To Linda

" Choose thou the darkness where our
light must shine, lighting the path,
however dimly, to those who follow
after "

Sanskrit Prayer

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ABSTRACT

A new direct colorimetric assay for adenosine-3', 5'-phosphate (3', 5'-AMP) is described. It is applicable to adenyl cyclase from brain and 3', 5'-AMP phosphodiesterase (PDEase). The procedure involves the following steps: precipitation of the proteins, periodate oxidation of the nucleotide mixture which leaves intact 3', 5'-AMP, sodium borohydride reduction of the oxidation products and oxidizing reagents, precipitation of iodide with lead acetate, color development by the orcinol method, extraction of the color into butyl acetate and optical density reading at 670 mμ. Beer's law is obeyed over a wide range of concentrations. The assay is sensitive down to 2×10^{-6} M 3', 5'-AMP and is suitable for routine kinetic studies at the research and undergraduate teaching levels. Interfering substances are: RNA- and DNA-like structures, NO_3^-, excessive amounts of oxidizable materials such as Co^{++}, Mn^{++}, nucleotides and chromophore forming materials.

Using this assay, some of the basic kinetic properties of cyclase in the absence and presence of various modifiers have been studied. The enzyme from brain cortex is stimulated by fluoride, but not by epinephrine, 5-hydroxytryptamine, glucagon, ACTH, or prostaglandin E_1. Fluoride activation is accounted for both in terms of an increase in the V_{max} for ATP and a decrease in the K_m for ATP in some preparations. Solubilization of cyclase by Triton X-100 abolished the fluoride effect. Synthesis of 3', 5'-AMP was linear

against protein concentration up to a maximum of 1.2 - 1.5 mg/ml in the presence of 5×10^{-2} M caffeine and over a twenty minute period of incubation. Tetraphenylboron, a potassium chelating agent inhibits cyclase by approximately 50% at 10^{-3} M, but was without effect at 10^{-4} M. No dependency of the enzyme on potassium ions could nevertheless be demonstrated. However, ethyleneglycol-bis (β-aminoethyl ether)-N, N'-tetraacetic acid (EGTA), a selective calcium chelating agent, inhibits 80 - 85% of the basal cyclase activity at 4×10^{-5} M and 45 - 65% of the activity in the presence of fluoride; this inhibition is relieved by calcium chloride at 3×10^{-5} M, but concentrations of the latter cause marked inhibition of cyclase in a fluoride containing system. Manganous, cobaltous, and - less efficiently - strontium ions, substitute for calcium, while barium, zinc, nicklous and ferrous ions are ineffective.

It appears that different forms of the enzyme may be stablized by magnesium, fluoride, and calcium ions. Protein concentration may directly influence cyclase efficiency. It is concluded that a large component of brain cyclase activity may be dependent on both calcium and magnesium ions, while a second distinct component appears to be fluoride dependent. The overall results have been rationalized on the basis of a model incorporating the discriminator unit concept developed by Robison, Bär and Hechter, and others.

The new assay is also applicable to the study of 3', 5'-AMP phosphodiesterase from the same brain cortex preparations. Concentrations of 3', 5'-AMP one tenth lower than the inorganic phosphate concentrations determined by other methods can be assayed. In contrast to 3', 5'-AMP, non-enzymatic hydrolysis of 3', 5'-GMP appears to take place under the assay conditions. The new assay is advantageous especially for the study of low K_m phosphodiesterase ($K_m < 10^{-4}$ M). As seems to be the case for rat brain, the presence of at least two phosphodiesterases possessing distinct K_m values has been also demonstrated for bovine brain. Alternatively, PDEase may be a single oligomeric enzyme with multiple binding sites, which could explain the observation of an intermediary plateau region in its saturation curve. Such systems have been analyzed by Teipel and Koshland. Further purification should allow a decision between these two alternatives. This has already been done in the case of rat brain.

The relative inhibitory activities of xanthine and purine derivatives were evaluated and it was confirmed that caffeine behaves as a competitive inhibitor of phosphodiesterase. However, it was observed that the caffeine effectiveness as an inhibitor is strongly dependent on protein concentration. Some improved inhibitors have been uncovered.

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADH	Antidiuretic hormone
ADP	5'-Adenosine diphosphate
AMP	5'-Adenosine monophosphate
3', 5'-AMP	3', 5'-Cyclic adenosine monophosphate
ATP	5'-Adenosine triphosphate
ATPase	Adenosine triphosphatase
CAA	N^6-chloroacetyladenine
dATP	2'-Deoxy-5'-adenosine triphosphate
DBP	2, 6-Dibromopurine
DCP	2, 6-Dichloropurine
diBu3', 5'-AMP	N^6, O^2-Dibutyryl 3', 5'-adenosine monophosphate
DTT	1, 4-Dithiothreitol
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine-N, N'-tetraacetic acid
EEDQ	2-Ethoxycarbethoxydihydroquinoline
EGTA	Ethyleneglycolbis (β-aminoethylether)-N, N'-tetraacetic acid
FSH	Follicle stimulating hormone
GH	Growth hormone
3', 5'-GMP	3', 5'-Cyclic guanosine monophosphate
G-1-P	Glucose-1-phosphate

G-6-P	Glucose-6-phosphate
GTP	5', -Guanosine triphosphate
HQ	8-Hydroxyquinoline
ICSH	Interstitial cell-stimulating hormone
I.r.	Infra-red
3', 5'-IMP	3', 5'-Cyclic inosine monophosphate
LATS	Long-acting thyroid stimulant
LH	Leutenizing hormone
MSH	Melanocyte stimulating hormone
MSF	Methylsulphonylfluoride
MoBu3', 5'-AMP	N^6-Monobutyryl 3', 5'-adenosine monophosphate
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotidephosphate (reduced)
N.m.r.	Nuclear magnetic resonance
O.D.	Optical density
pCMB	Para chloromercuribenzoate
PDEase	3', 5'-AMP phosphodiesterase
PEP	Phosphoenolpyruvate
PG	Prostaglandin
6-P-gluconate	6-Phosphogluconate
p-n pentyl DPA	Para-n-pentyldimethylphenylaziridinium (chloride salt)
P_i	Orthophosphate
PP_i	Pyrophosphate

PTH	Parathyroid hormone
RNA	Ribonucleic acid
Sat.	Saturated
ScAMP-TME	3',5'-Cyclic adenosine monophosphate tyrosine methyl ester
T_3	Triiodothyronine
T_4	Thyroxin
TCT	Thyrocalcitonin
TLC	Thin layer chromatography
TPB	Tetraphenylboron (sodium salt)
TSH	Thyroid stimulating hormone
U.v.	Ultra-violet

INTRODUCTION

PART "A". SYSTEMS CONTROLLING 3', 5'-AMP SYNTHESIS AND DEGRADATION

I. 3', 5'-AMP*

In 1958, Sutherland and Rall for the first time isolated 3', 5'-AMP, while studying the action of epinephrine and glucagon on glycogenolysis in dog liver (1, 2). They showed that 3', 5'-AMP was the heat-stable intermediate in the hormonal activation of phosphorylase (3).

At the same time, Lipkin et al identified 3', 5'-AMP as a degradation product of ATP* by the action of barium hydroxide (4, 5).

Since then, this cyclic nucleotide has been found in different tissues of higher animals (6), in microorganisms such as E. Coli (7) and Brevibacterium liquefaciens (8), in human and bovine milk (9). As much as 7 μmoles can be recovered from human urine during a twenty-four hour period (10).

The structure of 3', 5'-AMP is shown in Figure I, page 7. It consists of adenosine monophosphate, in which the phosphate group has been esterified at both the 3' and 5' positions of the ribose moiety to form a six-membered ring, which suggested the trivial name "cyclic AMP". Its structure was elucidated by Lipkin et al (4, 5, 11)

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* Abbreviations used in this Section: see page xiv

by an unambiguous chemical synthesis. Smith et al have published a general method for the synthesis of related cyclic nucleotides (12) using dicyclohexylcarbodiimide. More recent methods have been described (13, 14), including one by Symons (15) for the synthesis of radioactive (P^{32}) 3',5'-AMP.

By comparison with the common 5' nucleotides, 3',5'-AMP is very stable to acid, alkali (16) and heat (12). For instance, the half-life of 5'-AMP in 1 N HCl at 100°C is approximately 2 - 4 minutes, while the half-life of 3',5'-AMP under the same conditions is thirty minutes (4, 5). Adenine is liberated by acid and base hydrolysis. Additionally, 1 N NaOH at 100°C results in the partial destruction of the chromophore [λ_{max} = 258 mμ pH 7.0. $\varepsilon = 14.65 \times 10^6$ cm^2/mole] (4). Barium ions can also catalyze the opening of the ring giving both 3'-AMP and 5'-AMP in a ratio of 5:1 (16). Interestingly, however, the free energy of hydrolysis of 3',5'-AMP is higher than that of ATP ($\Delta G \gtrsim 12$ kcal/mole) (17, 18).

Biologically, this nucleotide triggers a wide variety of adaptive responses in target cells at the levels of replication, transcription, translation and regulatory enzymes, the effects themselves varying qualitatively and quantitatively from tissue to tissue and from species to species (19). These control mechanisms will be discussed later.

However, cellular control of the 3',5'-AMP levels themselves is exerted by two enzymatic systems: adenyl cyclase and 3',5'-AMP phosphodiesterase.

II. Adenyl Cyclase

Membrane-bound adenyl cyclase, catalyzes the formation of 3', 5'-AMP in the presence of ATP and Mg^{++} ions as follows:

$$ATP \xrightarrow[Mg^{++}]{\text{adenyl cyclase}} 3', 5'\text{-AMP} + PP_i$$

The inner phosphate group of ATP is the precursor of the phosphate group of 3', 5'-AMP (20) and the pyrophosphate group is evidently the leaving group (20). Cyclase is widely spread in nature (3, 6). Activity is found in brain, heart, liver, kidneys, adipose tissues (6), red blood cells (21), blood platelets (22) of higher organisms, flat and segmented worms, insects, amphibians, reptiles, birds and in bacteria (7, 8). Its presence in slime moulds (23) and myxamoebae (24) is implicated by the presence of 3', 5'-AMP in these organisms. Little literature has appeared which would suggest the presence of cyclase in plant tissues. However, Pollard (25) showed that 8-^{14}C-adenine was incorporated into 3', 5'-AMP in barley aleurone layers and the rate of incorporation was increased by the plant hormone gibberellic acid.

Generally, the specific activity of adenyl cyclase is relatively low; membrane-bound adenosine triphosphatase (ATPase) and 3', 5'-AMP phosphodiesterase (PDEase) associated with cyclase and are about fifty times more active than the latter. The highest cyclase activities occur in brain (6), in the parasite Fasciola hepatica (26) and in brown adipose tissue (27).

Adenyl cyclase of higher organisms does not exist as a single entity but rather as a system of proteins consisting of one or more catalytic units and one or more regulatory units (28, 29, 30) whose specificities vary from tissue to tissue and are presumably under genetic control. The action of biogenic amines and peptide hormones on target cells as reflected in cyclase activity has suggested the role of receptors for the regulatory units, a concept which has stimulated a great deal of research on the molecular mechanisms underlying chemical signal reception and readout (28). Adenyl cyclase which is bound to plasma membranes (6, 21, 31) and whose activity sediments most rapidly in cell-free preparations (2000 g) (6, 29), is activated by both biogenic amines and polypeptide hormones. Response selectivity of the enzyme to the chemical effectors is tissue dependent (32). Most cell-free preparations are also activated by the fluoride ion (6).

III. 3', 5'-AMP Phosphodiesterase

Cyclic AMP phosphodiesterase or 3', 5'-AMP phosphodiesterase (PDEase) catalyzes the breakdown of 3', 5'-AMP by hydrolysis of the 3' esteratic group, thus yielding 5'-AMP (2, 10, 33) as in the following equation:

$$3', 5'\text{-AMP} \xrightarrow[Mg^{++}]{\text{PDEase}} 5'\text{-AMP}$$

This Mg^{++} requiring enzyme is specific for cyclic nucleotides (10), and degradation of 3', 5'-AMP under physiological conditions is accomplished only by this enzyme. Non-enzymatic hydrolysis of 3', 5'-AMP is negligibly small owing to the marked chemical stability of the molecule (4, 5).

Similar to adenyl cyclase, PDEase is widely distributed in nature and indeed is found in all tissues of higher organisms where cyclase is present (10). In slime moulds (23, 34) and in myxamoebae (24), it is believed that control by PDEase on 3', 5'-AMP excretion is responsible for the aggregation phenomenon characteristic of these species. In higher organisms, there is no doubt that PDEase plays an important role in controlling the intracellular level of 3', 5'-AMP; however, it has yet to be demonstrated that this degradative enzyme may also be under hormonal control. On the other hand, an endogenous protein activator of membrane bound PDEase from bovine, human, pig and rat brain has been purified by Cheung (35, 36).

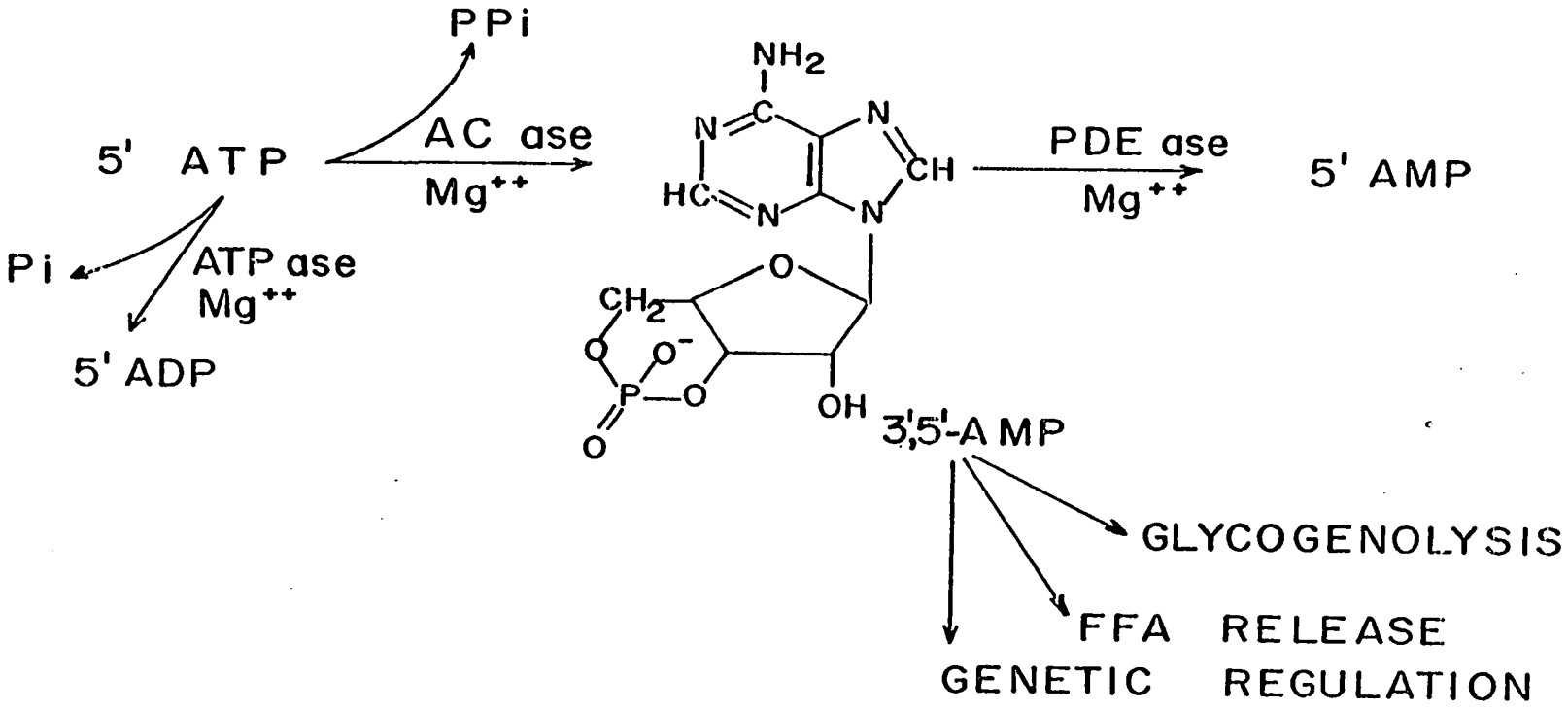
Insulin (37), nicotinic acid and imidazole (10) stimulate PDEase activity. Nucleoside triphosphates, 3',5'-AMP, pyrophosphate, citrate, and methyl xanthines are well-known inhibitors (10, 38, 39). Recently, both central nervous system depressants like chlorpromazine and promazine (40) and the antidepressants imiprimine and desmethylimiprimine (41) have been claimed to act as PDEase inhibitors.

FIGURE 1

Adenyl Cyclase and Related Membrane Systems

The relationship between membrane-bound adenyl cyclase (A.Case), 3', 5'-AMP phosphodiesterase (PDEase) and adenosinetriphosphatase (ATPase) and further systems affected by 3', 5'-AMP is shown diagrammatically.

ADENYL CYCLASE and RELATED MEMBRANE SYSTEMS



IV. In Vitro Enzymological Problems

It should be emphasized here that both adenyl cyclase and a large part of the 3',5'-AMP phosphodiesterase activity are found in the same particulate cellular fractions and their activities are not readily separable (6, 10, 20). Hence, when studying adenyl cyclase activity, it is important to include a phosphodiesterase inhibitor to prevent degradation of enzymatically synthesized 3',5'-AMP. The methyl xanthines, caffeine and theophylline are most commonly used for this purpose, as they do not appear (at appropriate concentrations), to affect the cyclase system (6, 20).

In the major fractions where cyclase is concentrated, there is also ATPase activity which rapidly consumes ATP, the substrate for adenyl cyclase.

$$ATP \xrightarrow[Mg^{++}]{ATPase} ADP + P_i$$

Both Mg^{++} dependent and Mg^{++}, Na^{+}, K^{+} dependent ATPases are present in these preparations (42); however, the latter ATPase can be made inoperative by the exclusion of K^{+} ions or by the addition of the specific inhibitor, ouabain (43). There is not, to date, any specific inhibitor[1] for the Mg^{++} dependent ATPase (sometimes called apyrase)

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[1] Emmelot and Bos (44), showed that appropriate concentrations of bile, deoxycholate, lubrol W, deoxycortisone acetate and progesterone inhibited apyrase and activated K^{+}, Na^{+}, Mg^{++}, ATPase. No specific and complete apyrase inhibition could, however, be obtained.

(42) whose specific activity can be as high as fifty times that of the cyclase. Nevertheless, one can account separately for the percentage of ATP hydrolyzed during the period of 3',5'-AMP synthesis (an aspect of the problem which many investigators seem to neglect) or, better, one can design an ATP-regeneration system (28, 45) so as to overcome the problem of unproductive substrate consumption.

Purification of adenyl cyclase has been a forebodingly difficult task, largely owing to the great instability of cyclase from higher organisms (20). Further, the physical attachment of this enzyme to plasma membranes usually demands the use of detergents or chaotropic agents (46) for detachment. However, some notable exceptions are known[2]. Most physical treatments are drastic and frequently result either in complete inactivation of adenyl cyclase [such as the action of deoxycholate on bovine brain cyclase (20)] or loss of hormone sensitivity[3].

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[2] Tao and Lipmann (47) succeeded in one hundredfold purification of E. Coli adenyl cyclase, most of which occurred in soluble form, the remainder being readily solubilized mechanically. This exceptional cyclase was hormone insensitive and inhibited by fluoride ions. Kamejiro and Field (48) were able to purify bovine thyroid membrane cyclase tenfold using a sucrose gradient only and actually achieved an increased activation by TSH.

[3] Levey (49) obtained a 90-100% solubilization of myocardial adenyl cyclase which remained fluoride sensitive but suffered loss of norepinephrine, glucagon and thyroxine sensitivity. Use of Triton X-100 abolished both fluoride and hormone activation (20, 49).

Finally, associated activities such as ATPase and PDEase tend to be solubilized by the same extraction techniques, thus defeating the purpose[4].

Phosphodiesterase, however, has been relatively easy to purify. Butcher and Sutherland (10) achieved an eightyfold purification of solubilized beef heart PDEase. This preparation is now commercially available.

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[4] When Rosen and Rosen (50) purified frog erythrocyte adenyl cyclase two hundredfold by lysis, sephadex and centrifugal fractionation, total ATPase activity was purified fortyfold: however, all 3',5'-AMP phosphodiesterase activity was lost in the process.

PART "B" ASSAY PROCEDURES

I. Experimental Objective

One of the major difficulties in the study of adenyl cyclase and 3', 5'-AMP phosphodiesterase mechanisms of action has been accessibility to simple and economical chemical assay methods.

In spite of the relatively large number of available assays for the cyclase system, research into the kinetic and allosteric properties of this complex system has been hampered for lack of a facile, sensitive, direct, inexpensive assay requiring a minimal of technical skills, training and special equipment. Such an assay would be useful for teaching purposes at the undergraduate level and for the routine screening of drugs and other effectors at the cyclase and PDEase levels.

It is a primary purpose of this thesis to describe such an assay and to illustrate its general usefulness.

II. Adenyl Cyclase Assays - A Summary of the Literature

Before presenting a complete description of the type of rationale used for the development of a direct chemical assay for 3', 5'-AMP as applied to cyclase and PDEase, an outline of previous and presently popular assay methods will be given. Much of the literature purports to express general dissatisfaction with a number of earlier methods. As many as five different classes of assay to measure adenyl cyclase activity are known:

(a) indirect or coupled enzymatic systems;

(b) chromatographic procedures;

(c) combination systems of (a) and (b);

(d) an immunological method;

(e) a microbiological method.

(a) Coupled enzymatic systems

The indirect system is designed to amplify the effects of small quantities of 3', 5'-AMP by coupling its synthesis to a series of enzymatic reactions, yielding a final reaction product which is more readily measurable.

Such coupling is accomplished by causing the initial reaction product (3', 5'-AMP, for example) to take part in a second enzymatic reaction, yielding increased equivalents of a second product. This product can be a substrate for a third biochemical reaction, and so forth, until a final product becomes readily measurable by

conventional, spectrophotometric or fluorometric techniques. It is sometimes possible to carry the assay by mixing all components (coenzymes, ions, etc.) so that a single overall step is involved.[5] However, all such indirect types of assays for 3',5'-AMP require more than one enzymatic step[6], the reaction being terminated by heat denaturation.

Some of the indirect or enzymatic assays for adenyl cyclase developed over the last twelve years are briefly described on pages 14, 15, 16 , Table 1. The specific purposes for which these assay procedures were created and their range of sensitivity[7] are indicated in the column on the left.

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5 In such a system the initial enzymatic reaction must be the rate limiting step in a steady state process; all other enzymes and co-factors being in excess.

