required the presence of Mg^{++}, whereas stimulation of cyclic AMP accumulation by NaF was highest in the presence of Mn^{++}.

Studies with the soluble PDE preparation showed that ATP and ADP were potent inhibitors of PDE activity and PP_i was a weak inhibitor. The inhibition of PDE activity by ADP and ATP was recently reported by Dousa and Rychlik (1970a) in a soluble PDE preparation from rat kidney. On comparison of the effects of ATP, ADP, AMP and PP_i in this preparation these authors suggested that the chelating action of the PP_i group for Mg^{++} was necessary for PDE inhibition. This was postulated by Cheung (1970) as the mechanism of ATP inhibition of PDE activity in a soluble preparation of rat brain. The present studies do not support this hypothesis, because increasing the Mg^{++} concentration 5 times did not reverse the inhibitory effect of ATP on the PDE activity of this preparation. In the present experiments it was also found that α,β mATP and β,γ mATP were not as potent inhibitors of PDE activity as was non-substituted ATP; α,β mATP was $^1/8$th as potent an inhibitor of the PDE activity of this preparation as ATP and β,γ mATP was an even weaker inhibitor.

Figure 27 shows the structures of the various substances used in the study of this PDE preparation in the order of their inhibitory potency. It can be noted that:

1) The phosphate chain of these compounds appears more important than the adenine-ribose moiety in inhibition of PDE as adenosine (Shimizu et al., 1970b) and AMP are weak inhibitors of PDE activity and ADP and ATP are stronger inhibitors of PDE activity. It has also been reported (Cheung, 1970) that inorganic triphosphate and tetraphosphate are strong inhibitors of the PDE



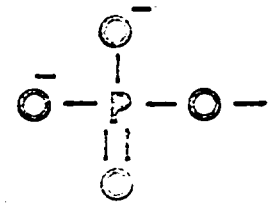
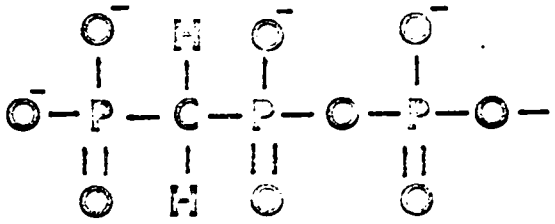
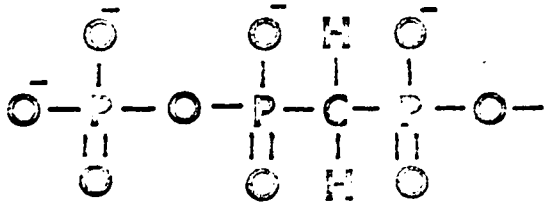
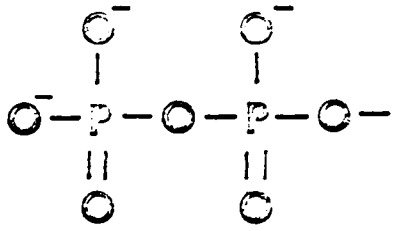
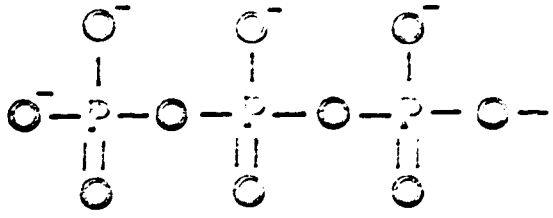
Substance	Nature of R Group
Adenosine	None
AMP	
β,ϒ mATP	
ϒ,β mATP	
ADP	
ATP	

FIGURE 27: The Structure of Various Substances in Order of Their Inhibitory Activity on a Partially Purified Preparation of PDE from Rat Cerebral Cortex

activity of this preparation.

2) A substitution of one of the bridging pyrophosphate oxygens by a methylene group weakens the inhibition of PDE activity by the triphosphate nucleotide. The methylene group substitution in the β,γ phosphate position produces a weaker inhibitor than substitution between the α and β phosphates.