6 In this case, the kinetics or requirements of subsequent coupled reactions will not permit a one step process. Coupled reactions must go to completion to obtain a reproducible standard curve.

7 In assays No. 3, 5 and 6 (Table 1, pages 14, 15, 16), not all portions of the standard curve are linear. Hence, even though the author may claim a low limiting concentration of sensitivity, the accuracy will only be optimal in the linear range.

Table I

COUPLED SYSTEMS (Type A)

No.	Purpose and Sensitivity	Basis of System	Reference
1	In vitro enzymatic studies	3', 5'-AMP, ATP, kinase, Mg^{++} liver dephosphophosphorylase $\rightleftharpoons$ liver phosphorylase phosphatase (inactive) (active)	Rall and Sutherland (1958) (31) (1960) (3) (1961) (51)
	$10^{-7}M \rightarrow 10^{-6}M$ 3', 5'-AMP	G-1-P $\xrightleftharpoons{\text{active liver phosphorylase}}$ glycogen + P_i Glycogen production from G-1-P is measured by the formation of a color iodine-glycogen complex aswell as by the appearance of P_i released	Butcher et al (1965) (52) Øye et al (1964) (53)
2	In vitro enzymatic studies	glycogen + P_i $\xrightleftharpoons{\text{active liver phosphorylase}}$ G-1-P	Brown et al (1962) (54)
	$10^{-7}M \rightarrow 10^{-6}M$ 3', 5'-AMP	G-1-P $\xrightleftharpoons{\text{phosphoglucomutase}}$ G-6-P	
	$10^{-7}M \rightarrow 10^{-8}M$ 3', 5'-AMP	2NADP + G-6-P $\xrightleftharpoons{\text{G-6-P dehydrogenase}}$ 2NADPH + 6-P-gluconate NADPH formation is measured by ultra violet absorption at 340 mμ.	Scott and Falconer (1965) (55)

Table I (ctd.)

COUPLED SYSTEMS (Type A)

No.	Purpose and Sensitivity	Basis of System	Reference
3.	Measurement of endogenous 3', 5'-AMP 2×10^{-8} M → 2×10^{-7} M 3', 5'-AMP	Activation of skeletal muscle phosphorylase (b) to active phosphorylase (a) analogous to system No. 1	Posner et al (1964) (56) Namm and Mayer (1968) (57)
4.	Measurement of endogenous 3', 5'-AMP 5×10^{-10}M - 3', 5'-AMP (and upward)	Activation of liver protein kinases (analogous to System 1)	Castagna (1970) (58)

Table I (ctd.)

COUPLED SYSTEMS (Type A)

No.	Purpose and Sensitivity	Basis of System	Reference
5.	Endogenous 3', 5'-AMP 10^{-8}M → 10^{-5} M 3', 5'-AMP (linear range of standard curve 10^{-7}M→10^{-5}M)	Destruction of ATP, ADP, and AMP using apyrase and phosphatase. Destruction of phosphatase with pepsin. 3', 5'-AMP $\xrightarrow{\text{phosphodiesterase}}$ 5'-AMP 5'-AMP + PEP $\xrightarrow[\text{ATP}]{\text{PEP kinase}}$ ATP ATP + glucose $\xrightarrow{\text{hexokinase}}$ G-6-P + ADP G-6-P + 2 NADP $\xrightarrow{\text{G-6-P dehydrogenase}}$ 6-P-gluconate + NADPH NADPH is measured fluorometrically	Breckenridge (1964) (59)
6.	In vitro cyclase activity, or endogenous levels of 3', 5'-AMP, 7×10^{-9}M→ 10^{-5}M, 3', 5'-AMP (linear range of standard curve from 10^{-1}→10^{-5}M)	The conversion of 3', 5'-AMP to ATP by the Breckenridge method (59) (see No. 5); measurement of ATP by a luminescent reaction with firefly luciferase	Johnson et al (1970) (60)

(b) and (c) Chromatographic and combination systems

Procedures based on the actual separation of 3', 5'-AMP [including combination systems group (c)] are listed on pages 19-23, Table II. (Since some of these assays make use of radioactive material such as ^{32}P, ^{3}H, or ^{14}C-ATP or ^{14}C-adenine[8]) as substrates, one can detect less than 10^{-12} M of 3', 5'-AMP, the sensitivity depending upon the specific activity of the substrate. Invariably, these techniques require separation of 3', 5'-AMP from other nucleotides and other by-products [either substrate or products resulting from the presence of ATPase, PDEase, 5'-nucleotidase (44) or other unknown enzymatic activities present in the systems]. Separation techniques involve either ion exchange resins (anionic or cationic) or molecular sieving processes (usually thin layer chromatographic plates or paper). Another technique, employed by Krishna, makes use of gas-liquid chromatography (see Table II, pages 19-23).

Some attention should be paid to the principles on which the different types of chromatographic methods rest before surveying each individual method for 3', 5'-AMP determination.

Cellulose fibers, silica or the ecteola particles of thin layer plates or columns have a high affinity for the water of the solvent phase, but a very low affinity for the organic solvent. Essentially the solid adsorbent (cellulose, silica etc.) behaves as an inert support for

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[8] Labeled adenine is preincubated with whole cells or tissue slices so that ^{14}C is incorporated into its nucleotide derivatives, including 3', 5'-AMP (61, 62). Hence cyclase activity can be studied in cruder systems, avoiding the problem of the lack of permeability of ^{14}C-ATP into whole cells.

a stationary aqueous phase. As the solvent moves over the area where the solute is adsorbed, a partitioning occurs between the stationary aqueous phase and the mobile organic phase. When the mobile organic phase containing the solute reaches an area of the support where no solute is present in the water phase, partitioning is resumed. Hence, the separation of nucleotides will depend on differences in their individual partition coefficients (63) between the organic and aqueous phases. The process is analogous to fractional distillation or continuous liquid-liquid extraction, the longer the adsorbing support the better the separation.

A similar principle applies to gas-liquid phase chromatography except that partition occurs between material in the vapor phase and adsorbed material in the liquid phase. The solid support should possess adsorbing properties such that it is capable of holding the stationary liquid without becoming greasy and incapable of binding sample components by secondary valence bonds.(64). The compounds to be separated must obviously be volatile.

An ion exchange resin, such as Dowex, is able to bind materials even more tightly through ionic interactions. This permits compounds with a lesser affinity for the resin to be washed out; the desired compound can then be eluted by increasing the ionic strength of the solvent(increasing competition for the ionic binding sites) or by altering the pH (which changes the degree of ionization, hence the affinity for the resin). Resins may be cationic or anionic. Both types have been successfully used to separate nucleotides and isolate 3', 5'-AMP (see Table II, pages 22, 23), the sharpness of separation depending on differences in the affinities for the ionic groups of the resin.

Table II

Chromatographic and Combination Systems (types b and c)

No.	Purpose of system	Mode of separation	Substrate	Basis of System	Reference
1.	In vitro cyclase studies	One ion exchange resin (H^+), one TLC.	^{14}C-ATP	Dowex-2 formate column - elution with formic acid - lyophilization, silica gel plates acetone: isopropanol: n-butanol:NH_4HCO_3 (2:2:3:2) pH 8.6	Jungas 1966 (65)
1'.	In vitro cyclase studies	One resin (H^+). One selective precipitation	^{14}C-ATP ^{32}P-ATP ^{3}H-ATP	Dowex-5 (H^+) column- elution with water - $ZnSO_4$ and $Ba(OH)_2$ precipitation	Weiss and Costa 1967 (66) Krishna et al 1968 (67) Chase and Aurbach 1968 (68) Cryer et al 1969 (69) Rodbell 1967 (70) Zor et al 1969 (71)
1''.	In vitro cyclase activity in brain	Two ion exchange resins	Unlabeled ATP	Dowex 2x8 (anionic) - Dowex 50Wx8 (cationic) - elution of each with formic acid, spectrophotometric measurement of 3',5'-AMP (260 mμ)	Bradham and Woolley 1964 (72) Bradham et al 1970 (73)
1'''.	In vitro studies in whole cells and tissue slices	One resin (H^+), One selective precipitation	^{14}C-adenine	Preincubation with ^{14}C-adenine - procedure is essentially the same as in 1'.	Kuo and Dill 1968 (61) Humes et al 1969 (62)

Table II (ctd.)

No.	Purpose of system	Mode of separation	Substrate	Basis of System	Reference
2.	In vitro cyclase studies	One paper chromatography system	^{14}C-ATP	Whatman 3MM filter paper 1M NH_4OAc:EtOH (30:75)	Hirata and Hayaishi 1965 (8)
2'.	In vitro cyclase studies	One paper chromatography system	^{14}C-ATP	Whatman 3 MM paper - isopropanol: NH_4OH (25%):H_2O (7:2:1)	Ho et al 1967 (14)
2''.	In vitro cyclase studies	One paper chromatography system	^{14}C-ATP	Whatman 40 filter paper. EtOH:0.1 M boric acid (3.5:1) (adjust to pH 4.0 with glacial acetic acid)	Streeto and Reddy 1967 (75)
2'''	In vitro cyclase studies	2D-paper chromatography system	^{3}H-ATP	Whatman 3MM filter paper - Isopropanol:NH_4OH:H_2O (7:2:1) - isopropanol:conc. HCl:H_2O (65:16.7:18.3)	Rabinowitz et al 1965 (76) Marinetti et al 1969 (77)
2''''	In vitro cyclase studies	Two conjunctive paper chromatography systems	^{14}C-ATP	Identical to system 2'''	Dousa and Rychlik 1970 (78)

Table II (ctd.)

No.	Purpose of system	Mode of separation	Substrate	Basis of System	Reference
3.	3', 5'-AMP levels and <u>in vivo</u>	Two identi-TLC systems, Two enz-ymatic systems	α^{32}P-ATP	NM cellulose 300 HR plates - (1-butanol:acetone:glacial acetic: 14.8N NH_4OH) (90:30:1:60) elution with EtOH and evaporation 3', 5'-AMP $\xrightarrow{\text{PDEase}}$ 5'-AMP 5'-AMP $\xrightarrow{\text{myokinase}}$ 5'-ADP Repetition of chromatographic system	Turtle and Kipnis 1967 (79)
3'.	<u>In vitro</u> studies	One selec-tive preci-pitation, One TLC system	Unlabeled ATP	$Ba(OH)_2$, $ZnSO_4$, precipitation - cellulose plates - isopropanol:H_2O: NH_4OH (7:1.5:1.5) - fluorometric measurement of 3', 5'-AMP	Goldberg <u>et al</u> 1969 (80)
3''.	<u>In vitro</u> studies	Two TLC systems	^{14}C-ATP	Ecteola plates - propanol:NH_4OH: H_2O (6:3:1) - glacial acetic: butanol:H_2O (1:2:1)	Bitensky <u>et al</u> 1968 (81)

Table II (ctd.)

No.	Purpose of system	Mode of separation	Substrate	Basis of System	Reference
3''	In vitro studies	One 2D TLC system, One ion exchange resin	α^{32}P-ATP	Cellulose plates - isopropanol:NH_4OH:H_2O (7:1:2) - polyethyleneimine ion exchange sheets - n-propanol:NH_4OH:H_2O (2 mls:60 g:100 ml)	Bär and Hechter 1969 (82)
4.	Tissue levels of 3', 5'-AMP	Double isotope dilution and derivative assay: Two resin, two paper chromatography systems, one chemical manipulation	^{14}C-ATP	Dowex 1 x -8 formate - elution with formic and evaporation - Whatman 3MM - isopropanol:NH_4OH:H_2O (7:1:2) - acetylation with titrated $(AcO)_2$ - repetition of column and paper chromatography. - for paper; isopropanol: acetic:H_2O (6:3:1)	Pauk and Reddy 1967 (83)
5.	Tissue levels of 3', 5'-AMP	Esterification and GLC	Labeled or unlabeled ATP	3', 5'-AMP is converted to its trimethylsilyl ester by treatment with bistrimethylsilyl acetamide - gas-liquid chromatography	Krishna 1968 (84)

Table II (ctd.)

No.	Purpose of system	Mode of separation	Substrate	Basis of System	Reference
6.	In vivo studies, detection in tissues and urine	One selective precipitation, one resin column, four coupled enzymatic systems (one step)	Unlabeled ATP	$ZnSO_4$, $Ba(OH)_2$ treatment - Dowex 50, 100 or 200 mesh (H^+) - enzymatic conversion of 3', 5'-AMP to ATP by the Breckenridge method (59) glyceraldehyde phosphate dehydrogenase ATP $\rightleftharpoons$ $AT^{32}P$ phosphoglycerate kinase P-glycerate, $^{32}P_i$. Rate of $^{32}P_i$-exchange is a direct function of (3', 5'-AMP)	Aurbach and Houston 1968 (85)
7.	Tissue levels of 3', 5'-AMP	An enzymatic radioactive displacement assay. Two steps and differential absorption and quenching on ion exchange resin	^3H-ATP	PDEase 3', 5'-AMP $\longrightarrow$ 5'-AMP (partial) 5'-AMP $\xrightarrow{\text{snake venom}}$ adenosine+P_i 5'-nucleotidase Addition of AG1-X2 400 mesh to quench the reaction. The amount of radioactive 3', 5'-AMP detected by liquid scintillation is an inverse function of the concentration of unlabeled 3', 5'-AMP (standard or unknown) added initially.	Brooker et al 1968 (86)

Assay methods of classes (b) and (c) outlined in Table II (pages 19-23) have been somewhat arbitrarily divided into seven categories according to the type of chromatographic technique employed. Some of them involve two types of chromatographies in conjunction as in the case of methods No. 1 Jungas(65) and No. 3" ' Bär and Hechter (71). Others [type (c)] utilize an enzymatic system[9], in conjunction with a chromatographic procedure, as is the case for example technique No. 3 Turtle and Kipnis (79), and No. 6 Aurbach and Houston (85). Many assays involve selective precipitation of noncyclic nucleotides and P_i and PP_i with barium hydroxide and zinc sulfate, as was originated by Weiss and Costa (66) and Krishna et al (67). Since selective precipitation is never quantitative (67, 68), it must be supplemented with a chromatographic technique.

Not all of the chromatographic assays (see Table II) require labeled substrate. Bradham and Woolley (72) attained a range of sensitivity of $10^{-6} \rightarrow 10^{-5}$M 3', 5'-AMP by directly measuring the U.v. absorption at 260 mμ, a technique sufficiently sensitive for application to brain cyclase activity. Goldberg et al (80) claim a sensitivity ranging from 5×10^{-9} M to 5×10^{-8} M 3', 5'-AMP by measuring 3', 5'-AMP fluorometrically. Aurbach and Houston (85) devised an ingenious enzymatic $^{32}P_i$ exchange system allowing them to measure 3', 5'-AMP in the range of 5×10^{-8}—10^{-6} M.

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[9] In contrast to the enzymatic procedures on Table I, both these systems were one step steady state processes (see footnotes 5 and 6, page 13).

All chromatographic assays generally require the use of internal and frequently external standards and controls (67, 83) in order to estimate the percentage of 3',5'-AMP lost in the manipulations. Some limitations of these methods will be examined in the Discussion Section.

Some chromatographic assays utilize an ATP regeneration system, usually consisting of phosphoenol pyruvate (PEP) and phosphoenol pyruvate kinase (PEP kinase) which is designed to maintain the substrate concentration constant during the period of 3',5'-AMP synthesis.

$$ATP \xrightarrow{\text{ATPase}} ADP + P_i$$

$$ADP + PEP \xrightarrow{\text{PEP kinase}} ATP + \text{Pyruvate}$$

See references (28, 45, 75, 76, 80, 82).

(d) A specific radioimmunological method

This method, developed by Steiner et al (87) is based on the in vivo formation of an antibody to an antigen formed upon binding of human serum albumin to an appropriate derivative of 3',5'-AMP [2' -succinyl 3',5'-cyclic adenosine monophosphate tyrosine methyl ester (SCAMP-TME)]. This antibody will precipitate in vitro by complexing with the antigenic protein; it will also bind 3',5'-AMP or ^{125}I iodinated SCAMP (fixed concentration). Since these two nucleotides

are, in effect, competing with the antigenic protein for antibody-antigen complex formation, the greater the amount of labeled nucleotide in the complex, the less unlabeled 3', 5'-AMP is present in the system. An inverse linear relationship between the specific activity of the precipitate and the $\log_{10}$[3', 5'-AMP] was found in the range of 10^{-9} M → 10^{-7} M 3', 5'-AMP. This assay was specifically designed to measure 3', 5'-AMP levels in tissues.

(e) A microbiological method

This microbiological method developed by Konijn (24) is based on the fact that myxamoebae are attracted to a source of 3', 5'-AMP. By measuring the percentage of a given population of cells moved by chemotaxis towards a source of 3', 5'-AMP, the nucleotide concentration can be estimated from an appropriate standard curve. This assay is sensitive down to 10^{-12} grams 3', 5'-AMP and is best applied to the qualitative detection of very small amounts of 3', 5'-AMP in crude tissue extracts.

III. 3', 5'-AMP Phosphodiesterase Assays: A Brief Survey

Because a direct chemical assay for 3', 5'-AMP is not only applicable to adenyl cyclase studies but also to 3', 5'-AMP phosphodiesterase, the types of systems presently used to study the latter enzyme should be included in our survey. Although less research has been carried out on developing assays for phosphodiesterase, recent investigations have emphasized the biological significance of this area of enzymology (35, 36). As many as four or five basic assay systems are presently favored (see pages 28 to 30). Similar to the cyclase assays, enzymatic methods and separation techniques have been employed. Recently Cheung, has introduced an ingenious titrimetric technique which is convenient for 5'-nucleotidase-free systems [see Table III, page 30].

It is difficult to compare the sensitivity of some of the various assays for 3', 5'-AMP-phosphodiesterase since different parameters are sometimes employed (Assays Nos. 2 and 6). It should,perhaps, be pointed out that sensitivity is not as critical as in the case of the cyclase assays owing to the relatively high specific activity of phosphodiesterase.

Table III

3', 5'-AMP Phosphodiesterase Assays

No.	Sensitivity range	Basis of Assay	Reference
1.	10^{-5} - 10^{-4}M	one step: 3', 5'-AMP $\xrightarrow[Mg^{++}]{\text{PDEase}}$ 5'-AMP; 5'-AMP $\xrightarrow[\text{5'-nucleotidase}]{\text{snake venom}}$ adenosine + P_i. Colorimetric measurement of P_i. (see refs. 91, 91).	Butcher and Sutherland (1962) (10) Cheung (1967) (88) Change (1968) (34) Weiss et al (1968) (89) Fiske and Subbarow (1925) (90) or Nakumura and Mori (1958) (91)
1'.		Same enzymatic system as Butcher and Sutherland used except ^{32}P-3', 5'-AMP is used as substrate and $^{32}P_i$ is measured.	Schonhofer et al (to be published) (92)
1''.	5×10^{-8} - 10^{-6} M or down to 5 μμmoles/ .15 mls.	Same enzymatic system as 1 and 1''. Measurement of ^{3}H-adenine by differential absorption on resin (see Table II).	Brooker et al (1968) (86)

Table III (ctd.)

No.	Sensitivity range	Basis of Assay	Reference
2.	10^{-6} - 10^{-5} M	$ATP + 5'\text{-}AMP \xrightarrow{Mg^{++},\ myokinase} 2(5'\text{-}ADP)$	Adam (1963) (93)
		$5'\text{-}ADP + PEP \xrightarrow[PEPkinase]{Mg^{++},\ K^{+}} pyruvate + ATP$	Cheung (1966) (94)
		$NADPH + pyruvate \xrightarrow{LDH} Lactate + NADP$	Cheung (1967) (88)
3.	Hydrolysis of .2 µmoles 5'-AMP hydrolyzed/30 secs (in a volume of 1.5 mls) were accurately measured	$5'\text{-}AMP \xrightarrow[deaminase]{Adenylic\ acid} 5'\text{-}IMP$	Drummond and Perrotyee (1961) (95)
		5'-IMP has a lower ε value than 5'-IMP or 3', 5'-AMP at 265 mµ. Measurement of a decrease in absorption - ΔA at 265°mµ.	Echstein and Bar (1969) (96)

Table III (ctd.)

No.	Sensitivity range	Basis of Assay	Reference
4.	$5 \times 10^{-8} \rightarrow 10^{-6}$ M	Identical to the TLC assay outlined in Table II, page 21 but measurement of 5'-AMP fluorometrically.	Goldberg et al (1969) (80)
5.	10^{-6}M $\rightarrow$ 10^{-5} M	Paper chromatographic separation of 3', 5'-AMP and 5'-AMP, using Whatman No. 1 or 3 MM paper. Isopropanol:NH_4OH:H_2O (7:1:2). Measurement of absorbance at 258 mμ.	Drummond and Powell (1970) (97)
6.	Generation of 10 m moles protons/min. is accurately measured	Titrimetric assay: release of one proton during the hydrolysis of 3', 5'-AMP.	Cheung (1969) (98)

IV. Criteria for Evaluation of Assay Methods

As can be seen in Tables I, II and III, only a limited number of basic types of assays are known, but many variations on the same themes are available.

It is not a particularly easy task to evaluate the practicality of the procedures in the absence of direct personal experience with all of them. However, some of the criteria which one may bear in mind before choosing one particular method are: convenience, efficiency (work accomplished/man-hour), accuracy, cost (chemicals, equipment and technical assistance), sensitivity (depending on the order of magnitude of the concentrations that are of interest to the individual), specificity, limitations (including interfering materials), and the purpose of the research (whether in vivo or in vitro studies are involved). In the latter case, whether 3', 5'-AMP synthesis and/or degradation is studied in tissue slices, individual whole cells, cell free homogenates or highly purified systems will determine the choice. One must also distinguish between assays designed to detect gross levels of 3', 5'-AMP in tissues (under various conditions) from those designed for the study of 3', 5'-AMP metabolism in vitro. Detection methods may frequently be adapted to dynamic studies, the main difference being only in the concentration ranges of 3', 5'-AMP to be assayed. The reverse, of course, is not necessarily true.

V. Rationale for the Development of a Direct Chemical Assay

One feature which does become painfully obvious after a rapid scan of Sections II and III, is the absence of any simple direct chemical method applicable to both the cyclase and PDEase systems.

A very simple rationale was applied in our search for such a method: periodate oxidation of a nucleotide mixture resulting from enzymatic attack of ATP should destroy the ribose moiety of all non-cyclic nucleotides, leaving 3',5'-AMP intact. Suitable, but by no means obvious manipulations may then allow selective detection of unoxidized 3',5'-AMP by the orcinol method. Clearly, the same rationale may form the basis of a direct chemical assay for the specific 3',5'-AMP phosphodiesterase which is generally found associated with cyclase. It is the purpose of this thesis to report on our success in attaining these goals, and to describe applications of the new assay to a preliminary study of the kinetic effects of known modifiers on both adenyl cyclase and 3',5'-AMP phosphodiesterase from brain tissue. Although the method is not yet applicable to preparations possessing low specific activities, it is quite suitable for studies with brain adenyl cyclase, a feature which should facilitate routine evaluations of the effects of central nervous system drugs at this level. Moreover, the techniques employed are eminently suitable for teaching at the undergraduate level where the questions of cost, equipment and convenience are limiting.

PART "C". HORMONAL CONTROL SYSTEMS INVOLVING 3', 5'-AMP

I. The Second Messenger Hypothesis

Undoubtedly, the most fascinating aspect of the adenyl cyclase system is its susceptibility to modulation by hormone action at the cellular level. Ever since Rall and Sutherland first showed that glucagon and epinephrine activated liver phosphorylase only when the rapidly sedimenting particulate fraction containing adenyl cyclase was present (99), it became evident that a large number of other physiological and biochemical effects of hormones could be mediated by 3', 5'-AMP.

In higher organisms, 3', 5'-AMP has been implicated as a "second messenger", the first messenger being the circulating hormone which is released by specific endocrine glands and which ultimately reaches the "target tissue(s)". There, it binds on specific receptors which influence membrane bound adenyl cyclase (3). Thus, it is thought that control can be exerted by a primary event on the outside of the plasma membrane, rapidly causing some form of "allosteric transition"[10] in the cyclase complex, thus leading to activation

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[10] The term "allosteric transition" has been loosely applied to mean any conformational change in a protein which significantly alters its biological properties. However, more specifically, Monod et al (100) and Koshland et al (101) have constructed models based on changes in subunit interaction as a function of the binding of ligand and effector molecules. Such models have been useful in explaining the kinetic behavior of feed-back inhibited enzymes such as aspartic transcarbamylase (102, 103) whose physical properties Sutherland et al compare to those of adenyl cyclase (104).

or inhibition of the rate of 3', 5'-AMP synthesis inside the cell. Many secondary events at multiple levels of control in different types of cells are determined by the amount of 3', 5'-AMP available (and its cellular localization). Thus, simply stated, this "second messenger" hypothesis has been formulated largely on the basis of the pioneering work of Sutherland and his co-workers (3, 105, 106) and has more recently been reviewed and elaborated upon in three different languages (104, 107, 108). Every aspect from the control of carbohydrate metabolism (109) to the mechanisms of drug action (110, 111) converges on the profound significance of these control mechanisms for the survival of the organism.

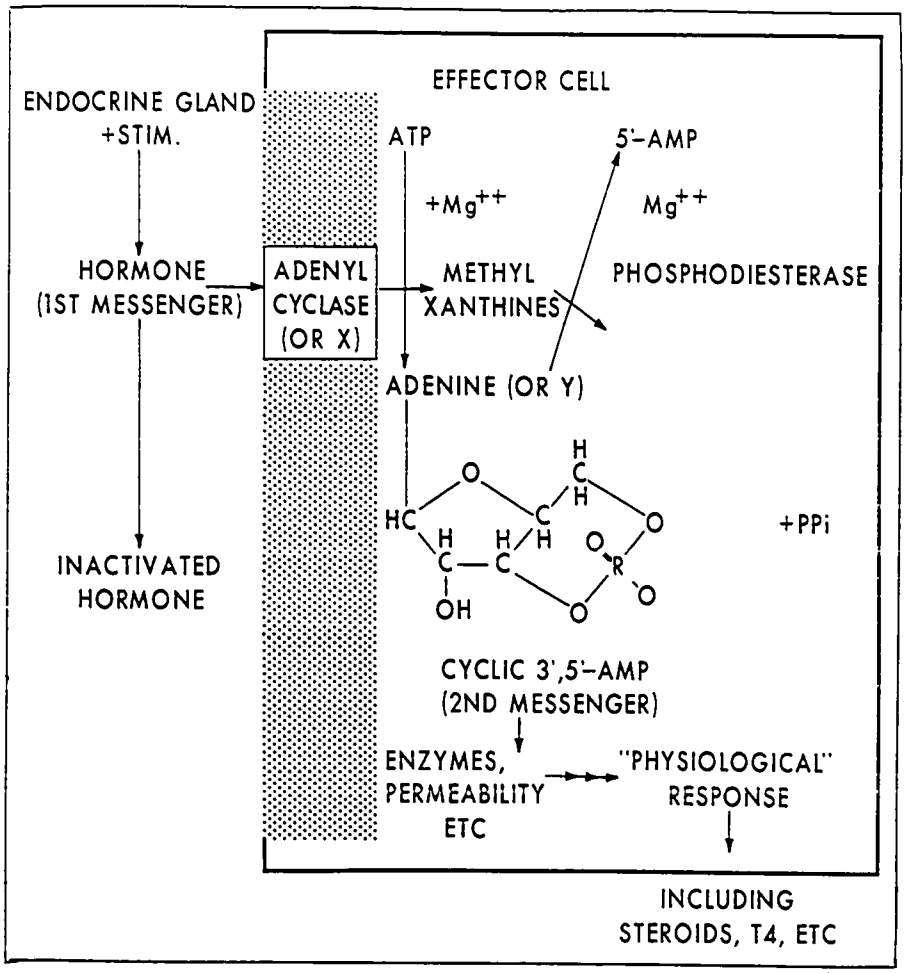
Since the late fifties, when glucagon and epinephrine had already been shown to behave as primary messengers or hormones, the list of different cyclase effectors has been growing equally rapidly as the list for assay methods for this system. Today well over twenty distinct effector molecules have been demonstrated to modulate adenyl cyclase.

Although the hormones and their target tissues are well-known (112, 113), a few recent additions taken from the literature of the past two years must be made in order to completely update the significance of the "Sutherland hypothesis". In Table IV, page 36 the effector molecules have been classified into three different categories, according to their structure, although the third group is very heterogeneous since most members are metabolites rather than hormones.

FIGURE 2

The Second Messenger System involving Adenyl Cyclase

A model depicting the second messenger concept as proposed by Sutherland and co-workers, diagrammatically illustrated, was reproduced from a paper by Sutherland et al (112).



THE SECOND MESSENGER SYSTEM INVOLVING ADENYL CYCLASE.

Table IV — Effectors of Adenyl Cyclase

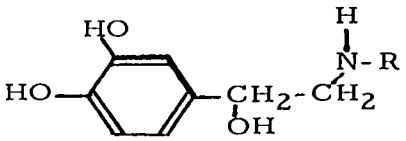
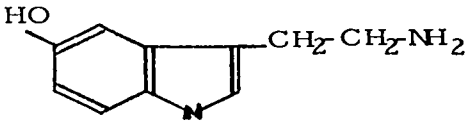
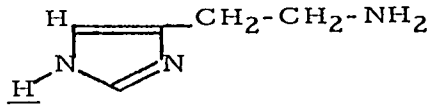
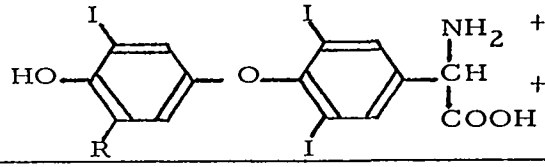
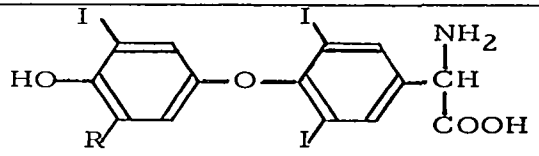
1. Biogenic amines and amino acids	General structure	Effect*	Systems affected	Reference
epinephrine ($R=CH_3$) norepinephrine ($R=H$) (isoprenaline $R = -CH(CH_3)_2$)		Usually +ve sometimes -ve. (α-component)	Heart; Liver; Kidney: Brain slices. Adipose tissue, Erythrocytes, Blood platelets.	Reviews: (105, 106, 112) Murad and Vaughan (114) Kakuichi and Rall (115, 116) Davoren and Sutherland (21) Robison et al (117)
Serotonin (or 5-hydroxy-tryptamine)		+ve	Fasciola hepatica, rabbit cerebellum slices.	Mansour (118) Kakuichi and Rall (115) Mansour and Stone (119)
histamine		+ve +ve -ve	Brain slices, Gastric mucosa, Pancreas	Kakuichi and Rall (115, 116) Harris and Alonso (150) Feldman and Lebovitz (120)
triiodothyronine R=H (T_3)		+ve +ve	Adipose tissue, Monkey sperm	Brodie et al (121) Mandel and Kishl (122) Casillas and Hoskins (124)

Table IV (ctd.)

I. Biogenic amines and amino acids	General structure	Effect*	Systems affected	Reference
thyroxin R=I (T_4)		+ve [11]	Monkey sperm	Casillas and Hoskins (124)
		-ve	Pituitary	Wilber et al (125)

* +ve = An increase in levels of 3', 5', AMP in the presence of a specific effector, either in an organelle, intact cells or homogenates or in an in vitro adenyl cyclase system.

-ve = A decrease in levels of 3', 5', -AMP in these systems.

11 Frazer et al (123) found that although thyroxin increased the phosphorylase (b) kinase activity, and phosphorylase (a) content of rat heart, it did not raise the level of 3', 5'-AMP.

Table IV (ctd.)

2. Polypeptide hormones	Effect	Systems affected	Reference
Glucagon	+ve	Liver, Heart, Adipose tissue.	Ray et al (126) Murad and Vaughan (114) Birnbaumer et al (127, 128).
ACTH (corticotropin)	+ve	Adrenal cortex Adipose tissue Dorsal frog skin	Graham-Smith et al (129) Grower and Branson (130) Hechter et al (131) Swislocki (132) Birnbaumer et al (127, 128) Abe et al (133)
LH or ICSH (or gonadatropin)	+ve	Corpus leuteum, Testes.	Marsh (134) Murad et al (135)
TSH (thyrotropin) or LATS	+ve	Thyroid, Adipose tissue	Kamejiro and Field (48) Burke (1936) Desbarats et al (137) Zor et al (71) Ahn and Rosenberg (138)
PTH	+ve[12]	Kidney cortex, Fetal rat calvaria, Rat bone, Rat lymphocytes	Chase and Aurbach (68, 140, 141) Murad et al (141) Whitefield et al (142)

Table IV (ctd.)

2. Polypeptide hormones	Effect	Systems affected	Reference
MSH	+ve	Dorsal frog skin	Abe et al (133) Bitensky and Burstein (143)
GH	+ve	Adipose tissue	Swislocki (132)
FSH	+ve	Rat and dog testes	Murad et al (135)
Vasopressin (ADH)	+ve	Toad bladder, Kidney	Orloff et al (144) Orloff and Handler (145) Chase and Aurbach (68)
Insulin	-ve	Adipose tissue, Liver	Butcher et al (146) Fain and Reed (147) Bishop and Larner (148) Ray et al (126)
Secretin	+ve	Adipose tissue	Rodbell et al (149)
Melatonin	-ve	Dorsal frog skin	Abe et al (133)
Thyrocalcitonin (TCT)	+ve	Rat kidney and bone	Murad et al (141)
Gastrin	+ve	Gastric mucosa	Harris and Alonso (150)

Table IV (ctd.)

2. Polypeptide hormones	Effect	Systems affected	Reference
Pancreozymin	+ve	Rat and mouse pancreas	Knodell et al (151) Kulha and Sternlicht (152)
Calcitonin	+ve	Fetal rat calvaria	Chase and Aurbach (140)
"Hypothalamic releasing factors"	+ve	Anterior pituitary	Zor et al (153)
"Thyroxin releasing factors"	+ve	Thyroid	Zor et al (153)

12 J. D. Sallis (139), however, could find no evidence supporting the 3', 5'-AMP mediation of PTH stimulated phosphaturia in rat kidney.

Table IV (ctd.)

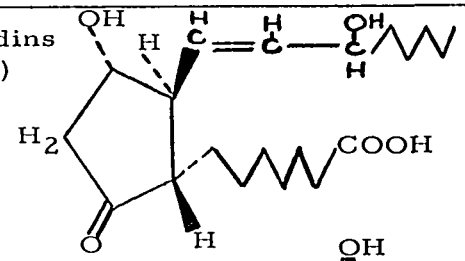
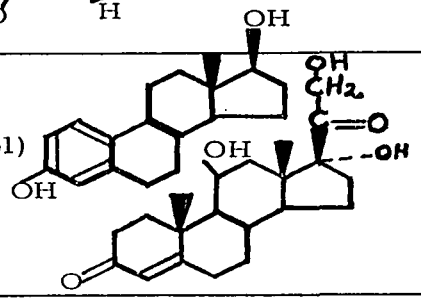
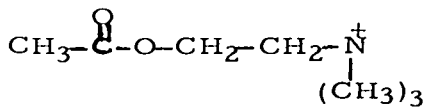
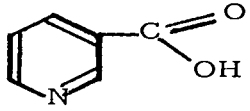
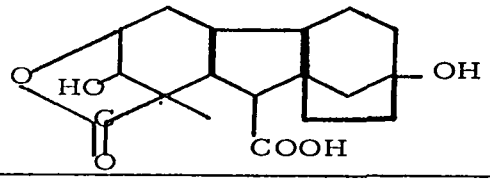
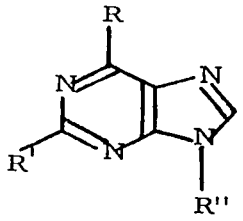
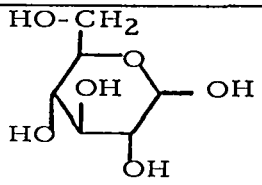
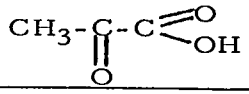
3. Miscellaneous Metabolites	Structure	Effect	Systems affected	Reference
Prostaglandins e.g. (PGE)		Usually -ve sometimes +ve (α-component)	Adipose tissue, Thyroid Fetal rat calvaria, Toad bladder, blood platelets, lung, spleen, diaphragm, kidney.	Steinberg and Vaughan (154) Ahn and Rosenberg (138) Wolff and Jones (155) Chase and Aurback (140) Orloff et al (145) Robison et al (117) Butcher and Baird (156)
Estrogen, (estradiol-17β) (diethylstilbestrol)		+ve	Castrated rat uterus	Szego and Davis (157) Rosenfeld and O'Malley (158)
corticosteroids (corticosterone)		+ve	adipose tissue of adrenalectomized rats	Braun and Hechter (159) Friedman et al (160)
Acetyl choline		-ve	Guinea pig heart	Vincent and Ellis (161)
Nicotinic acid		-ve	Adipose tissue	Butcher et al (146)

Table IV (ctd.)

3. Miscellaneous Metabolites	Structure	Effect	Systems affected	Reference
Gibberellic acid		+ve	Barley aleurone layers	Pollard (25)
GTP: R=OH, $R'=NH_2$ R''=ribose		+ve	Rat corpus leuteum	Franks and Stansfield (45)
dATP: $R=NH_2$, R'=H, R''=deoxy-ribosetriphosphate		-ve	Sheep thyroid, rat liver, mouse adrenal tumours	Burke (136) Mariwaki and Foa (162) Taunton et al (163)
Adenosine, $R=NH_2$, R'=H, R''=ribose and		-ve		
Adenine, $R=NH_2$, R'=H, R''=H		-ve		
Glucose		+ve	Adipose tissue	Meng and Ho (164)
Pyruvate		+ve	Brevibacterium liquifactiens	Hirata and Hayaishi (8).

Several question, however, remain to be answered:

(a) what physiological responses arise from increasing intracellular levels of 3', 5'-AMP in various cells ?

(b) what is the mechanism of such responses ?

(c) what determines which responses will occur in which cells ?

(d) what criteria should be examined to show that a given physiological response to a hormone is mediated by 3', 5'-AMP and that this effect is mediated by modulation of adenyl cyclase (rather than 3', 5'-AMP phosphodiesterase or ATPase, etc.) ?

The first three questions can only be partially answered at this time. Lastly, there is one question, of considerable relevance to the field of molecular pharmacology :

(e) what is the molecular mechanism of cyclase action and control ?

It is in pursuit of this mechanism, at the heart of so many control systems and drug interactions, which has stimulated much research into developing assay tools.

II. The Biochemical and Physiological Response to Increased Cellular 3',5'-AMP Levels

In higher organisms the final physiological consequence of an increase in the cellular concentration of 3', 5'-AMP will depend on the enzymatic profile (104) and ultimately on the functional DNA of the cell. Indeed, some effects such as induction of enzymes may depend on a mechanism of interaction directly at the DNA level (Table V, No. 6, page 46). The exact mechanisms of many of the biochemical and associated physiological changes mediated by 3', 5'-AMP are not well understood. The better understood ones will not be elaborated on here to any extent, but rather will be used as prototypes that are well documented (see Table V, page 45). The primary function of this section is to indicate the relative importance of the "second messenger" in different biological systems, which explains the large volume of research on the metabolism of 3', 5'-AMP.

All these 3', 5'-AMP mediated processes are very selective with regard to the first messenger and the type of biochemical response in different organisms and tissues. For instance, epinephrine in the heart increases phosphorylase(b) kinase activity, phosphorylase and ultimately glycogenolysis, as well as inotropism. In the adrenal cortex, ACTH may initiate the series of steps required for steroidogenesis, and so forth. To quote Orloff and Handler (144) : "Since epinephrine, ACTH glucagon and vasopressin all exert their specific effects via the intermediacy of 3', 5'-AMP, the release of 3', 5'-AMP by the 'wrong' hormone or a non-specific burst of activity in all sensitive tissue would be a catastrophic effect".

Table V

Biochemical Processes Affected by 3', 5'-AMP

No.	Enzymes affected	Effect	Mechanism	Reference
1.	(a) phosphorylase kinase (liver, kidney, muscle) (b) phosphorylase (indirectly in same tissue) (c) glycogen → G-1-P	+ve	Cascade process: Inactive form → active form	Haugaard and Hess (165) Krebs et al (166) Riley et al (167) Shaeffer et al (168) Louisot (169)
2.	glycogen synthetase (liver, heart, muscle)	-ve	Active form → inactive form	Larner (170) Buschiazzo et al (171)
3.	phosphofructokinase (Fasciola hepatica, pig liver)	+ve	Inactive form → active form	Stone and Mansour (172) Mansour (173)
4.	fructose-1, 6-diphosphatase (kidney and liver)	-ve	Active form → inactive form	Mendiceno et al (174)
5.	tryptophane pyrolase	+ve	Inactive form → active form	Chrytil and Skrivanova (175)
6.	histone kinases (many tissues and organisms)	+ve	Inactive form → active form	Kuo and Greengard (176) Meyamoto et al (177) Langan (178)

Table V (ctd.)

No.	Enzymes affected	Effect	Mechanism	Reference
7.	glutamate dehydrogenase	+ve		Erwin (179)
8.	tyrosine amino transferase active (rat liver) e.g. (tyrosine, α-keto-glutarate transaminase) (rat liver)	+ve	Both: Inactive form → active form, and induction (requiring a de novo protein synthesis)	Tryfiates and Litwack (180) Hager and Kenney (181) Fuller and Snoddy (182) Wicks (183)
9.	serine dehydratase (rat liver)	+ve	Induction (at level of transcription)	Jost et al (184)
10.	galactokinase (E. Coli)	+ve	Induction (derepression of glucose effect) level of action	Tuo and Schweiger (185)
11.	β-galactosidase (E. Coli)	+ve	Induction (activation at level of transcription by increasing the rate of mRNA produced)	Pastan and Perlman (186) Pastan and Perlman (187) Varmas et al (188) Chambers and Zubay (190)
12.	tryptophanase (E. Coli)	+ve	Induction (activation at level of translation (see No. 1(b)).	Del Campo et al (191) Pastan and Perlman (186) Pastan and Perlman (187)

Table V (ctd.)

No.	Processes affected (incorporation studies etc.)	Effect	Mechanism	Reference
13.	amino acids → proteins[13]			
	(a) rat liver	-ve	level of action ?	Pryor and Berthet (192)
		+ve	level of action ?	Grand and Gross (193)
	(b) E. Coli	+ve	binding to G factor during translocation of charged tRNA on messenger	Kuwano and Schlessinger (194, 195)
14.	acetate → liver fatty acids	-ve		Berthet (196)
15.	uridine → RNA (uterine hornes)	+ve	activation at level of transcription (not affected by cycloheximide)	Sharma and Talwar (197)
16.	Permeability to water, sodium and certain small molecules. (toad skin and bladder)	+ve	"third messenger" effects on the "porosity" of a membranous barrier ?	Orloff and Handler (146)
17	Melanocyte dispersion (frog skin)	+ve	a "third messenger" effect mediated by α receptors	Bitensky and Burstein (144) Novales and Fujii (198) Abe et al (133)
18.	Blood platelet aggregation	-ve	mediated by α receptors	Ardlie et al (199) Robison et al (117)

Table V (ctd)

No.	Processes affected (incorporation studies etc.)	Effect	Mechanism	Reference
19.	Release of insulin (pancreas)	+ve		Sutherland (109) Sussmann and Vaughan (200) Turtle and Kipnis (201) Feldman and Lebovitz (120)
20.	Release of TSH and GH (anterior pituitary)	+ve	mediated by TRF	Wilber et al (125) Zor et al (153)
21.	K^+ release (liver)	+ve		Tsujimoto et al (202) Exton and Park (203)
22.	Release of amylase (a) rat paratoid (b) barley endosperm (c) pancreas	+ve	does not require de novo protein synthesis	Grand and Gross (194) Solomon and Schramn (204) Galsky (205) Kulka and Sternlicht (152)
23.	Uptake and release of thyroxin (thyroid)	+ve	closely associated with glucose oxidation	Shishiba et al (206)

Table V (ctd)

No.	Processes affected (incorporation studies etc.)	Effect	Mechanism	Reference
24.	Secretion of trypsin, chymotrypsin (pancreas)	+ve	mediated by pancreozymin ?	Knodell et al (152)
25.	HCl secretion	+ve	mediated by an ion transport system supported by oxidative phosphorylation.	Harris and Alonso (150)
26.	Glucose oxidation (adipose tissue, rabbit brain, thyroid)	+ve	activation of certain enzymes. ?	Blecher (207) Dittman and Herrmann (208) Shishiba et al (206)
27.	2-ketoglutarate oxidation (adipose tissue)	+ve	closely related to increased lipolysis ?	Skosey (209)
28.	$^{32}P \rightarrow$ ATP, GTP, GDP. (intact rat diaphragm)	+ve	changes in ion (P_i) transport due to changes in membrane permeability ?	Walaas and Walaas (210)
29.	Gluconeogenesis (liver) [a slow process compared to glycogenolysis - see no. 1]	+ve	acceleration of rate limiting step between oxaloacetate and triosephosphates	Exton et al (211) Exton and Park (212) Pagliara and Goodman (213)

Table V (ct)

No.	Processes affected (incorporation studies etc.)	Effect	Mechanism	Reference
30.	Ketogenesis	+ve	activation of certain enzymes ?	Bewsher and Ashmore (214)
31.	Steroidogenesis (adrenal cortex)	+ve	Either a de novo protein synthesis or inhibition of oxidation of NADH ?	Akhtar et al (215) Sutherland et al (104)
32.	Lipolysis	+ve	Cascade process. Inactive lipase → active lipase	Hales et al (216) Sutherland et al (104)
33.	Urea production (liver) [a slow process]		Increase deamination of: endogenous, exogenous amino acids	Exton and Park (212)
34.	Inotropism (perfused heart)	+ve	mediated by β receptors ?	Hauggaard and Hess (165)

[13] Adamson (192) obtaining only 20-50% stimulation of transport and incorporation of amino acids into protein by diBu-3',5'-AMP and no stimulation by 3',5'-AMP, discounts the mediation of TSH by 3',5'-AMP in embryonic bone

Sutherland et al (104) have outlined some of the criteria used to determine whether or not a given process is mediated by 3', 5'-AMP :

1. The "first messenger" should cause an increase in 3', 5'-AMP in broken cell preparations.
2. The same effect should take piace in intact tissue.
3. This effect should be potentiated by methyl xanthines (PDEase inhibitors).
4. The final effect of the first messenger in intact tissue should be mimicked by 3', 5'-AMP[14].