3) The fact that ATP is a stronger inhibitor of PDE than ADP and that α,β mATP is a better inhibitor of PDE than β,γ mATP indicates the importance of the terminal pyrophosphate bond in the polyphosphate for maximum inhibition of PDE activity.

Experiments on a more purified preparation of PDE with the use of other ATP analogs and polyphosphate chains might lead to a further understanding of the nucleotide inhibition of PDE activity and the nature of the PDE receptor (system).

SUMMARY

A method for measuring the rate of production of ^{14}C-labelled adenine nucleotides, from ^{14}C-ATP was developed and used to study the relationship of adenyl cyclase to other membrane-bound ATP utilizing enzymes and PDE. The preparations used were: 1) a rat cerebral cortex preparation of adenyl cyclase, 2) a guinea-pig pancreas preparation of adenyl cyclase, 3) a synaptic membrane preparation of rat cerebral cortex adenyl cyclase and 4) a soluble PDE preparation from rat cerebral cortex. The agents used to study this relationship were NaF, Mn^{++}, adrenaline and ouabain.

The results of these studies indicated that:

a) The method of adenine nucleotide determination developed in this study was accurate, and provided a useful procedure for the study of the relationship of adenyl cyclase to other membrane-bound ATP utilizing enzymes and PDE.

In the rat cerebral cortex preparation of adenyl cyclase:

b) NaF, 5 mM, 10 mM and 50 mM significantly inhibited ATP and ADP hydrolysis and AMP accumulation. The highest concentration of NaF, 50 mM, produced the greatest inhibition of ATP and ADP hydrolysis but failed to stimulate cyclic AMP accumulation. Ten mM NaF produced the highest amount of cyclic AMP accumulation, significantly higher than that accumulated in the presence of 5 mM and 50 mM NaF.

c) The ATP regenerating system used was unable to maintain the ATP concentration during the 10 minute incubation period; following this period there was no significant difference in the amount of ATP, ADP, AMP or cyclic AMP present in vessels containing the ATP regenerating system compared to the controls.

d) In the presence of 10 mM NaF and the ATP regenerating system the ATP concentration was maintained at a higher level than in the presence of the ATP regenerating system alone or NaF alone. The accumulation of cyclic AMP under these conditions was also significantly increased.

e) A time course of the metabolism of ATP revealed the following: 1) in the controls, ATP was rapidly hydrolyzed with most of the nucleotide following 2.5 minutes of incubation recovered as AMP, while cyclic AMP accumulation was positive for the first 2.5 minutes of incubation and negative from thereon; 2) ATP hydrolysis in the presence of NaF, 10 mM, was rapid during the first minute of incubation, ADP accumulation accounting for the majority of the ATP hydrolyzed during this time. ATP hydrolysis was then much slower throughout the rest of the incubation period while cyclic AMP accumulation was positive during the entire incubation period; 3) in the presence of the ATP regenerating system, the concentration of ATP, following the first minute of incubation was higher than in the controls or in the presence of NaF alone. Following the first minute of incubation the ATP concentration fell rapidly with AMP appearing as the major metabolite. Cyclic AMP accumulation was initially very rapid then destruction of cyclic AMP was greater than cyclic AMP accumulation throughout the rest of the incubation period.

f) Adrenaline (0.1 mM) and ouabain (0.1 mM) both significantly increased cyclic AMP accumulation though, following the 10 minute incubation period there was little difference in the amount of AMP, ADP and ATP recovered in the presence and absence of adrenaline or ouabain compared to the controls.

g) POB (0.1 mM), an α-adrenergic blocking drug, and pronethalol (0.1 mM) a β-adrenergic blocking drug inhibited adrenaline (0.1 mM) induced cyclic AMP accumulation.

h) Mn^{++} (3.5 mM) significantly increased cyclic AMP accumulation while significantly inhibiting ATP and ADP hydrolysis and AMP accumulation. The addition of 10 mM NaF to the Mn^{++} incubated tissue further increased cyclic AMP accumulation while further decreasing, though not significantly, ATP hydrolysis and AMP accumulation.

i) Adrenaline (0.1 mM) and ouabain (0.1 mM) did not stimulate cyclic AMP accumulation in tissue incubated in the presence of Mn^{++}.