Not in all the processes outlined in Table V have all four criteria been applied satisfactorily up to now. Fulfilment of the second but not the first criterion is an indication of the relative lability of hormone sensitivity which ultimately dictates the type of models adopted to explain these effects (see Part D, page 54).

It should be added that not all the effectors outlined in Table IV necessarily act directly at the cyclase level[15]. For example, although only the +ve and -ve catacholamine effects are normally blocked

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[14] Many of the metabolic effects outlined in Table V are not mimicked by 3', 5'-AMP itself, especially with intact systems. However, an analog N^6, O^2-dibutyryl 3', 5'-adenosine monophosphate (diBu-3', 5'-AMP) frequently mimicks the effect, thus serving to fill the fourth criterion (104, 213). The lack of effect of 3', 5'-AMP is usually attributed to its relative inability to penetrate membranes compared to the more lipophilic diBu-3', 5'-AMP.

[15] Krishna et al (218) showed that thyroid hormone (TSH) enhances the action of 3', 5'-AMP mediated lipolysis in adipose tissue by increasing the adenyl cyclase content. This process requires a de novo protein synthesis as it is blocked by puromycin and cycloheximide.

by the action of β and α adrenergic blocking agents[16] respectively, the TSH stimulation in sheep thyroid mitochondrial cyclase preparations (220) is nevertheless blocked by the same agents. However, the MSH response in frog skin is inhibited only by α blockers (133). Hence it would appear that the TSH and MSH effects could modulate adenyl cyclase via either local epinephrine release or by binding at the same site. However, these two examples appear to be exceptions rather than the rule.

To further hinder our understanding, the "third messenger" in one system may act as a "first messenger" in another. For example, insulin released from the pancreas (200, 201) would have a negative effect on the epinephrine stimulation of adipose tissue and liver cyclase (106, 112). It appears that negative first messengers (e.g. prostaglandins and insulin) and adrenergic antagonist do not function by lowering the basal rate of 3', 5'-AMP synthesis, but rather by inhibiting the stimulation of cyclase by a positive "first messenger" (catecholamines, and ACTH). In every case, the stimulation by fluoride is not affected by these agents.

Lastly it has been observed that in those tissues where catecholamines exert a negative effect, this effect can be at least partially reversed by the presence of an α adrenergic blocking agent, [e.g. in the aggregation of blood platelets (117), darkening of frog skin (133), and insulin released from rat pancreas (109)]. Catecholamine effects in these tissues are blocked by α-adrenergic reagents. In such tissues,

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[16] Pharmacologically, sympathetic enervation has been classified into α and β adrenergic types (219). Accordingly, catecholamines elicit a negative response from the former and a positive response from the latter. Thus the definition has been extended to include the response elicited on the "cyclase receptor".

cyclase (or its mediated final physiological event) is said to be "mediated by adrenergic α receptors" (133) (as opposed to the more usual "β adrenergic mediation" (105) (see Table V). This aspect will be discussed later when models are constructed to explain cyclase action (see Part D).

It is worthy of mention presently that 3',5'-AMP is probably not the only "second messenger"[17]. In fact, 3',5'-GMP has also been isolated from tissues and urine (222, 223) and guanyl cyclase activity [exactly analogous to adenyl cyclase] has been demonstrated in the soluble fraction of several tissues, the highest activity occurring in lung (224). However, this enzyme is not stimulated by epinephrine and glucagon (225). Hence 3',5'-AMP and 3',5'-GMP are under separate biosynthetic control.

Although the specific activity of guanyl cyclase is too low for the application of the periodate-orcinol assay (see below), it is anticipated that the assay should be useful in the determination of PDEase activity using 3',5'-GMP as substrate.

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[17] A 3',5'-GMP-dependent histone kinase has been demonstrated in lobster muscle (221)

PART "D". IONIC EFFECTS AND MECHANISTIC CONCEPTS OF CYCLASE ACTION

I. The Molecular Problem and Ion Effects

It is evident that nearly all hormone and metabolite effects are exerted at the cyclase level, rather than mediated by the diesterase, although some drugs are capable of acting at multiple levels to alter the 3', 5'-AMP concentration of tissues (110). This central role of cyclase has triggered a "literature explosion" over the last decade. Because hormone effects are qualitatively and quantitatively so varied in their nature from one tissue to another, it is impossible to devise any single rigid model to explain the nature of cyclase action. More general models, however, have been applied and their verification and modification must await further refinements in present research techniques (see Part A). Before pursuing any physical or chemical visualization of cyclase action, it is necessary to examine first some specific ion effects, as an understanding of this aspect of the problem may pave the way to general molecular concepts of hormone-receptor interaction.

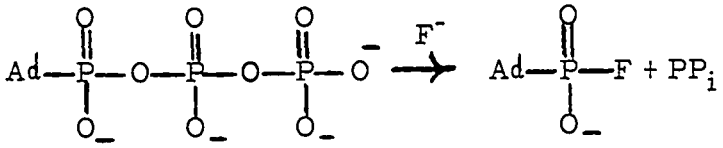
II. Fluoride Effects

Stimulation by the fluoride ion is a curious feature of adenyl cyclase which makes it unique among the ATP-requiring enzymes. Guanyl cyclase (224), ATPase (44) and many other functionally related enzymes are inhibited by sodium fluoride. The degree of activation of cyclase activity varies from two-fold (and less) in brain preparations (20) to

five- or ten-fold in fat cell ghosts (28, 127)[18]. However, pigeon and turkey erythrocytes do not respond to fluoride (21, 229). Øye et al (229) on the other hand, showed that after homogenization of these cells, the enzyme became fluoride sensitive although the epinephrine sensitivity of the intact cells was now lost. Curiously, purified adenyl cyclase from frog erythrocytes (50) appears to have an absolute requirement for fluoride ions !

Fluoride elicits an effect at an optimal concentration of about 10^{-2}M; higher concentrations are usually inhibitory. These effects are not additive with those of epinephrine, ACTH and other hormones. When fluoride is present in a cyclase preparation, the hormone effects are no longer visible (106, 111, 113).

Bloom and Goldman (230) attempted to explain the fluoride effect by a nucleophilic attack of fluoride on the innermost phosphate group of ATP to give the fluorophosphate corresponding to ATP. Presumably the phosphoryl transfer reaction would be enhanced when this fluorophosphate is substituted for ATP:



Other hypotheses invoking a greater affinity of the enzyme for an ATP-Mg^{++}-F^- complex or a Mg^{++}-F^- complex etc., can be envisaged. These ideas stemmed from the observation of fluoride effects on adrenergic receptors (231)(not unlike the effect on cyclase) and have been extended to encompass fluoride action on cyclase.

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[18] This activation effect was accounted for as a direct effect and not merely due to the inhibition of ATPase activity.

Belleau (232) interpreted the fluoride effect " in terms of a 'fit' of this ion into the lattice of water coordinated to divalent metal ions; thus, displacement of a water molecule of coordination would produce electric field effects and induce proton mobility about key anionic charges on the enzyme-bound ATP ".

This is an appealing concept, since the fluoride ion is small, mobile, with a high electric field and capable of exerting a "structure making" effect in the water lattice (46, 233). However, the exact site(s) and mode of binding of fluoride ion remains to be elucidated not only for cyclase but for many other enzymes.

Table VI

Specific Ion Effects on Adenyl Cyclase

Ion	Effect	System	Comments	Reference
F^-	+ve (10^{-2} M)	Practically all broken cell systems from tissues of higher organisms	Usually increases the V_{max} for ATP Can lower the Km for Mg^{++} There is no substitute for F^-	Bär and Hechter (82) Birnbaumer et al (127) Reviews (106, 113)
Mg^{++}	+ve (mM range)	Cyclases from all known sources	Mg-ATP is thought to be substrate Mn^{++} and Co^{++} will frequently substitute	Birnbaumer et al (127) Reviews (106)
Ca^{++}	-ve (mM range)	Adipose tissue Adrenal tumour	Competition for Mg^{++} site ? In adipose tissue, ACTH and NaF increase sensitivity of cyclase to Ca^{++} inhibition	Birnbaumer et al (127) Taunton et al (163)
	+ve (mM range)	Liver plasma membranes,	Stimulation of basal rates	Marinetti et al (77)
	+ve (10^{-5} → 5×10^{-5}M)	Brain homogenates,	Mn^{++} and Sr^{++} substitute for Ca^{++}	Bradham et al (73, 226)
	+ve (mM range)	Brain slices	Ca^{++} is required for effects of depolarizing agents but not for histamine stimulation	Shimizu et al (227)
	+ve (mM range)	Adipose plasma membranes and whole fat cells	Increased affinity for ACTH, LH, and TSH, but not for glucagon and epinephrine	Bär and Hechter (28) Kuo 30)

Table VI (ctd.)

Ion	Effect	System	Comments	Reference
K^+	+ve (mM range)	Brain slices	Not documented in homogenates	Shimizu et al (227) Sattin and Rall (228)
		Fat cells	Basal activity stimulation, Na^+ and Rb^+ substitute	Birnbaumer (127)
Zn^{++}	-ve (mM range)	Brain, skeletal muscle, fat cells	Competition for Mg^{++} site ?	Rall and Sutherland (20) Birnbaumer et al (127)

III. Calcium Effects

The effects of calcium on cyclase preparation have received a great deal of attention in recent years. Evidence has accumulated indicating the involvement of adenyl cyclase in the transport of Ca^{++} ions through membranes; hence, the possibility that control mechanisms involving the translocation of Ca^{++} ions could be coupled with the synthesis of 3', 5'-AMP (234, 235, 236). Loosely speaking, Ca^{++} may act as a "third messenger" (preferably "satellite messenger") in unbroken cells.

The calcium effects on cyclase turn out to be quite varied as shown in Table VI. However, with the exception of liver plasma cyclase, high concentrations of calcium ions ($>10^{-4}$ M) will inhibit the basal rates of synthesis, probably as a direct result of competition with Mg^{++} ions. More significantly, through the use of the calcium chelating agent EGTA[19], Bradham et al (73, 226) showed a partial dependence of brain cortex fluoride-stimulated cyclase on Ca^{++} ions. Similarly 3', 5'-AMP mediated α amylase secretion (238) by parathyroid glands and the PTH mediated gluconeogenesis in rat kidney cortex (239) are both Ca^{++} dependent.

It is significant that only the ACTH, TSH and LH responses of adipose tissue cyclase require Ca^{++} ions for their stimulatory effects. In contrast the epinephrine and glucagon responses are greater in a Ca^{++} free medium (28, 30). It was this discovery that led to the concept

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[19] EGTA has been documented as a powerful bivalent ion chelator more selective for calcium than EDTA (237) and is useful in showing calcium dependency of a biochemical activity.

of a "cyclase receptor" or "discriminator unit" controlling cyclase responses. This concept infers that adenyl cyclase may exist in different forms in the same tissue, each one having an identical catalytic unit but a different receptor[20] or discriminatory unit, subjection of the former to modulation. Three such different "receptor" units in adipose tissue have been detected by virtue of their different responses to specific effectors:

Table VII

A Receptor Model for Adipose Cyclase

No.	Receptor	Effector	Property
1.	Catecholamines	(nor)epine-phrine, isoprenaline	inhibited by β adrenergic blocking reagents. response is greater in the absence of Ca^{++}.
2.	Polypeptide hormones 1.	ACTH TSH LH	require Ca^{++} for response - no effect of β blockers.
3.	Polypeptide hormones 2	Glucagon	greater response in the absence of Ca^{++} no effect of β blockers

[20] Robison et al (105) conceived of adenyl cyclase as containing one catalytic and one regulatory receptor unit; the latter may be identical to or a close analog of an α or β adrenergic receptor. More recent studies of potentices of cyclase and PDEase effectors, and the relative potentices of these compounds as β adrenergic agonists and antagonist support the concept of mediation by 3', 5'-AMP of β adrenergic effects (240). See also Item no. 34, Table V, page 50, and footnote 16, page 52.

IV. Alternative Models

As yet, no experimental evidence, however, is available which allows a distinction between this model and the alternative possibility that a single catalytic unit may be equipped with all three receptor units. Evidence such as differences in sedimentation behavior (81) and identification of separate anatomical or cytochemical sites (68, 145, 241) of differently responding cyclases appear to support the first hypothesis which, however, contradicts earlier speculations on the mode of catecholamine binding at the active site (232, 242).

The interesting model suggested by Maddaiah (243) which assumes that polypeptide hormones may bind at the epinephrine site by the stereopositioning of a tyrosyl residue in catecholamine-like conformation, is not very convincing in the light of recent developments. The differential Ca^{++} requirement is not satisfactorily explained yet and the model may apply only to those exceptional cases where polypeptide hormone effects are inhibited by adrenergic blocking agents (217, 133) (see page 51).

It is clear that at present our understanding of the molecular interactions modulating the synthesis of 3', 5'-AMP is very limited. Future developments will depend largely on improved techniques for handling tissue preparations and on the accessibility of convenient assay methods.

MATERIALS AND METHODS

PART "A". MATERIALS

Disodium ATP, monosodium 3', 5'-AMP, epinephrine bitartrate and Triton X-100 were obtained from Sigma; glycylglycine, 5-hydroxytryptamine , adenine, kinetin dithiothreitol CMB and ouabain from Nutritional Biochemicals Corp.; xanthine, methyl xanthines and EGTA, from Eastman-Kodak; purine derivative (excepting adenine, kinetin and 2,6-diamino purine sulphate) and tetraphenylboron from Raylo Chemicals; magnesium sulphate, sodium fluoride, nicklous, zinc cupric and ferrous chlorides, isopropanol and sodium silicotungstate from Baker; sodium metaperiodate perchloric acid from Analar; sodium borohydride from British Drug Houses and orcinol monohydrate, lead acetate, sodium molybdate and sodium, calcium strontium, barium and cobalt chlorides from Fisher. 3', 5'-GMP and 2,6-diaminopurine sulphate were obtained from Calbiochem; manganous chloride from Merck and 2-mercaptoethanol and methyl sulphonylchloride from Aldrich. Glucagon and ACTH were obtained from Mann and Nordic chemicals respectively. Prostaglandin E was a gift from Dr. I. H. Hooper, Bristol Laboratories; EEDQ was obtained from Mr. W. T. Robinson, and the n-pentyl DPA from Dr. V. DiTullio, Department of Biochemistry, University of Ottawa, and butoxamine was a gift from Burroughs Welcome, New York.

Stock solutions containing ATP 10^{-2} M and 3', 5'-AMP 5×10^{-4} M were prepared and the pH adjusted to 7.5 with sodium hydroxide. They were stored frozen at -20°C.

PART "B". METHODS

I. Preparation of Adenyl Cyclase from Brain

Whole cow brains were obtained within 30 minutes of killing from a local slaughter-house. The tissue was immediately immersed in ice-cold standard buffer consisting of glycylglycine 2×10^{-3} M (pH 7.6), $MgSO_4$ 10^{-3} M, and NaCl 2×10^{-2} M. The cortex was cut out, minced, and the tissue disrupted in a Teflon-glass Potter-Elvehjem homogenizer according to a modified procedure of Bitensky and co-workers (81). Five to ten grams of tissues were homogenized at a time by 9 passes through the apparatus, at the rate of 210 rmp[20]. The resulting homogenate was processed by a slight modification of the procedure of Sutherland and his associates (6). At this stage, eight volumes of homogenate in standard buffer were obtained from one volume of tissue. The suspension was strained through a double layer of No. 35 Curity gauze and centrifuged again at 600 g for 15 minutes. This procedure was repeated once more, and the sediment was re-suspended and stored frozen at -20° in 15 ml portions in sealed polyethylene tubes.

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[20] The number of revolutions per minute of the apparatus was estimated using a Smith ATH_4 Tachometer.

II. Solubilization of Adenyl Cyclase

"Solubilization" of the sediment with Triton X-100 was accomplished using a method similar to that of Sutherland and co-workers (6); 10 mls of triton solution was added slowly to 50 mls of washed 50% diluted membrane particles while stirring constantly at 0°C to bring the final triton concentration to 1.8%. After stirring for 10 minutes the suspension was centrifuged at 40,000 g for 20 minutes in a Spinco model "L" centrifuge; the supernatant was recentrifuged at 100,000 g for 2 hours. The pellet formed was resuspended in 5 mls of ice-cold standard "wash buffer" which contained additional glycylglycine (.2 M at pH 7.5). "Solubilized" preparation was stored frozen at -20°C or -70°C.

III. Cyclase Assay

The standard incubation medium (4 ml) consisted of ATP 10^{-3} M, $MgSO_4$, 3.6×10^{-3} M, caffeine 5×10^{-2} M (or 3×10^{-2} M), glycylglycine 6×10^{-2} M (pH 7.5) and NaF 6×10^{-3} M. The latter was omitted when potential activators of cyclase were tested. The cyclase-catalyzed reaction was initiated by the addition of 0.2 ml (containing 4 - 5 mg total protein) of particulatesuspension from brain tissue. The incubation mixtures were shaken for 20 minutes at 25° in a Dubnoff incubator and the reaction stopped by rapid immersion for 3.5 minutes in boiling water using 15 ml cellulose nitrate centrifuge tubes as containers.

After cooling to 0°, the tubes were centrifuged at 10,000 g for 40 minutes in a Spinco model "L" centrifuge to sediment all proteins. The clear supernatants were transferred to 25 ml flasks and 0.2 ml of $NaIO_4$ solution (100 mg/ml) added. The flasks were stoppered and allowed to stand at 40 - 45° in the dark for 18 - 22 hours. After this time, 0.2 ml portions of freshly prepared sodium borohydride solution[21] (60 mg/ml) were added at 25°, the mixtures gently shaken and allowed to stand for 30 minutes. Portions of 1 ml of 0.2 M lead acetate solution were then added and occasionally a small drop of 2-octanol added to prevent frothing when excessive. The suspensions were centrifuged in 15 ml tubes at 1000 g for 20 minutes at 25° to remove lead iodide and 4 ml portions of the clear supernatants withdrawn with 5 ml Hamilton syringes equipped with polyethylene needles (contact with metallic surfaces must be avoided at this stage). The clear solutions were transferred to 25 ml boiling tubes and the orcinol test for ribose applied according to a modification of Mejbaum's technique (244): 4.0 ml portions of the aqueous solution were transferred to the boiling tubes, then 4.0 mls of a .2% solution of ferric chloride in concentrated hydrochloric acid were added. Finally, 0.4 mls portions of an aqueous solution of orcinol hydrate were added (100 mg/ml). The tubes were stirred for 15 seconds (vortex mixer), heated to 100° for 40 minutes, and cooled to 25°. The color was extracted into 4.5 ml of redistilled n-butyl acetate (vortex mixer) and the lower phase was allowed to settle for 3 minutes. Gentle tapping aided phase separation, or in the case of assays with detergent treated enzyme, low gravitational centrifugation was necessary. The upper phase was transferred to quartz cuvettes (pasteur pipettes) and the absorbance read at 670 mμ (a scale-expander was used for low optical densities). Appropriate blanks were run in parallel.

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[21] The sodium borohydride should be stored in several small vials in a desiccator, as otherwise moisture absorption leads to incomplete reduction of the oxidation products.

IV. 3',5'-AMP Phosphodiesterase Assay

The same detailed technique described above was applied, except that 3',5'-AMP (5×10^{-5} M) was substituted for ATP and caffeine was omitted, unless otherwise designated in the incubation medium. Less protein was used for K_i determinations. In this case, the rate of disappearance of absorbance at 670 mμ was followed, instead of the rate of appearance.

V. Phosphodiesterase Inhibition

Under conditions of higher protein concentration (4 - 5 mg total protein), caffeine, theophylline, DBP, DCP, adenine was added to the incubation mixtures prior to 3',5'-AMP addition. The respective range of concentrations were: 10^{-3} to 3×10^{-2} M, 5×10^{-4} to 10^{-2} M, 5×10^{-4} to 5×10^{-3} M, 10^{-3} to 3×10^{-2} M and 1 to 6×10^{-3} M.

VI. ATPase Activities

These were measured by modification of the method of Post and Sen (245) as recently described by us (246), except that all reaction mixtures had a volume of 4 ml. The reactions were quenched by the addition of 1.35 ml of perchloric acid solution (245).

VII. Protein Concentration

This was done according to Lowry et al (247)

FIGURE 3

The Direct Chemical Assay

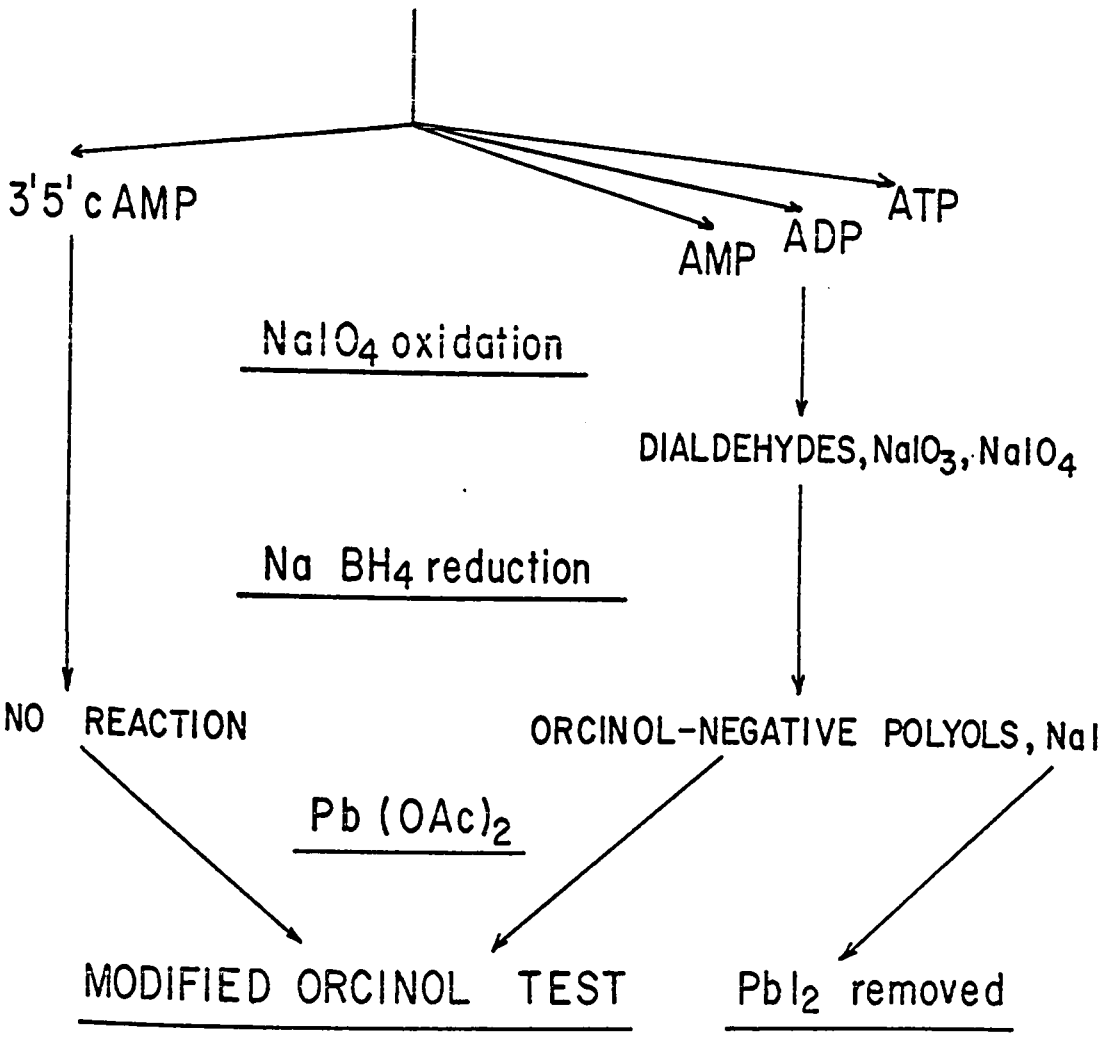
The chemical discrimination between 3', 5'-AMP and non-cyclic nucleotides by periodate oxidation and subsequent chemical manipulations is depicted diagrammatically.

DIRECT CHEMICAL ASSAY FOR 3' 5' c AMP

1) Enzyme reaction stopped by heating for 3½ mins at 100°C

2) Protein removal by centrifugation

3) Product analysis



VIII. Calcium and Manganous Ion Effects

Concentrations of Ca^{++} and Mn^{++} in standard assay mixtures containing all ingredients except protein or up to 4.0 mg of protein were measured on a type AA5 techtron atomic adsorption spectrophotometer. For Ca^{++} an acetylene/nitrous oxide flame (10 psi and 27 psi pressures) and for Mn^{++} and air/acetylene flame (25 psi each) were respectively used and absorption at 422.8 mμ and 279.5 mμ were measured respectively.

The solution for bivalent ion measurement was prepared exactly as the standard incubation mixture (protein ion measurement excepted). Membranous protein (8.0 mgs) was solubilized by gently heating in 1 ml of concentrated nitric acid and the final volume increased to 10 mls by addition of ethanol. Appropriate blanks and standards were measured in parallel.

IX. Synthesis of N^6-chloroacetyladenine (CAA)

The method of synthesis was adapted from that of Craveri and Zoni (248). Adenine (1.5 g) was dried at 78°C in vacuo overnight and added to 100 mls of toluene (distilled over sodium) and 25 mls of dimethylformamide (distilled over CaH_2); 5 g of chloroacetylanhydride (Eastman) was added and the mixture heated under reflux for 3-1/2 hours. On cooling to room temperature, a mass of crystals separated, were filtered out and identified by TLC as starting material (650 mgs). The filtrate was evaporated in vacuo to a volume of about 12 mls. On standing several hours a second mass of crystals was collected and washed with methanol then dried in vacuo. Yield = 15%.

X. Identification, Purity and Properties of CAA

1. Thin layer chromatography ($CHCl_3$:MeOH, 3:1) yielded one spot only. R_f 6.6.

2. The melting point was 225 - 228°C [reported 200°C; (248)].

3. Crystals were pale ivory fine prisms which became pink on standing several months [reported as pink (248)], or red when allowed to stand for several hours in solution. (Dimethylsulphoxide or water).

4. N.m.r. spectrum (in dimethylsulphoxide) :

   a) δ = 4.6, singlet integrating for 2 protons ;

   b) δ = 8.6, singlet integrating for 1 proton ;

   c) δ = 8.8, singlet integrating for 1 proton.

5. I.r. spectrum :

   a) broad band 3600 - 3400 Å

   b) strong peak 1700. Å

RESULTS

The standard curve for 3',5'-AMP shown in Figure 4 was constructed in both the absence and the presence of a 100-500-fold excess of ATP. No difference was noticeable. As can be seen, Beer's law applies over a wide range of concentrations. The limit of sensitivity is reached at about 10^{-6} M 3',5'-AMP. The same curve can be reproduced using either ribose or 3',5'-AMP, in the absence of ATP, but with the omission of the periodate oxidation, subsequent reduction and precipitation, thus showing that no detectable quantity of the cyclic nucleotide is lost during the chemical manipulations. Higher concentrations of borohydride (2-fold) tend to decrease the standard absorbance. The orcinol blanks were not significantly increased by proteins at 1.0 mg/ml, caffeine at 5×10^{-2} M or epinephrine at 10^{-4} M. Complete removal of the nuclear fraction is essential in order that low blanks are obtained. This is readily accomplished at the centrifugation step preceding periodate oxidation.

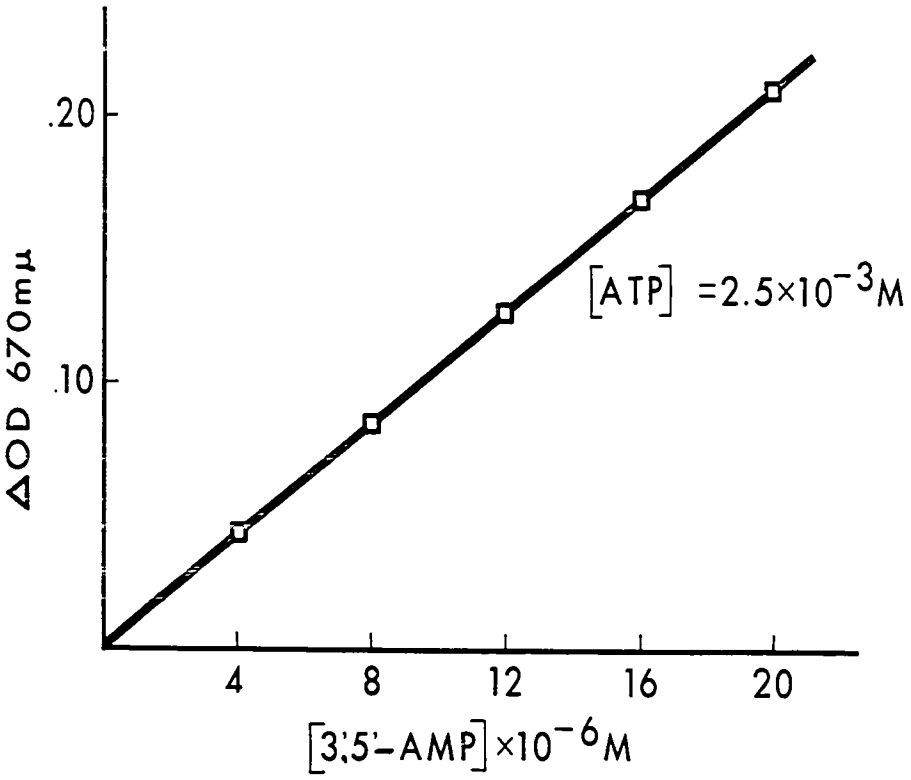
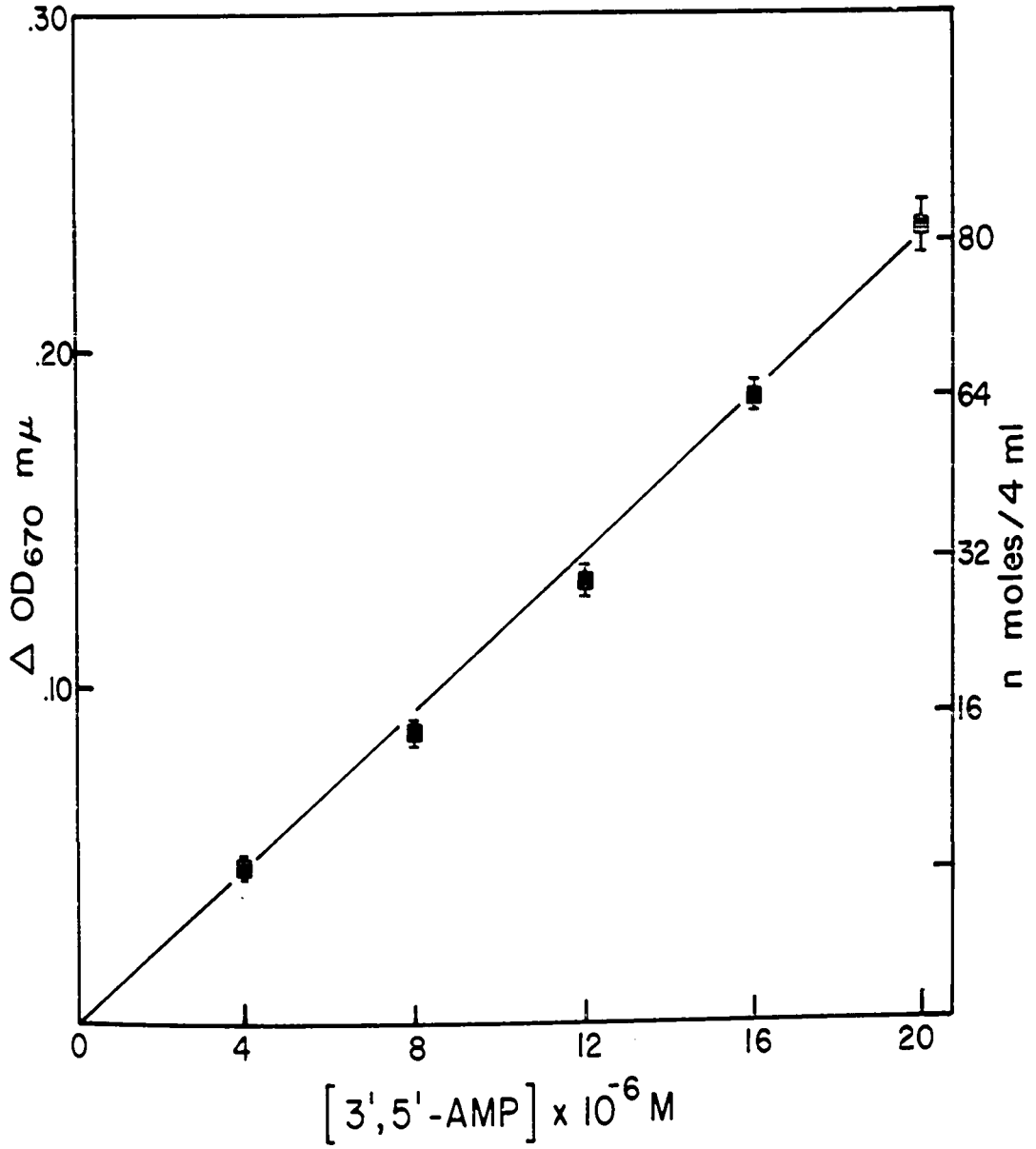

.20
.10
ΔOD 670mμ
[ATP] =2.5×10⁻³M
4
8
12
16
20
[3',5'-AMP]×10⁻⁶M

FIGURE 4

Standard Curve for 3', 5'-AMP in the Presence of Excess ATP *

The assay medium consisted of ATP 2.5×10^{-3} M, glycylglycine 0.06 M (pH 7.5), caffeine 5×10^{-2} M, $MgSO_4$ 3.6×10^{-3} M, and variable concentrations of 3', 5'-AMP in a total volume of 4 ml. The color was developed by the periodate-orcinol technique and absorbance read at 670 mμ.

* Data presented are the average readings for three typical determinations.


.30
.20
.10
Δ OD670 mμ
80
64
32
16
n moles/4 ml
0
4
8
12
16
20
[3',5'-AMP] x 10-6 M

PART "A". STATISTICAL STUDY OF BLANKS

In order to determine the statistical error derived from random fluctuations in the blanks (including the protein preparation), a series of determinations were made (using the standard assay procedure with all ingredients except where noted on page 64).

As can be seen in Tables VIII and IX, the average contribution due to the addition of protein (.0006 O.D. units) is insignificant when the assay is carried out under the recommended conditions. [Clearly, the complete removal of protein and nuclear fractions before the periodate addition is an essential feature of the assay].

I. Sample Blanks

Table VIII

Blanks containing 5×10^{-4} M ATP and no Protein

No.	O. D.	No.	O. D.
1	.022	6	.024
2	.024	7	.023
3	.024	8	.023
4	.023	9	.021
5	.025	10	.022

Mean blank reading = .0229 O. D. units

Table IX

Blanks containing 1.0 mgs Protein/ml and no ATP

No.	O. D.	No.	O. D.
1	.021	7	.021
2	.024	8	.022
3	.023	9	.024
4	.025	10	.024
5	.024	11	.023
6	.025	12	.026

Mean blank reading = .0235 O. D. units

II. Standard Deviations

Applying the formula

$$S_D = \sqrt{\frac{\sum [x - \bar{x}]^2}{N-1}} \qquad (249)$$

where $\bar{x} = \sum \frac{x}{N}$

S_D is the standard deviation

x is a single value of one reading

$\bar{x}$ is the mean value of all readings

N is the number of readings taken.

For values of Table VIII (ATP containing blanks) :

S_D = .0027.

For values of Table IX (protein containing blanks) :

S_D = .0048.

Table X

Percentage Error due to Fluctuation in Blanks

	Blank	Control Reaction Mixture	Fluoride containing Reaction Mixture
Typical O. D. reading	.023	.105	.225
Error in ATP containing systems	± 6. 0%	± 1. 2%	± 0. 6%
Error in protein containing systems	± 10%	± 2. 3%	± 1. 1%

Because of the extreme stability of the chromophores the error due to blank fluctuations is minimal, thus permitting kinetic studies in the low optical density range.

PART "B". 3',5'-AMP SYNTHESIS

I. Effects of Protein Concentration

In Figure 5 the relation between 3',5'-AMP synthesis[22] and cyclase concentration in the absence of activators is shown. The relationship is adequate until protein concentration reaches 2 mg/ml. However, linearity is observed only at protein concentrations less than 1.2 mg/ml.

Deviation from linearity at high protein concentrations may be explained by ATPase and residual PDEase activity. However, a useful range of protein concentrations is 0.8 - 1.2 mg/ml for studies of straightforward kinetic effects on cyclase. Another factor which may be contributing to the deviations from linearity is the direct effect of protein concentration on the physical state of cyclase. Therefore, the kinetic effects of potential modifiers might be strongly dependent on protein concentration.

II. Time Course Studies on 3',5'-AMP Synthesis

The amount of 3',5'-AMP produced over a 20 min period was reasonably linear for all practical purposes and this at varying initial concentrations of ATP both in the presence or absence of fluoride. Thus again, it is seen that the effects of ATPase and residual PDEase activities do not appear to excessively obscure cyclase kinetics.

---

[22] The level of 3',5'-AMP, as evaluated by our procedure, was kindly verified by A. D'Iorio and D.M. Horwood according to their own assay based on the thin layer chromatographic procedure of Randerath (250), and using radioactive substrate.

FIGURE 5

The Effect of Protein Concentration on the Yield of 3', 5'-AMP

The media consisted of ATP 5×10^{-4}, glycylglycine 0.06 M (pH 7.5), caffeine 3×10^{-2} M (●—●) or 5×10^{-2} M (▲—▲) $MgSO_4$ 3.6×10^{-3} M. Incubations lasted 20 minutes at 25°.

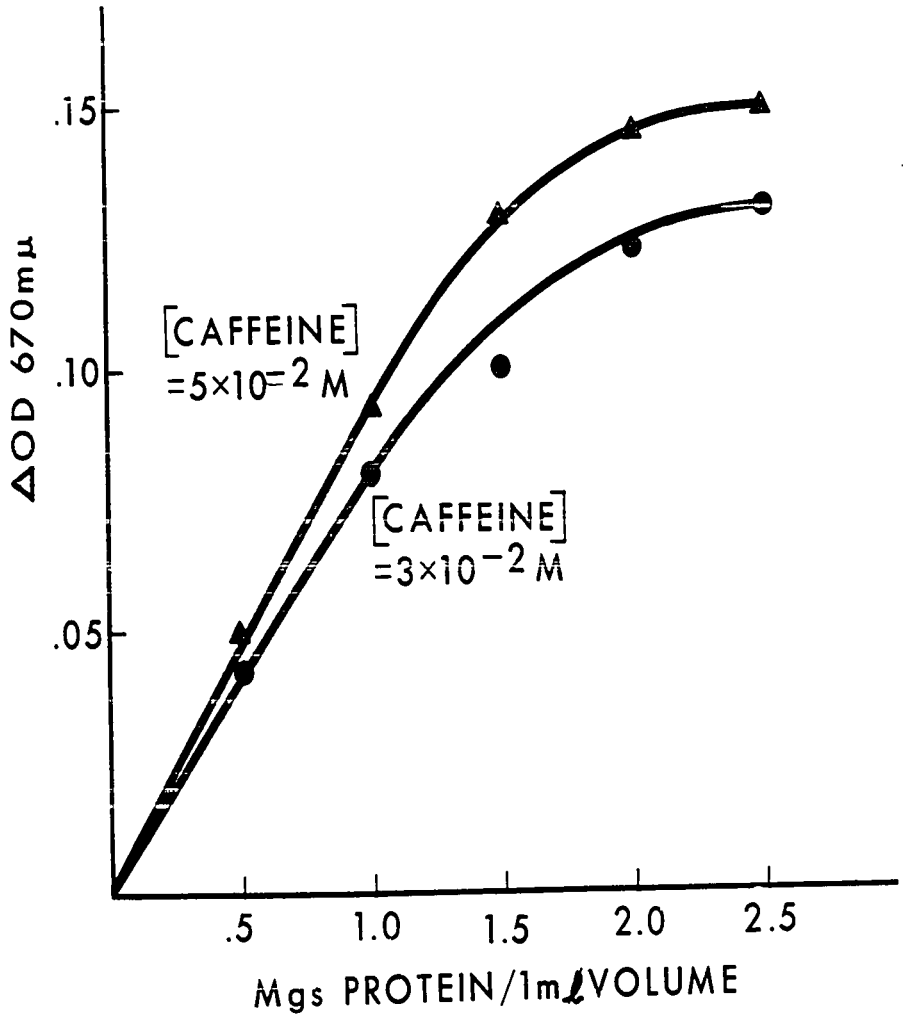
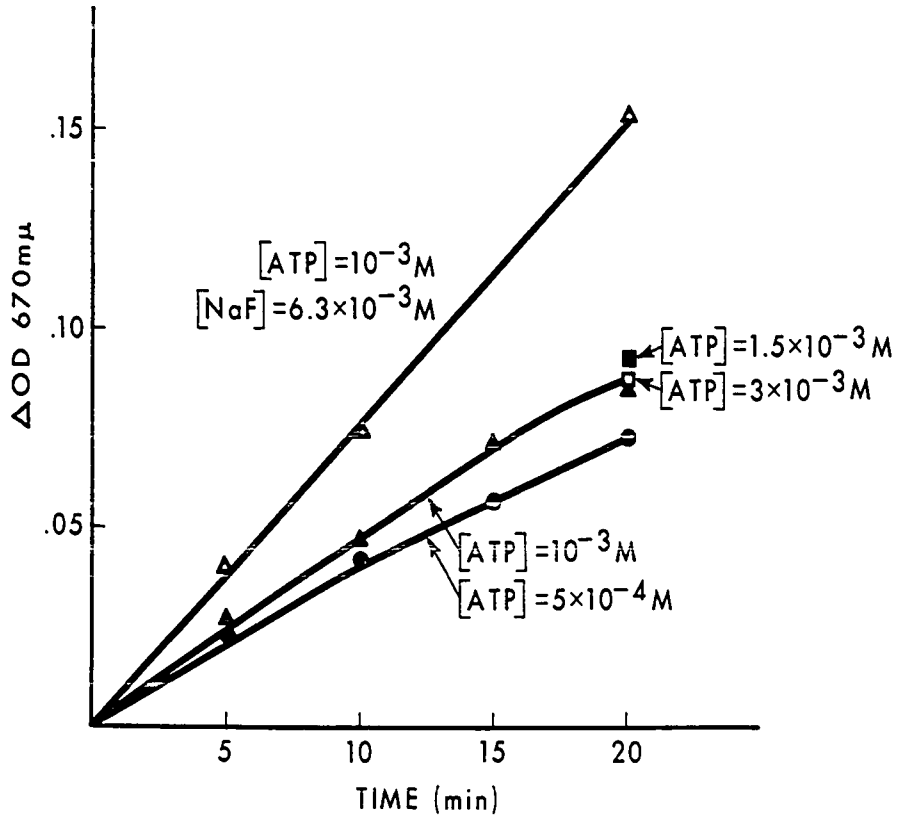

.15
.10
.05
ΔOD 670mμ
[CAFFEINE]
=5×10⁻² M
[CAFFEINE]
=3×10⁻² M
.5
1.0
1.5
2.0
2.5
Mgs PROTEIN/1mℓVOLUME

FIGURE 6

Time Course Curves of 3', 5'-AMP Synthesis

3', 5'-AMP synthesis was studied at 25°C in the presence of 10^{-3} M ATP and 6.3×10^{-3} M sodium fluoride (Δ—Δ) and in the presence of 3×10^{-3} M ATP (□), 1.5×10^{-3} M ATP (■), 10^{-3} M ATP (▲—▲), and 5×10^{-4} M ATP (●—●) all in the absence of fluoride ions. Reagents $MgSO_4$ 3.6×10^{-3} M, caffeine 5×10^{-2} M, glycylglycine .06 M pH 7.5, proteins 1.0 mg/ml ATP and sodium fluoride.