In the guinea-pig pancreas preparation of adenyl cyclase:

j) NaF significantly increased cyclic AMP accumulation, although ATP hydrolysis, ADP hydrolysis and AMP accumulation were not significantly reduced in the presence of NaF compared to the controls.

k) Carbachol (1 mM and 0.1 mM), adrenaline (0.1 mM and 1 mM), pancreozymin (0.9 and 1.8 I.U.) and secretin (1.0 and 3.0 I.U.) did not stimulate cyclic AMP accumulation in this preparation.

In the synaptic membrane preparation of adenyl cyclase:

l) NaF, 10 mM, significantly increased cyclic AMP accumulation, while inhibiting significantly the accumulation of AMP and inhibiting, though not significantly, the hydrolysis of ATP.

m) The ATP regenerating system used was unable to maintain the ATP concentration during the 10 minute incubation period; following this period there was no significant difference in the concentration of ATP, ADP or cyclic AMP in vessels containing the ATP regenerating system compared to the controls while AMP accumulation in the presence of the ATP regenerating system was significantly decreased.

n) In the presence of 10 mM NaF and the ATP regenerating system the concentration of ATP following the 10 minute incubation period was significantly higher

than that observed in the controls, in the presence of NaF (10 mM) alone and in the presence of the ATP regenerating system alone, however, cyclic AMP accumulation was not significantly increased compared to that accumulated in the presence of 10 mM NaF alone.

A time course of the metabolism of ATP revealed the following:

o) 1) In the controls, more than 50% of the ATP present was hydrolyzed during the first 6 minutes of incubation, with ADP the major metabolite; from 6-10 minutes no further ATP hydrolysis was observed. The rate of accumulation of cyclic AMP was positive for the first 3 minutes and negative from thereon; 2) in the presence of 10 mM NaF, ATP hydrolysis was rapid during the first minute of incubation, ADP accumulation accounting for the majority of the ATP hydrolyzed during this period. Following this period ATP hydrolysis was reduced. Cyclic AMP accumulation was positive throughout the incubation period; 3) in the presence of the ATP regenerating system, there was no fall in the ATP concentration during the first 6 minutes of incubation following which the ATP concentration fell rapidly and ADP accumulated. The rate of accumulation of cyclic AMP was greatest during the first 3 minutes of incubation but remained positive throughout the incubation period; 4) in the presence of both 10 mM NaF and the ATP regenerating system there was no fall in the ATP concentration during the 10 minute incubation period. Cyclic AMP accumulation was rapid during the first 6 minutes of incubation while no accumulation of cyclic AMP occurred during the 6-10 minute period.

p) In the absence of Ca^{++} in the preparation media, adrenaline (0.1 mM) had no significant effect on cyclic AMP accumulation or ATP, ADP or AMP levels. With the addition of 0.01 mM Ca^{++} to the preparation media, 1) ADP accumulation in the controls was significantly increased, 2) ATP hydrolysis

ADP hydrolysis and AMP accumulation in the presence of adrenaline (0.1 mM) were significantly increased compared to adrenaline-induced levels in the absence of Ca^{++} in the preparation media and 3) cyclic AMP accumulation in the presence of adrenaline (0.1 mM) was significantly increased compared to the controls. Ouabain had no effect on ATP, ADP, AMP or cyclic AMP levels either in the presence or absence of Ca^{++} in the preparation media.

q) POB (0.1 mM) and pronethalol (0.1 mM) significantly inhibited adrenaline (0.1 mM) induced cyclic AMP accumulation.

r) Increasing the Mg^{++} concentration in the incubation medium increased cyclic AMP accumulation both in the presence and absence of (0.1 mM) adrenaline. In the presence of high concentrations of Mg^{++}, adrenaline was not able to further stimulate cyclic AMP accumulation. In the presence of adrenaline (0.1 mM) decreasing the Mg^{++} concentration of the incubation medium significantly increased ATP hydrolysis.