.15
.10
.05
ΔOD 670mμ
[ATP]=10⁻³M
[NaF]=6.3×10⁻³M
[ATP]=1.5×10⁻³M
[ATP]=3×10⁻³M
[ATP]=10⁻³M
[ATP]=5×10⁻⁴M
5
10
15
20
TIME (min)

III. Effects of ATP Concentration

Cyclic AMP synthesis as a function of ATP concentration gave rise to a simple Michaelis curve (Fig. 7 and 8). It can be seen that fluoride activation of cyclase is the result of a two-fold increase in the apparent V_{max} of ATP without a significant change in Km (Figure 7). Yet large physical and/or biological variations are evident when Figure 7 is compared to Figure 8. Under identical conditions, fluoride V_{max} effects cannot be observed but a significant shift in Km is apparent (Figure 8).

Table XI

Apparent Values of Kinetic Parameters for ATP as Substrate

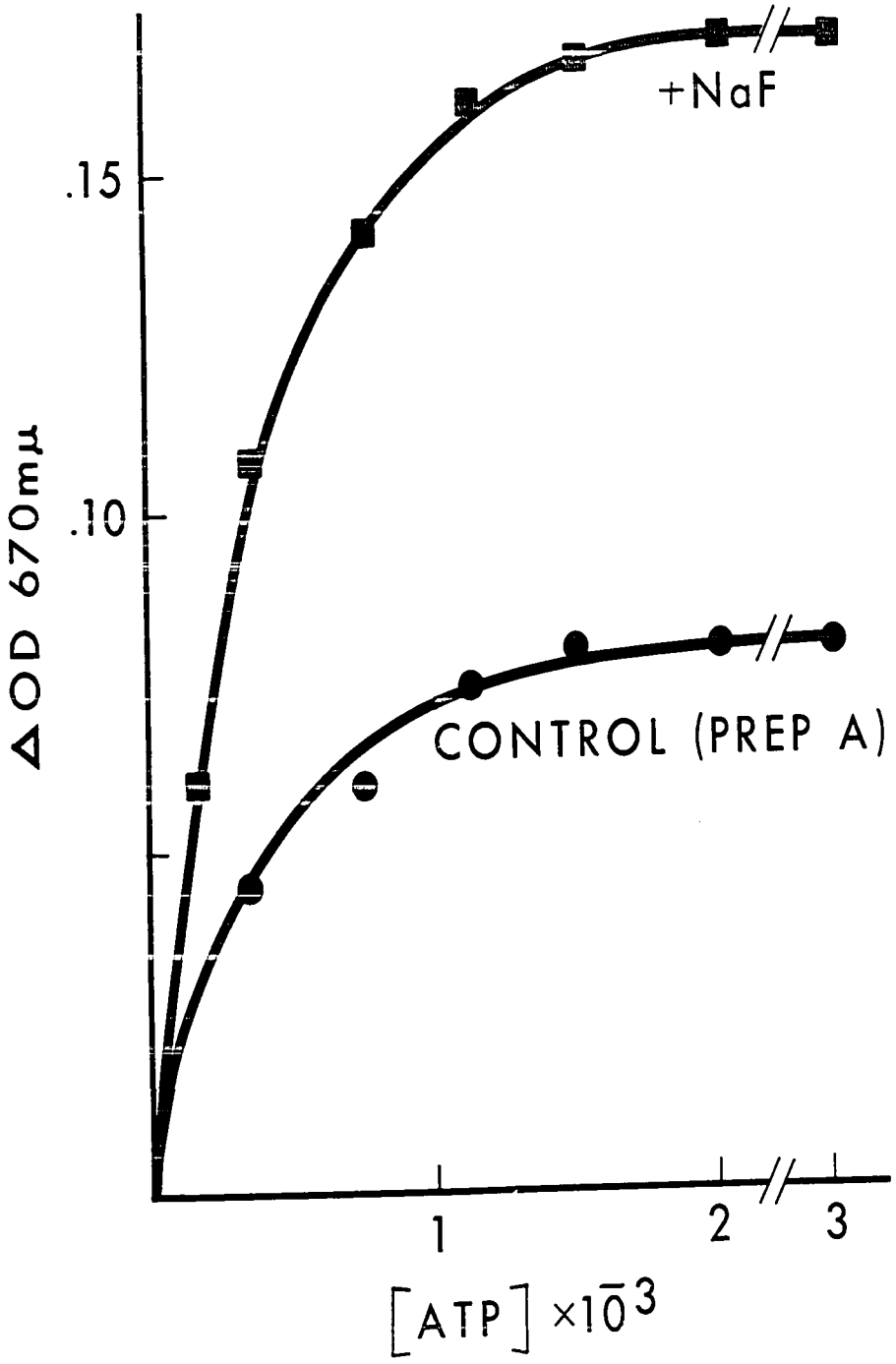
	-NaF		+NaF	
	Km	V_{max} ([caffeine] $5x10^{-2}M$)	Km	V_{max} ([caffeine] $5x10^{-2}M$)
Fig. 7, prep. (a)	$3.0x10^{-4}M$	$3.7x10^{-10}$ moles/mg protein/min.	$2.5x10^{-4}M$	$7.7x10^{-10}$ moles/mg protein/min.
Fig. 8,	$3.0x10^{-4}M$	$3.9x10^{-10}$ moles/mg protein/min.	$0.5x10^{-4}M$	$4.2x10^{-10}$ moles/mg protein/min.

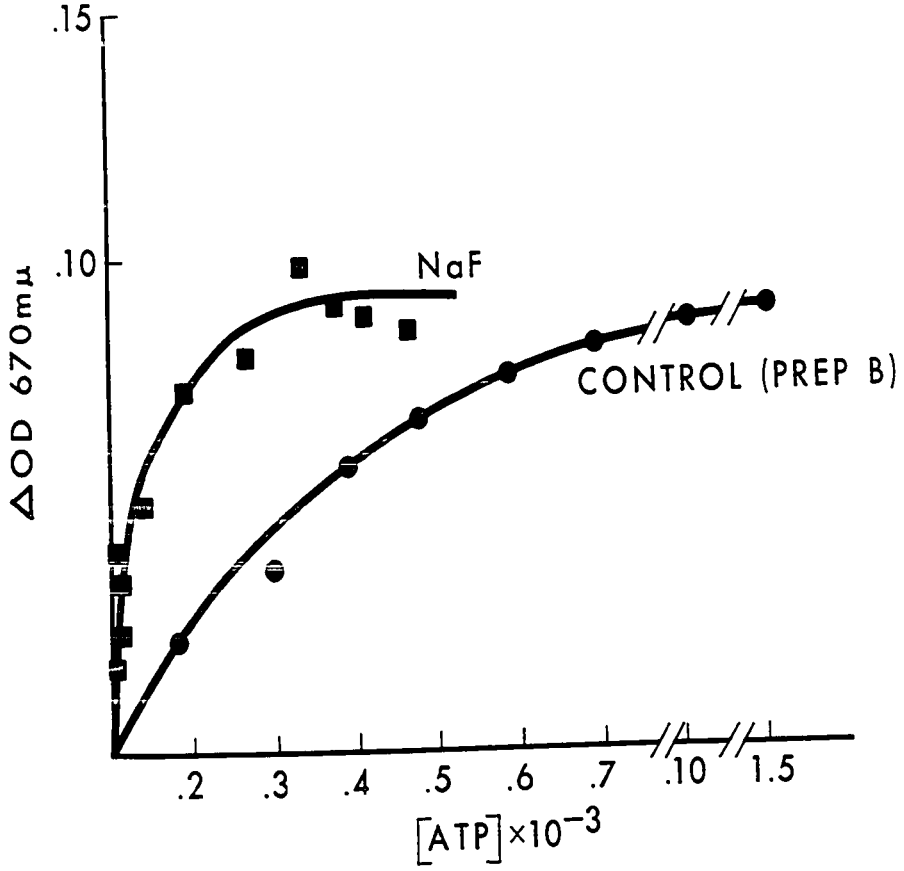
It becomes evident upon superficial examination of fluoride effect on 16 brain cyclase preparations, under saturating concentrations of Mg^{++} and ATP, that the V_{max} effect may vary from 0% to 300%.

FIGURES 7 and 8

3', 5'-AMP Synthesis versus ATP Concentration

The same incubation media as described in Figs. 4 and 5 were used. [caffeine] = 5×10^{-2} M. [$MgSO_4$] : 3.6×10^{-3} M. The protein concentration was 1 mg/ml; (■—■) with NaF 6×10^{-3} M and (●—●) without fluoride.


+NaF
.15
.10
ΔOD 670mμ
CONTROL (PREP A)
1
2
3
[ATP] ×10⁻³


.15
.10
ΔOD 670mμ
NaF
CONTROL (PREP B)
.2
.3
.4
.5
.6
.7
.10
1.5
[ATP]×10⁻³

IV. Effects of Mg^{++} Concentration

The dependence of cyclase on Mg^{++} was readily confirmed as illustrated in Figure 9. The apparent Km for Mg^{++} was 3×10^{-4} M. Some fluoride effect occurred at lower Mg^{++} concentrations (6×10^{-4} M) which was not evident at 2×10^{-3} M when relatively unresponsive preparations were used [PREP B], thus verifying Figure 8.

V. Effects of ATP Concentration under Conditions of Low $[Mg^{++}]$

As already shown in Figure 9, the absence of activation by fluoride ions was notably absent or minimal at higher Mg^{++} concentration in some preparations. However, it was found that fluoride activation in the same preparations is markedly influenced by Mg^{++} concentration. As can be seen in Figure 10, at low $[Mg^{++}]$, fluoride (6.3×10^{-3} M) causes both a decrease in the Km for ATP and an increase (about 1.5-fold) in V_{max}, the latter disappearing at higher Mg^{++} concentrations. It is evident, even in preparations which display a significant V_{max} effect by fluoride a higher Mg^{++} (3.6×10^{-3} M) that a significantly greater effect is observed at lower Mg^{++} (6×10^{-4} M).

VI. Effects of Fluoride vs. ATPase:

The activating effect of fluoride on cyclase was found to be quite complex. Not only does activation depend on fluoride concentration, but it is also influenced both qualitatively and quantitatively by Mg^{++} concentration, by "solubilization" of the enzyme, and sometimes by the number of washings with standard buffer of the first

FIGURE 9

3', 5'-AMP Synthesis versus $[Mg^{++}]$

The same conditions described in the preceding Figure were used; [ATP] was 1×10^{-3} M and [protein] 1 mg/ml; (▲—▲) with NaF 6×10^{-3} M and (●—●) without fluoride.

A preparation (PREP. A) (Δ) sensitive to fluoride at $[Mg^{++}] = 3.6 \times 10^{-3}$ M is contrasted with fluoride insensitive preparation (PREP. B) (▲—▲) under identical conditions.

FIGURE 10

3', 5'-AMP Synthesis versus [ATP] at low (Mg^{++}] in the Presence of NaF 6×10^{-3} M (●——●) and Absence of Fluoride (▲——▲).

Reagents: $MgSO_4$ 6×10^{-4} M, caffeine 5×10^{-2} M, glycylglycine 0.06 M, proteins 1.0 mg/ml. Incubation for 20 minutes, temperature 25°.

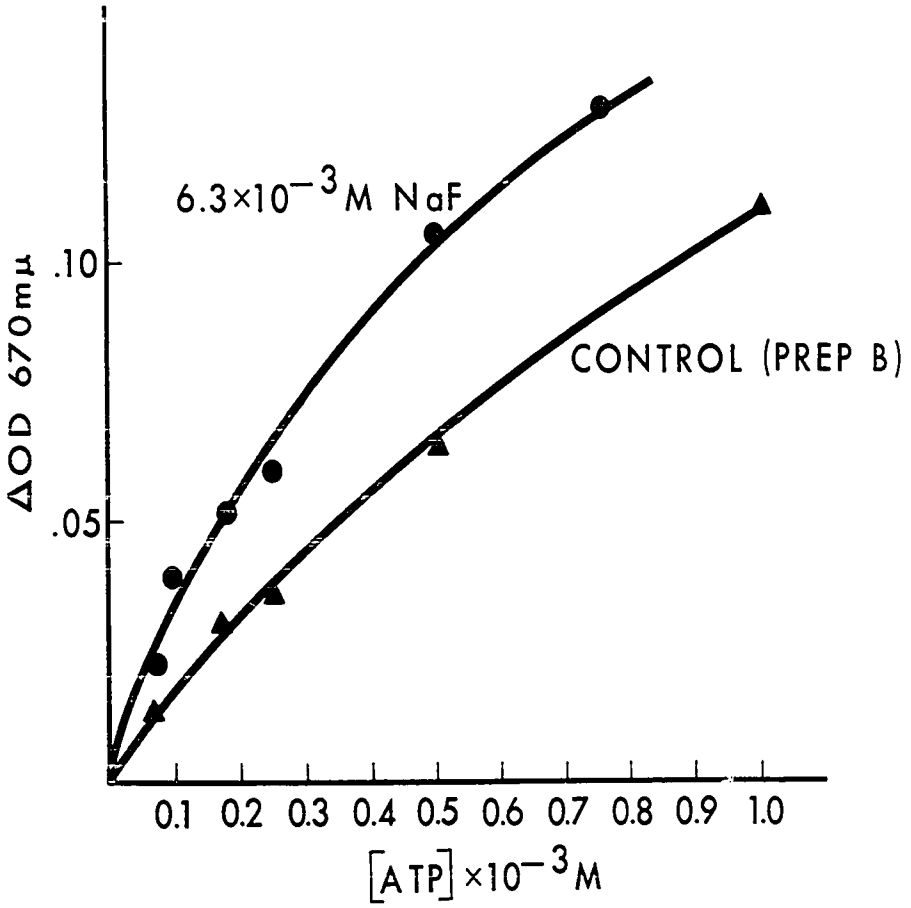
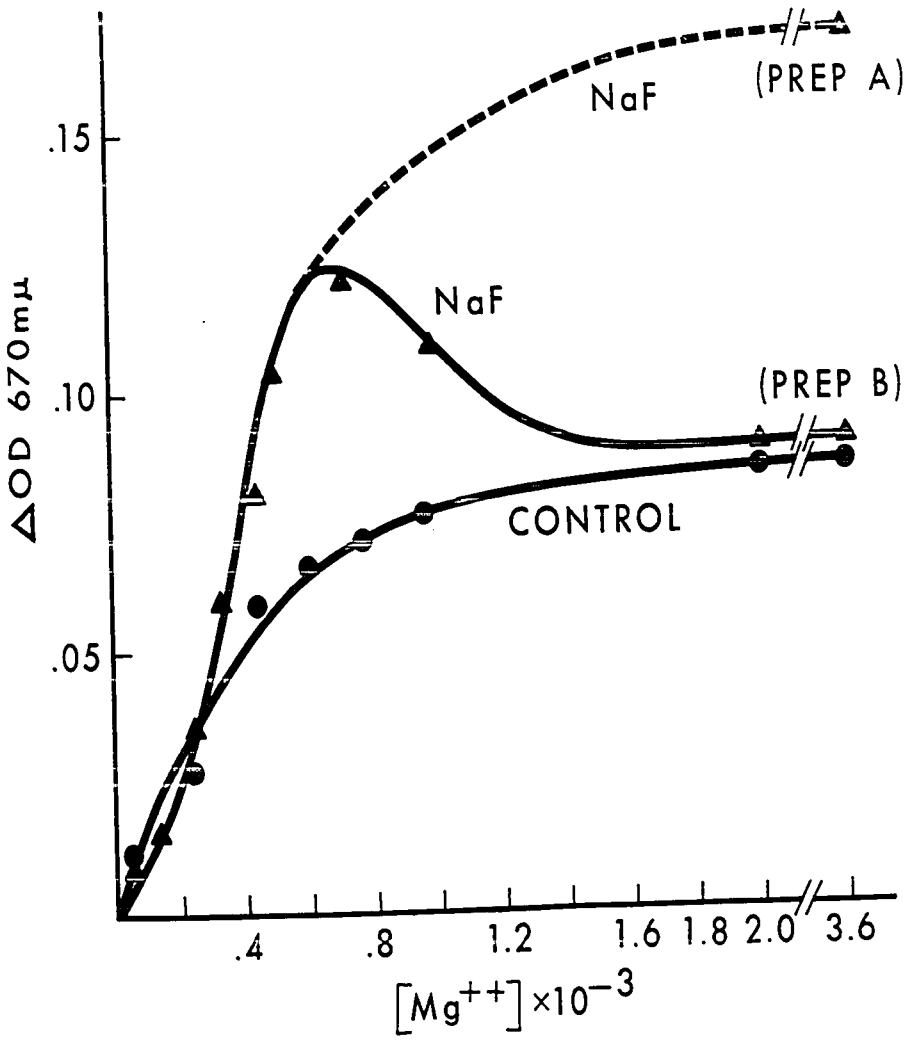

6.3×10⁻³M NaF
CONTROL (PREP B)
ΔOD 670mμ
.10
.05
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
[ATP]×10⁻³M

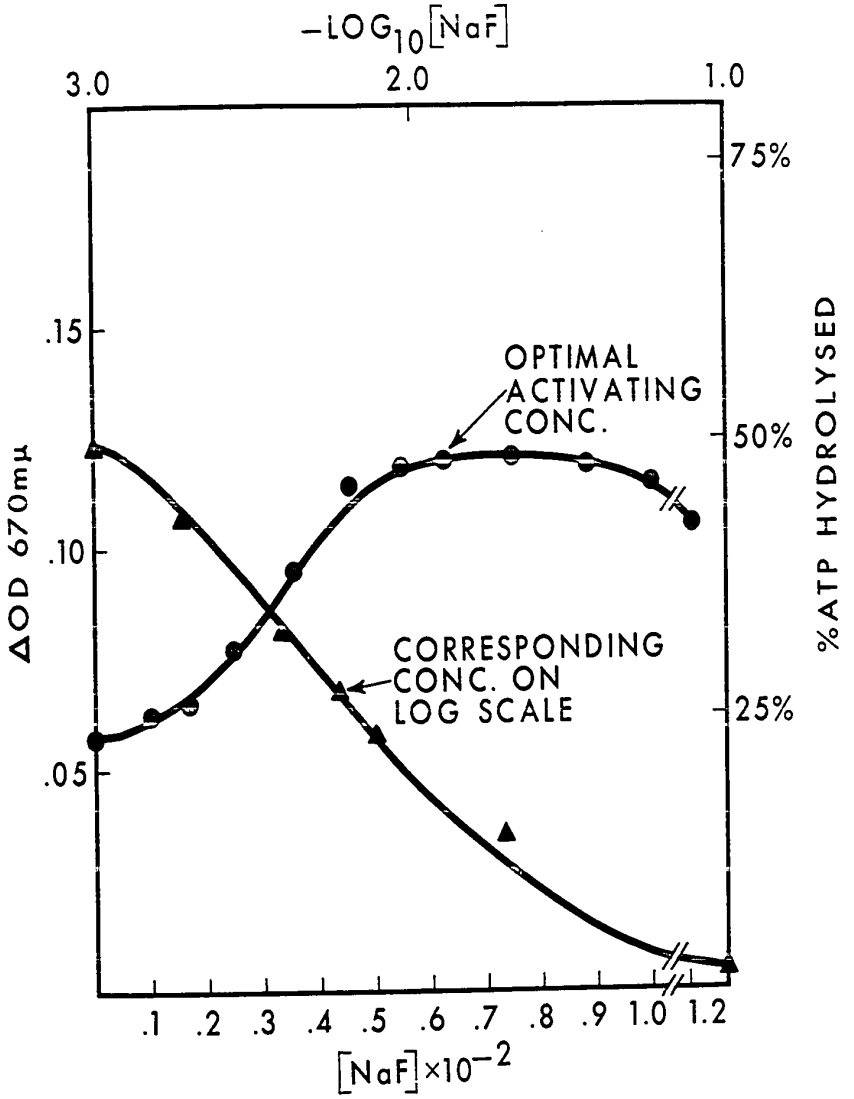
FIGURE 11

Effects of Fluoride on Cyclase and ATPase

Variations of cyclase activity (left vertical axis) with fluoride concentration (lower horizontal axis) (●—●) and variation of ATPase activity (right vertical axis) with log of fluoride concentration (upper horizontal axis) (▲—▲). Reagent concentrations for cyclase assay: ATP 3.5×10^{-4} M, $MgSO_4$ 3.5×10^{-3} M, glycylglycine 0.06 M, caffeine 5×10^{-2} M, proteins 1 mg/ml. Reagent concentrations for ATPase assay: ATP 5.0×10^{-4} M, $MgSO_4$ 3.6×10^{-3} M, glycylglycine 0.06 M, proteins 1.0 mg/ml.

Incubation time 20 minutes; temperature 25°.


NaF
(PREP A)
NaF
(PREP B)
CONTROL
.15
.10
.05
ΔOD 670mμ
.4
.8
1.2
1.6
1.8
2.0
3.6
[Mg++] ×10−3


$-LOG_{10}[NaF]$
3.0
2.0
1.0
75%
50%
25%
.15
.10
.05
ΔOD 670mμ
% ATP HYDROLYSED
OPTIMAL ACTIVATING CONC.
CORRESPONDING CONC. ON LOG SCALE
.1 .2 .3 .4 .5 .6 .7 .8 .9 1.0 1.2
$[NaF] \times 10^{-2}$

sediment containing cyclase activity. In Fig. 11 is shown the relation between 3', 5'-AMP synthesis and fluoride concentration in the presence of a saturating level of Mg^{++}. Maximum activity is obtained over an optimum range 5×10^{-3} M to 10^{-2} M of fluoride concentrations. Also shown in the same figure is the relation between ATPase activities over the range of fluoride concentrations. At the optimum of 6×10^{-3} M fluoride for cyclase activation about 45% of the total ATPase activity is inhibited, thus suggesting that the observed optimum in the fluoride concentration curve does not coincide with the repression of total ATPase activity. Total ATPase activity over a 20-minute period under the cyclase assay conditions caused 50 - 54% hydrolysis of ATP in the presence of 6.3×10^{-3} M sodium fluoride (Figure 12). At 6×10^{-4} M $MgSO_4$, the ATPase is saturated with respect to Mg^{++}; when the $MgSO_4$ concentration is raised to 6.3×10^{-3} M, only a slight decrease in activity was observed. It is reasonable to conclude that only a very small fraction of the fluoride effects on cyclase at different Mg^{++} concentrations may be accounted for solely in terms of ATPase effects.

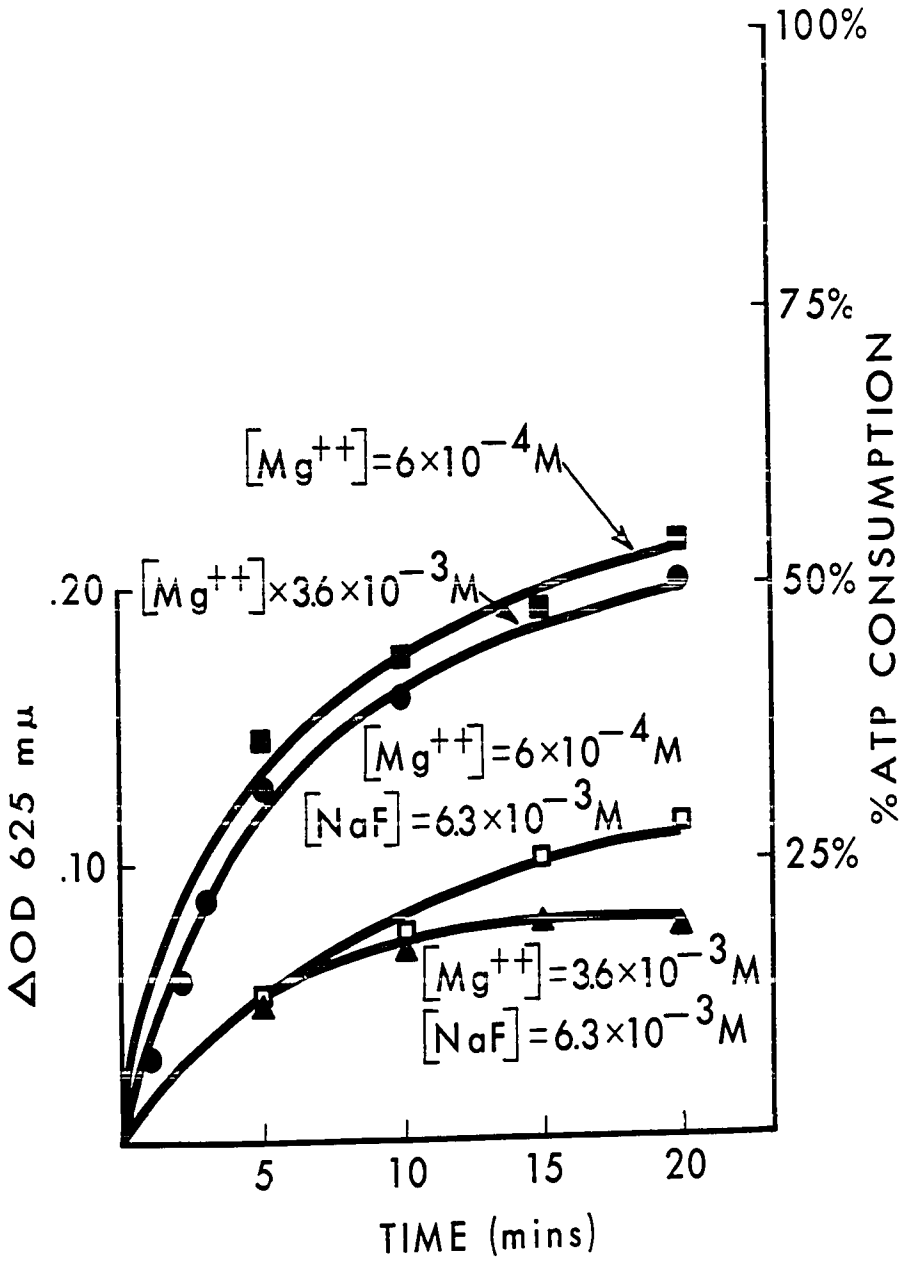
---

[23] Over a twenty-minute period the accumulation of 3', 5'-AMP (proteins = 1.0 mg/ml) showed a fairly good linear dependence on initial ATP concentration in agreement with this conclusion (see Figure 6).

FIGURE 12

Effects of Mg^{++} and Fluoride on ATPase

Time course of ATPase activity in the presence of high $[Mg^{++}]$ (3.6×10^{-3} M) with fluoride at 6.3×10^{-3}M (▲—▲) and without fluoride (●—●); also in the presence of low $[Mg^{++}]$ (6×10^{-4} M) with fluoride at 6.3×10^{-3}M (□—□) and without fluoride (■—■). Reagents present: caffeine at 5×10^{-2} M, glygly buffer 0.06 M (pH 7.5), ATP at 5×10^{-4} M, proteins 1 mg/ml; incubation at 25°.


100%
75%
50%
25%
% ATP CONSUMPTION
[Mg++]=6×10−4M
[Mg++]×3.6×10−3M
[Mg++]=6×10−4M
[NaF]=6.3×10−3M
[Mg++]=3.6×10−3M
[NaF]=6.3×10−3M
.20
.10
ΔOD 625 mμ
5
10
15
20
TIME (mins)

VII. Effects of EGTA, Calcium Ions and Fluoride Ion Cyclase Activity

Incubation of the cyclase preparation for 20 minutes at 25°C with EGTA at 4×10^{-5} M led to 85% inhibition of total activity in the absence of fluoride and 45 - 60% inhibition at a fluoride concentration of 6.3×10^{-3} M. Higher concentrations of EGTA produced no additional effect in either control or fluoride-containing reaction mixtures. Prior addition of calcium chloride at 4×10^{-5} M completely prevented EGTA inhibition and restored full sensitivity to fluoride activation (Figures 14 and 15); EDTA at 4×10^{-5} M under identical conditions had little effect on cyclase activity as previously reported (81, 251). Concentrations of Ca^{++} higher than 5×10^{-5} M were inhibiting to cyclase in the absence of EGTA. Epinephrine at 10^{-4} M had no stimulatory effect on the EGTA-inhibited cyclase. Results are qualitatively similar at either low (6×10^{-4} M) or high (3.6×10^{-3} M) $[Mg^{++}]$ (Fig. 13) and are entirely consistent with our observations on the $[Mg^{++}]$ effects on fluoride stimulation.

VIII. Substitution of Other Bivalent Metal Ions for Calcium

Preincubation of the cyclase preparation for 20 minutes at 25° with EGTA and 5×10^{-5} M divalent metal ions gave the results summarized in Figure 15 where it can be seen that Mn^{++}, Co^{++} and to a lesser degree Sr^{++} (see also Figure 14) can substitute for Ca^{++}. Moreover, at 5×10^{-5} M, Mn^{++} and Co^{++} lead to significantly higher fluoride activation effects (without affecting controls) than those achieved with 5×10^{-5} M Ca^{++} or fluoride containing controls. Other divalent ions, Ba^{++}, Zn^{++}, Ni^{++} and Fe^{++} were ineffective. These results are in complete agreement with those of Bradham and co-workers (73, 226).

FIGURE 13

Effects of EGTA and Fluoride on Cyclase Activity

A 20-minute preincubation at 25° with EGTA was used prior to ATP addition; 3', 5'-AMP synthesis at high [Mg^{++}] (3.6×10^{-3}M) in the presence of fluoride (6×10^{-3} M) (▲—▲) and the absence of fluoride (Δ—Δ) and at low [Mg^{++}] (6×10^{-4} M) in the presence of fluoride (6×10^{-3}M) (●—●) and the absence of fluoride (○—○) is plotted against EGTA concentration. Reagents: ATP 5×10^{-4} M, glygly buffer 0.06 M, pH 7.5, caffeine 5×10^{-2} M and protein 1.0 mg/ml, and $MgSO_4$. Incubations for 20 minutes at 25°.

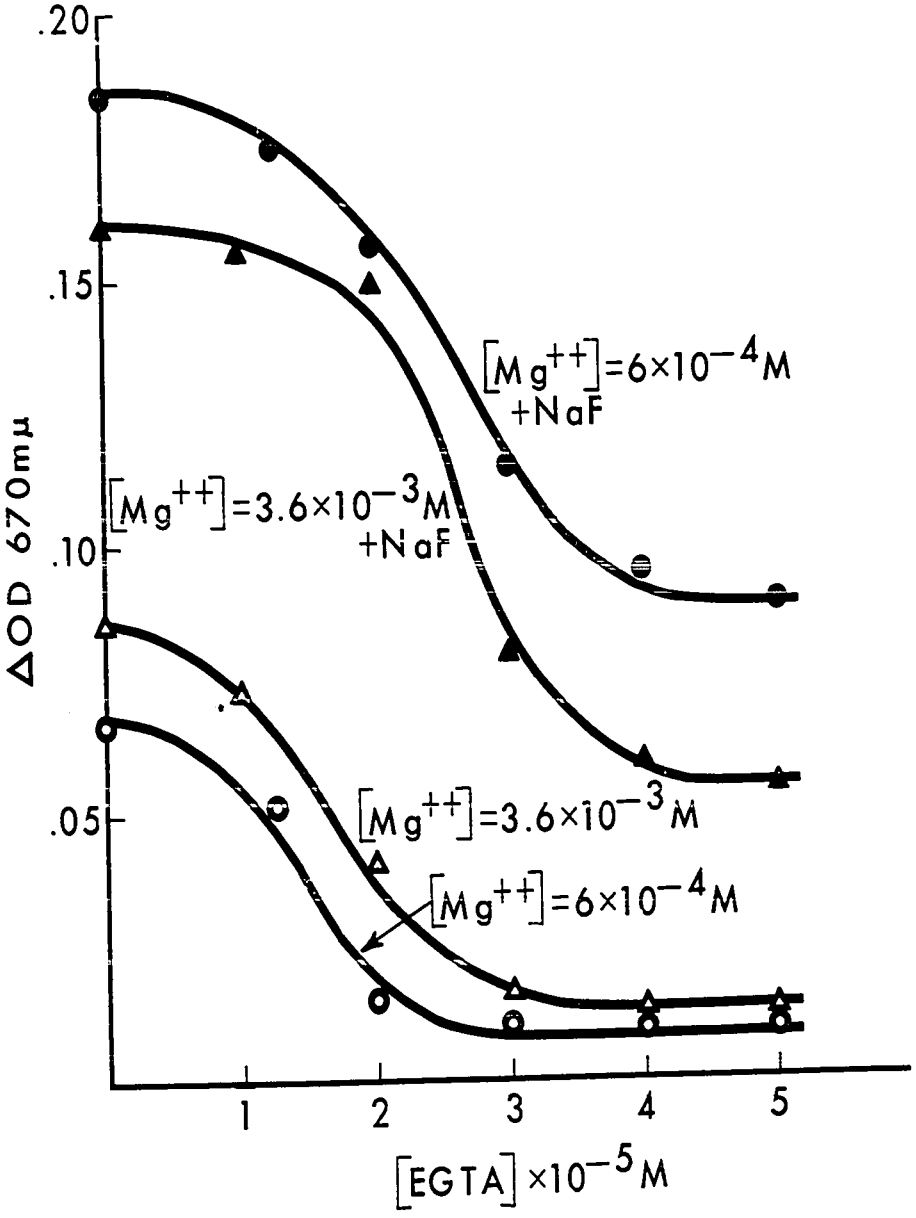

.20
.15
.10
.05
ΔOD 670mμ
[Mg++]=6×10−4M
+NaF
[Mg++]=3.6×10−3M
+NaF
[Mg++]=3.6×10−3M
[Mg++]=6×10−4M
1
2
3
4
5
[EGTA] ×10−5M

FIGURE 14

Protection by Ca^{++} and Sr^{++} ions against EGTA Inhibition

Cyclase activity in the presence of EGTA is plotted against bivalent metal ion concentration [Me^{++}] for both $CaCl_2$ in the presence of 6×10^{-3} M fluoride (▲—▲) and the absence of fluoride (△—△) and for $SrCl_2$ in the presence of 6×10^{-3} M fluoride (■—■) and its absence (□—□). Reagents: ATP 5×10^{-4} M, glygly 0.06 M, pH 7.5, caffeine 5×10^{-2} M, $MgSO_4$ 6×10^{-4} M, EGTA 4×10^{-5} M, protein 1.0 mg/ml, $SrCl_2$ and $CaCl_2$. Preincubation for 20 minutes at 25° with EGTA prior to ATP addition, followed by a 20-minute assay.

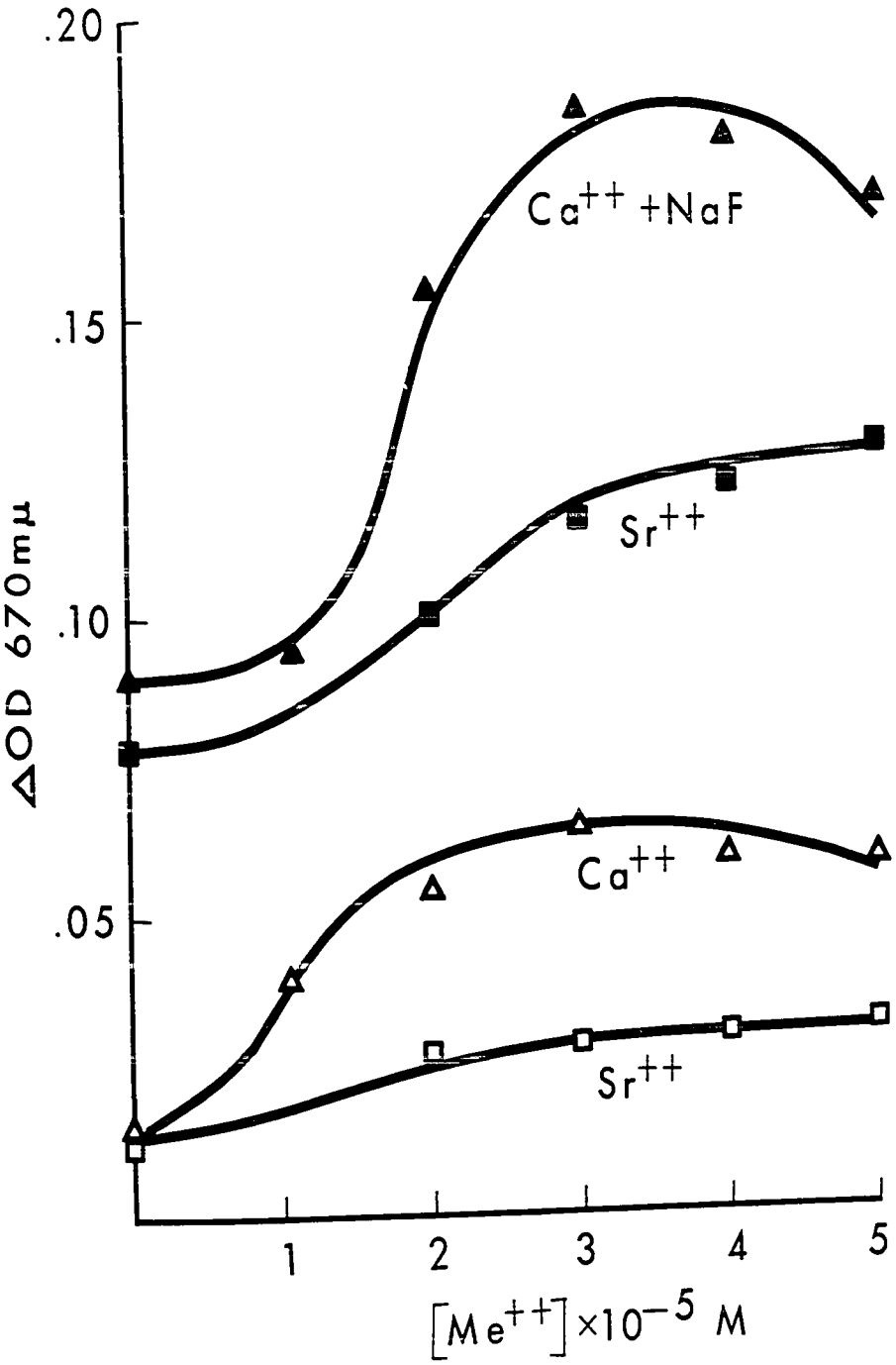
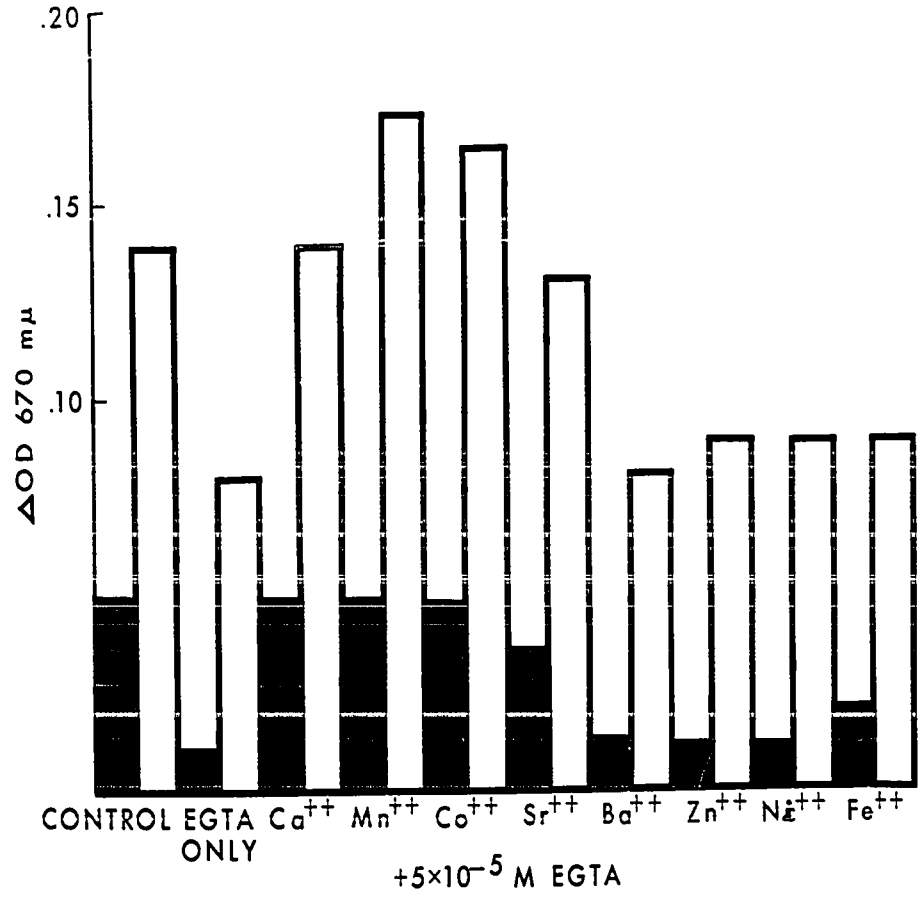

.20
.15
.10
.05
ΔOD 670mμ
Ca++ +NaF
Sr++
Ca++
Sr++
1
2
3
4
5
[Me++] ×10−5 M

FIGURE 15

Relative Ability of Various Divalent Metal Ions to Substitute for Calcium in the Presence of Fluoride (unshaded bars) and in the Absence of Fluoride (shaded bars).

Reagents ATP, 5×10^{-4} M, $MgSO_4$ 3.6×10^{-3} M, caffeine 5×10^{-2} M, glycylglycine .06 M, pH 7.5, proteins 1.0 mg/ml, EGTA 5×10^{-5} M (absent in the control, only), 5×10^{-5}M bivalent metal ion chloride (as designated) and 6.3×10^{-3} M sodium fluoride (unshaded bars only). A reincubation of the reaction mixtures in the presence of EGTA (as designated) 20 minutes at 25°C in the absence of substrate preceded the 20 minute 3', 5'-AMP synthesis period at 25°C.


.20
.15
.10
ΔOD 670 mμ
CONTROL
EGTA ONLY
Ca++
Mn++
Co++
Sr++
Ba++
Zn++
Ni++
Fe++
+5×10−5 M EGTA

XI. Calcium and Manganese Ion Concentrations in the Extracts

Concentrations of calcium and manganese in the final reaction mixtures containing 4.0 mgs of the particulate preparation (as used in the standard reaction mixture) were found to be 1.0×10^{-5} M and $< 5 \times 10^{-7}$ M[24] respectively. Thus, there appears a greater likelihood that the Ca^{++} ion may be a true co-factor in vivo. In the protein-free reaction mixtures (see page 73) the total calcium and manganous concentrations were 1.2×10^{-5} M and $< 10^{-7}$ M[24] respectively. Most of the calcium not initially associated with the protein is derived from the magnesium sulphate (as confirmed by the .01% Ca^{++} ion impurity according to the analysis given by Baker Chemicals) and from the glycyl glycine buffer (likely from the sodium hydroxide required to adjust the pH to 7.5). Less than 10% of the $[Ca^{++}]$ in the complete reaction mixture (total $[Ca^{++}]$ 2.2×10^{-5} M) was derived from the ATP[25]. The $[Ca^{++}]$ from the stock solutions of caffeine and sodium fluoride was immeasurably small. These results are also in accord with those of Bradham et al (73).