s) 1) In the absence of NaF, the highest amount of ATP hydrolysis was obtained in tissue prepared in Mn^{++} and incubated in Mg^{++}. Cyclic AMP accumulation under these conditions was equal to that produced under any other condition and significantly higher than that observed in the presence of Mg^{++} prepared and Mg^{++} incubated tissue. 2) In the presence of NaF (10 mM), the amount of ATP hydrolysis in Mn^{++} prepared and/or Mn^{++} incubated tissue was the same as in the presence of Mg^{++} prepared tissue but the amount of cyclic AMP accumulated in tissue prepared in Mn^{++} was considerably higher.

t) Ouabain (0.1 mM) and adrenaline (0.1 mM) both failed to stimulate cyclic AMP accumulation in the presence of Mn^{++} prepared and/or incubated tissue.

In a soluble preparation of PDE from rat cerebral cortex:

u) 1) ATP and ADP significantly inhibited PDE activity while AMP and PP_i inhibited PDE activity only slightly. NaF did not alter the inhibitory effects of ADP, ATP or PP_i but significantly increased the inhibitory effect of 1.0 mM AMP. Increasing the Mg^{++} concentration did not reverse the inhibitory effect of ATP on PDE activity. 2) α,β mATP and β,γ mATP were weak inhibitors of PDE activity; α,β mATP had $^1/8$th the inhibitory activity of ATP, β,γ mATP significantly less. 3) PDE activity was the same in the presence of Mn^{++} as in the presence of Mg^{++}.

In the cerebral cortex preparation of adenyl cyclase:

v) 1) In the presence of 6.7 mM theophylline, ADP (0.5 mM) and ATP (1.0 mM) significantly reduced PDE activity compared to that observed in the presence of theophylline alone. 2) PDE activity in the presence of Mn^{++} incubated tissue was significantly reduced compared to PDE activity in the presence of Mg^{++}.

In the synaptic membrane preparation of adenyl cyclase:

w) 1) Preparation and/or incubation of tissue in Mn^{++} significantly reduced PDE activity compared to PDE activity observed in the presence of Mg^{++} prepared tissue. 2) Adrenaline (0.1 mM), ouabain (0.1 mM) and Ca^{++} (5 mM) had no effect on PDE activity in this preparation.

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CLAIMS TO ORIGINAL RESEARCH

1. The method of determination of adenine nucleotides, including cyclic AMP in a single-step procedure using anion-exchange column chromatography and double label isotope procedures for monitoring recoveries and measuring the purity of the peak samples.
2. The observation that NaF inhibited PDE activity indirectly by inhibition of ATP and ADP hydrolysis. ADP and ATP were shown to be potent inhibitors of PDE activity even in the presence of theophylline.
3. The observation that Mn^{++} inhibited ATP and ADP hydrolysis in preparations of rat cerebral cortex adenyl cyclase.
4. The observation that Mn^{++} inhibited PDE activity in these adenyl cyclase preparations indirectly by inhibition of ATP and ADP hydrolysis and in a manner unrelated to nucleotide inhibition of PDE.
5. The observation that ouabain (0.1 mM) significantly stimulated cyclic AMP accumulation in the rat cerebral cortex preparation of adenyl cyclase and the observation that this stimulation was not due to an inhibition of ATP or ADP hydrolysis or an inhibition of PDE activity.
6. The observation that when Ca^{++} (0.01 mM) was added to the preparation media, adrenaline significantly stimulated cyclic AMP accumulation in the synaptic membrane preparation of adenyl cyclase and the observation that this stimulation was not due to an inhibition of ATP or ADP hydrolysis or an inhibition of PDE activity.
7. The observation that adrenaline (0.1 mM) did not stimulate cyclic AMP accumulation in the rat cerebral cortex or synaptic membrane preparation of adenyl cyclase in the presence of Mn^{++}.

8. The observation of adenyl cyclase activity in the guinea-pig pancreas that can be increased by NaF but not by carbachol, pancreozymin, adrenaline or secretin.
9. The observation in a partially purified PDE preparation of rat cerebral cortex that methylene analogs of ATP were much weaker inhibitors of PDE activity than non-substituted ATP.