X. Effects of Solubilization

The activating effects of fluoride were completely lost upon solubilization of cyclase with Triton X-100 (Fig. 16). While solubilization caused less than a two-fold increase in specific activity in the absence of fluoride and the presence of 5×10^{-2} M caffeine at saturating concentrations of substrates (20 minute incubations and

---

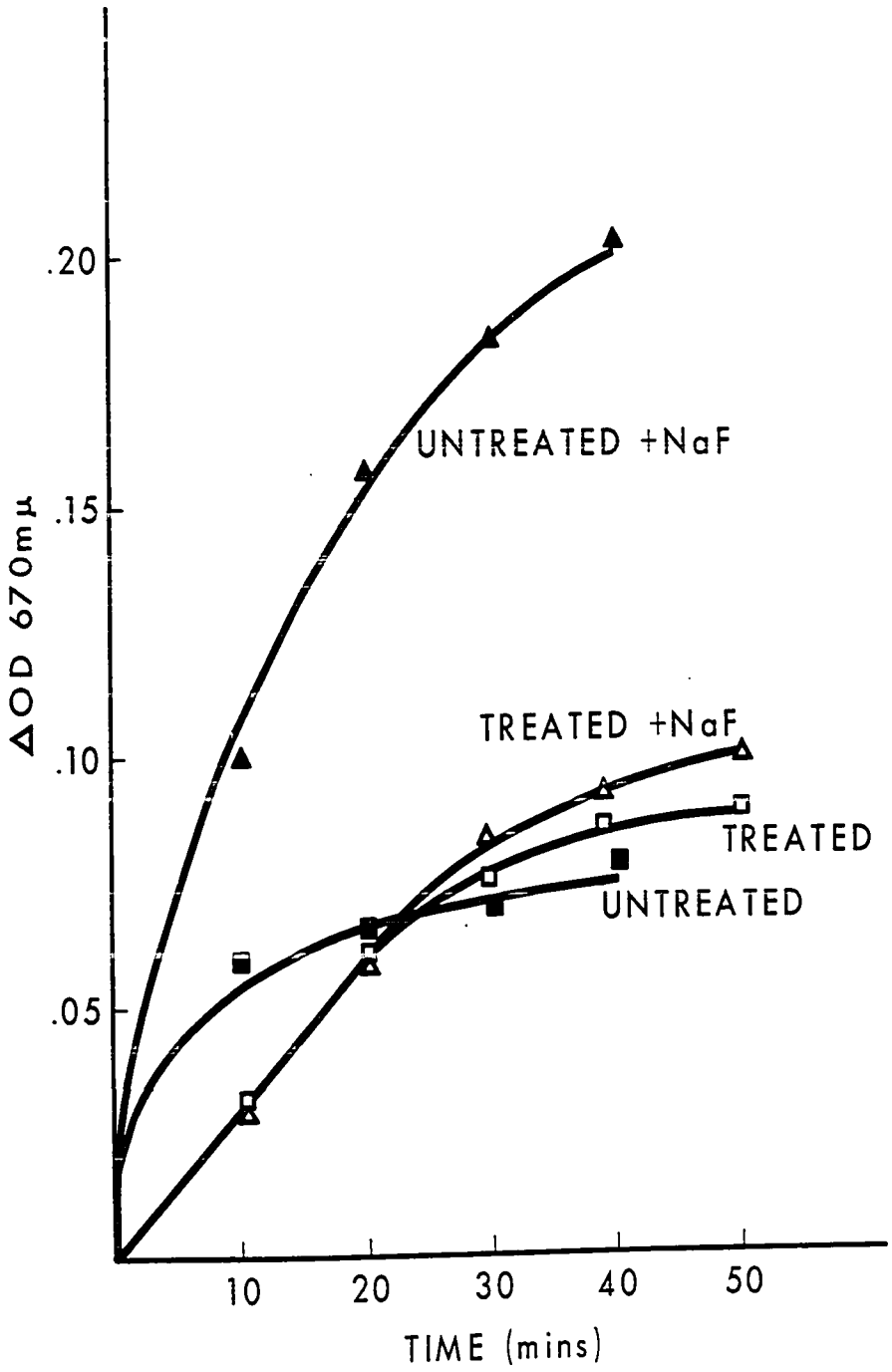
[24] The total $[Mn^{++}]$ in all solutions was too small for accurate determination by atomic absorption techniques.

[25] Sigma advertizes a decreased level of Ca^{++} impurity in their ATP by improved purification procedure (see catalogue, p. 32, Jan. 1969).

FIGURE 16

Effect of Triton X-100 on Cyclase Response to Fluoride

Control time-course of 3', 5'-AMP synthesis in the presence of fluoride 1×10^{-2} M (▲—▲) and absence of fluoride (■—■); after Triton X-100 treatment, assay in the presence of fluoride 1×10^{-2} M (△—△) and absence of fluoride (□—□). Reagents: caffeine 3×10^{-2} M and $MgSO_4$ 3.6×10^{-3} M respectively, glygly buffer 0.06 M, pH 7.5, and proteins 2 mg/ml for crude systems and 0.3 mg/ml for solubilized systems. Temperature 25°.


.20
.15
.10
.05
ΔOD 670mμ
UNTREATED +NaF
TREATED +NaF
TREATED
UNTREATED
10
20
30
40
50
TIME (mins)

protein = 1.2 mg/ml), it reduced the ATPase activity[26] by about one third. Typical purification data are summarized in Table XII:

Table XII

Solubilization of Plasma Membrane Preparations

Expt. No.	[Caffeine]	Specific activity of crude preparation (per 20 min. period)		Specific activity of "solubilized" preparation (per 20 min. period)	
		Adenyl cyclase	ATPase	Adenyl cyclase	ATPase
1.	5×10^{-2} M	4.7×10^{-9} moles/mg	3.8×10^{-7} moles/mg	5.8×10^{-9} moles/mg	2.2×10^{-7} moles/mg
2.	7×10^{-3} M	1.8×10^{-9} moles/mg		7.2×10^{-9} moles/mg	

It should be mentioned that an apparent four-fold purification can be obtained using 7×10^{-3} M caffeine. However, if the relationship between caffeine and the observed cyclase activity for particulate enzyme is examined closely (see Figure 18), much of this "purification" is attributable to a loss of residual PDEase activity.

26 ATPase assays were done under identical incubation conditions as those for the cyclase assays, except only .3 mgs protein/ml were used.

XI. Effects of Fluoride and EGTA on Treated and Particulate Enzyme

The loss of fluoride activation on "solubilization" with Triton X-100 (as seen in Figure 16) was verified by examining the effect of fluoride on the particulate and solubilized preparations (Fig. 17). Moreover, the presence of a fluoride ion-dependent, Ca^{++} ion-independent component of cyclase activity was demonstrated. Its presence is consistent only with a partial loss of fluoride stimulated activity through the action of EGTA (as shown in Figures 13 and 14). In contrast, preincubation of the solubilized preparation with EGTA resulted in complete deactivation. It should be noted that the fluoride concentration required for optimal activation of the treated enzyme is significantly greater than that for the untreated preparation.

XII. Stability of Adenyl Cyclase

As previously documented (6), cyclase was found to be a fairly unstable enzyme* in the crude state. However, the addition of the thiol reagent DTT, to the standard "wash buffer" (see page 63) increased the stability, as shown in the following table.

---

* Acceptable linearity was found over 20 minutes in the time course curves with 1.0 mg/ml proteins at 25°C (see page 78) but considerable deviations from linearity and little increase in initial activity was observed at 30° (Macdonald and Belleau, unpublished observations).

FIGURE 17

Effects of EGTA, and Fluoride on Particulate and Treated Preparations

Cyclase activity, varying the fluoride concentration was studied on particulate preparation (proteins 1.0 mg/ml) in the presence of 5×10^{-5} M EGTA (■—■) and in the absence of EGTA (▲—▲) and on "solubilized" preparations (proteins 1.1 mg/ml) in the presence of 5×10^{-5} M EGTA (●—●) and the absence of EGTA (○—○). Reagents: $MgSO_4$ 3.6×10^{-3} M, ATP 5×10^{-4} M, caffeine 5×10^{-2} M, glycylglycine.06 M, pH 7.5, treated and untreated preparations and sodium fluoride. All reaction mixtures were incubated 20 minutes at 25°C.

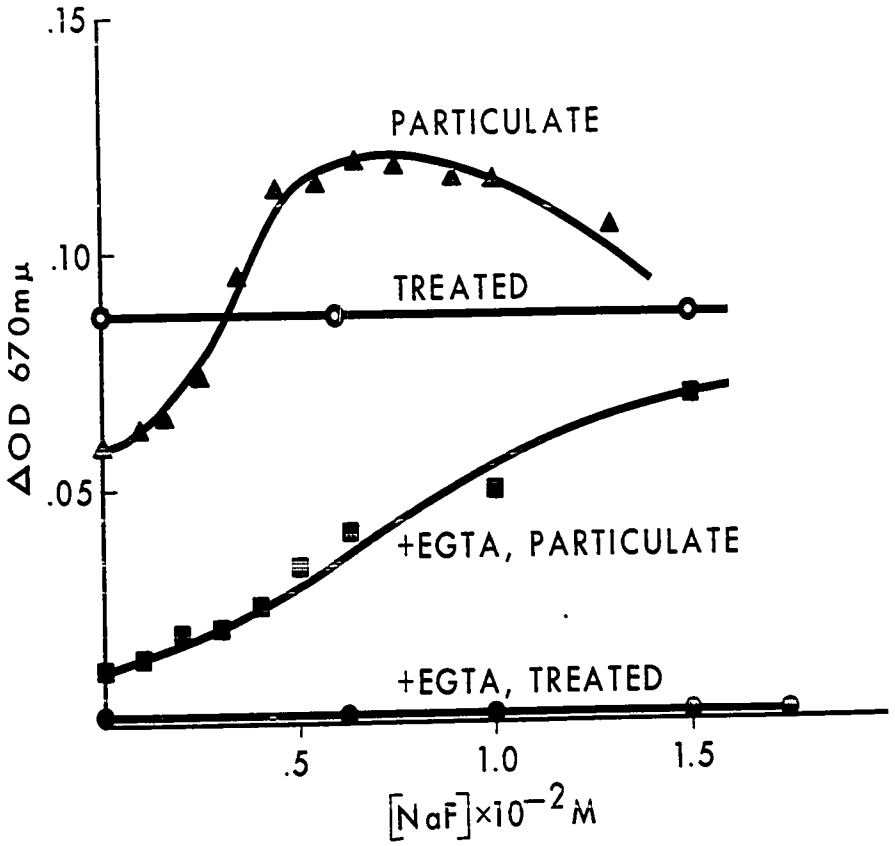

.15
.10
.05
ΔOD 670mμ
PARTICULATE
TREATED
+EGTA, PARTICULATE
+EGTA, TREATED
.5
1.0
1.5
[NaF]×10⁻² M

Table XIII

Stability Studies of Cyclase

Expt. no.	Preparation	[DTT] in preparation	Storage period at -20°C required for a 25% loss in activity
1.	Particulate	-	3-3 1/2 weeks
2.	Particulate	2×10^{-4} M	4-4 1/2 weeks
3.	Particulate	2×10^{-3} M[27]	6-7 weeks
4.	"Solubilized" with Triton X-100	-	5-6 days

XIII. Effects of other Ions, Chelators, Biogenic Amines, Adrenergic Blocking Agents, Polypeptide Hormones, Prostaglandins, Sulphydryl Reagents and Methyl Xanthines

The effects of various reagents on adenyl cyclase activity (conditions of reaction as on page 64 of Methods section) have been outlined in Table XIV, page 97 .

[27] Peculiar changes observed during the kinetic studies on ATP and fluoride effects were sometimes observed when cyclase preparations were stored in the presence of high concentrations of DTT. Activation effects by a downward shift in K_m for ATP as substrate and a biphasic fluoride response were reproducible within a single preparation but not from one preparation to the other. The observation of such "artifacts" discouraged the use of DTT for stabilization of activity during storage and purification.

Table XIV

The Effect on Adenyl Cyclase of Various Reagents

No.	Preincubation time and temp.	Conc.	Reagent	% Inhibition $(\frac{Vi}{Vc} \times 100)$ ± 5%
1.	-	control	-	0%
2.	-	10^{-4} M	$CaCl_2$	40%
3.	-	10^{-4} M	$ZnCl_2$	42%
4.	-	10^{-2} M	KCl	12%
5.	-	10^{-4} M	TPB *	5%
6.	-	10^{-3} M	TPB *	51%
7.	10 min. at 25°C	10^{-4} M	EDTA *	0%
8.	5 min. at 25°C	10^{-4} M	HQ *	19%
9.	5 min. at 25°C	$2x10^{-5}$M	HQ	.35%
10.	-	$2x10^{-5}$M	l-epinephrine	3%
11.	-	10^{-4} M	l-epinephrine	5%
12.	-	10^{-4} M	5-HT *	0%
13.	-	10^{-4} M	butoxamine	0%
14.	-	$2x10^{-5}$M	PGE_1 *	0%
15.	-	$1x10^{-5}$M	ACTH *	0%
16.	-	$1x10^{-5}$M	glucagon	0%
17.	-	10^{-4} M	DTT *	0%
18.	10 min. at25°C	10^{-4} M	pCMB *	85%
19.	2 min. at 25°C	10^{-4} M	n-pentyl-DPA *	80%
20.	3 h at 0°C	10^{-4} M	phenoxybenz-amine	70%
21.	10 min. at 25°C	10^{-4} M	EEDQ *	0%

* For abbreviations see Page xiv.

FIGURE 18

Apparent Effects of Caffeine and Theophylline on Cyclase Activity as a Function of Inhibitor Concentrations

Reagents: ATP 5×10^{-4} M, glygly buffer 0.06 M, pH 7.5, $MgSO_4$ 3.6×10^{-3} M, high protein concentration of 2 mg/ml; (■—■) with fluoride 1×10^{-2} M and (●—●) without fluoride for caffeine; and (□—□) with fluoride 1×10^{-2} M and (○—○) without fluoride for theophylline. Incubation for 20 minutes at 25°.

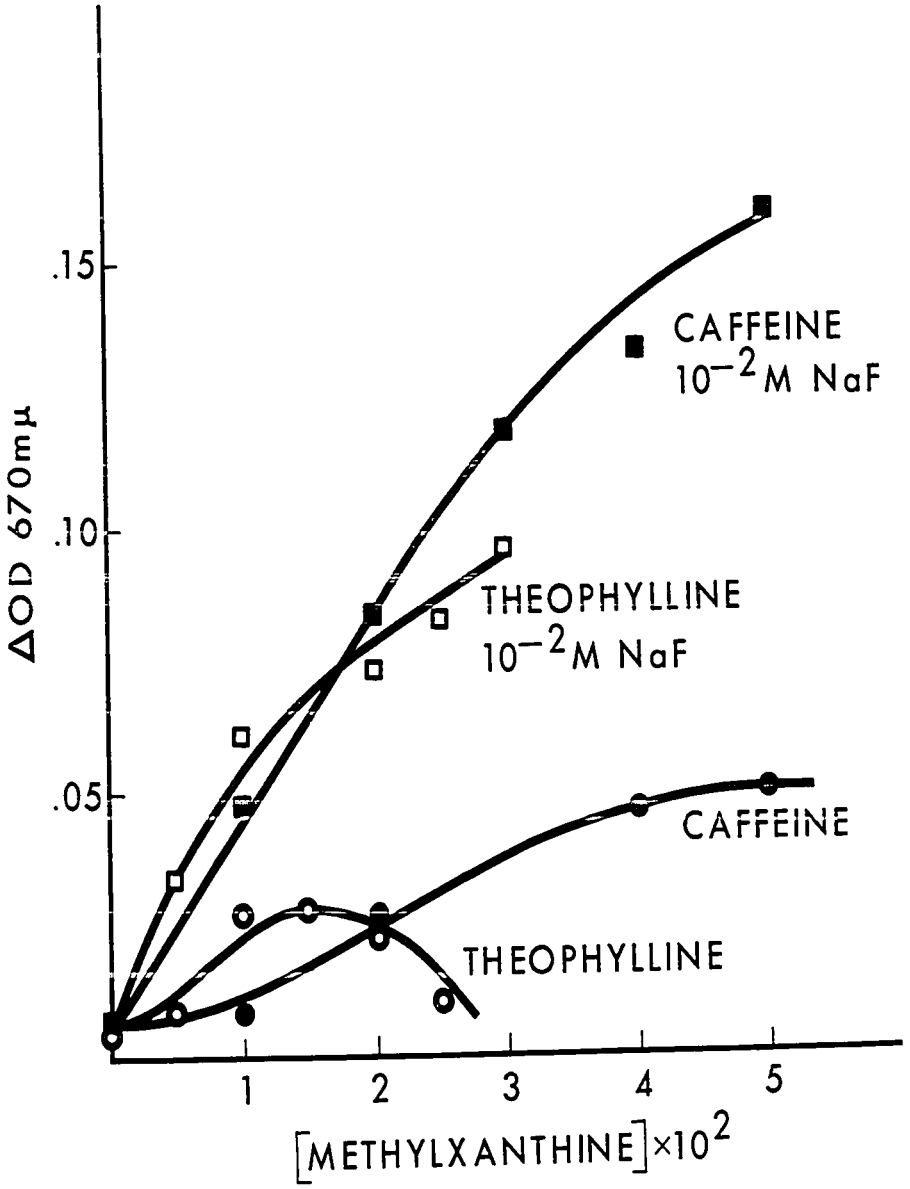
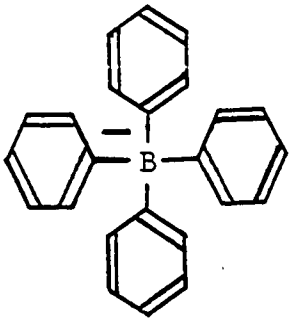
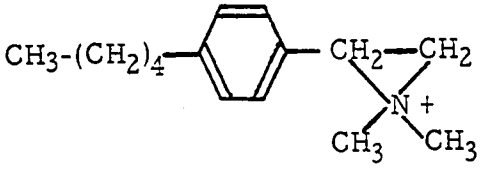

.15
.10
.05
ΔOD 670mμ
CAFFEINE
10⁻²M NaF
THEOPHYLLINE
10⁻²M NaF
CAFFEINE
THEOPHYLLINE
1
2
3
4
5
[METHYLXANTHINE]×10²

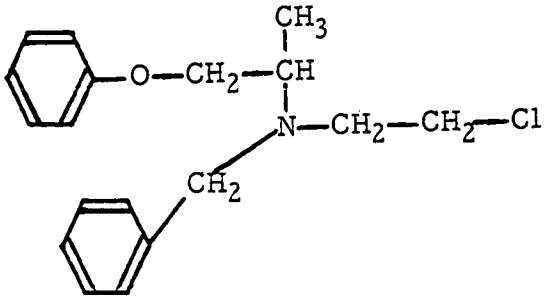
Figure 19. Structures of some Compounds tested on Adenyl Cyclase



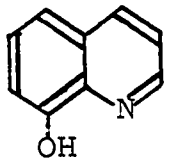
Tetraphenylboron (TPB) (Nos. 5 and 6)



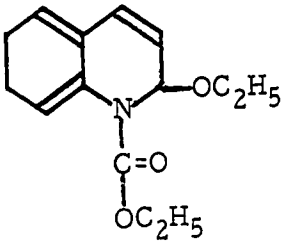
p-n-Pentyldimethylphenylazi ridinium (Pentyl DPA) (No. 19)



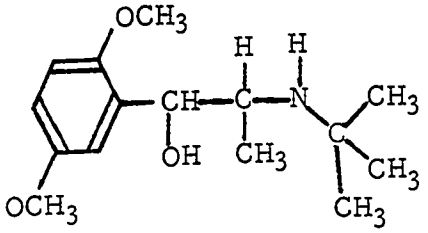
Phenoxybenzamine[28] (No. 20)



8-Hydroxyquinaline (HQ) (Nos. 8 and 9)



EEDQ (No. 21)



Butoxamine[28] (No. 13)

[28] The name of this compound in common use is the commercial name (not I.U.P.A.C.).

As can be seen, the potassium ion was without effect on cyclase (although it may exert an indirect effect by stimulating the K^+, Na^+, Mg^{++}-dependent ATPase activity). Zinc and calcium at 10^{-4} M were strongly inhibitory as previously noted [see Table Sodium tetraphenyl boron (TPB), a potassium chelating agent and known ATPase inhibitor (252) decreased cyclase activity by 50% at 10^{-3} M but was without effect at 10^{-4} M. Significantly, l-epinephrine at 10^{-4} M, 5-hydroxytryptamine at 10^{-4} M, prostaglandin E, at 2×10^{-5} M and the β blocking agent, butoxamine at 10^{-4} M were without effect on cyclase either in the absence of presence of fluoride. Even the first 600 g sediment from freshly disrupted cells was not responsive to epinephrine, regardless of incubation time, protein or substrate concentrations. Polypeptide hormones such as glucagon and ACTH were also ineffective at 10^{-5} M.

The presence of 8-hydroxyquinoline (HQ), a Mg^{++} and Zn^{++} chelating agent (253) did not alter the epinephrine response as previously suggested (254). Surprisingly this chelator was a more effective inhibitor than EDTA, but not as effective as EGTA (see Figures 13 and 17). Sulphydryl group reagents such as pCMB* and pentyl-DPA* were very strongly inhibitory suggesting an absolute requirement of free thiol groups for enzyme action. This is consistent with the stabilizing effects of DTT described above and elsewhere (50, 81). The irreversible α blocking agent phenoxybenzamine (219) effectively inhibited, but required a longer preincubation period than pCMB and pentyl-DPA for similar results. The effect of the new potent α blocker EEDQ* (255, 256) at 10^{-4} M was negligible after a 10 minute preincubation with adenyl cyclase at a pH of 7.5 (Table XIV) as with ATPase (246) and PDEase (Table XVI).

* for Abbreviations see page xiv

Methyl xanthines, as indicated in Figure 18, gave an apparent stimulatory effect on cyclase activity. As is well documented, and indicated in Figures 22 and 23, this effect is caused by the inhibition of 3', 5'-AMP phosphodiesterases. A high concentration of caffeine was generally used in all cyclase studies. Theophylline being less soluble, was not generally used for cyclase studies since it appears to cause some inhibition at 2×10^{-2} M in the absence of fluoride.

PART "C". 3',5'-AMP HYDROLYSIS AND ITS INHIBITORS

Using the same assay method, a time-course of phosphodiesterase catalyzed hydrolysis of 3',5'-AMP by the above enzyme preparation could be readily followed as illustrated in Figure 20. Also, it will be noted that fluoride at 10^{-2} M was without effect on the enzyme. On the other hand, we have confirmed earlier reports (10, 33) that caffeine and theophylline inhibit the enzyme. At 3×10^{-2} M, the former protected 3',5'-AMP against hydrolysis to the extent of 70% over a 20-minute period at a protein concentration of 1.0 mg/ml in the incubation mixture.

I. Saturation Curve for 3',5'-AMP Phosphodiesterase

Evidence was recently reported for the presence of more than one type of phosphodiesterase in rat brain (86). Our observation of a strong inflection in the 3',5'-AMP saturation curve for the bovine brain phosphodiesterase (Figure 21) does not contradict this hypothesis. However, single enzymes displaying an intermediate plateau in their substrate saturation curves have recently been documented by Teipel and Koshland (257). An explanation for the inflection must await investigations with the purified enzyme. For a comparative study of inhibitors only the "low Km" region of the saturation curve (Figure 21) was used in the computation of the data. Relative inhibitory potencies were established in terms of relative abilities to prevent hydrolysis of 3',5'-AMP at 5×10^{-5} M (low Km region) by 1.0 mg/ml protein reaction

mixture over a 12-minute interval (Figure 22). For purine derivatives the following order of potencies was obtained: 2,6-dibromopurine > caffeine > theophylline > dichloropurine>adenine (Figure 22).

II. Relative Strengths of some Potential PDEase Inhibitors

Phosphodiesterase was inhibited also by other purine derivatives, particularly those halogenated at the 6 position - see Table XV. However, 6-chloro- and 6-bromo purine were not as effective as the 2,6-dihalogenated analogs. The substitution of an amino group at the 2- position appears to reduce the effectiveness of these compounds as inhibitors. For example, adenine (6-aminopurine) was a superior inhibitor to 2,6-diaminopurine and 2-aminopurine did not inhibit; 2-amino-6-chloropurine was inferior to 6-chloropurine. Substitution of mercapto groups for the halogens at the 2 and 6 positions markedly decreases the degree of inhibition. Halogenation at the 8-position of 2-chloropurine (on the 5 membered ring) leads to the complete loss of effectiveness as an inhibitor.

Xanthine[29] was without significant effect on phosphodiesterase, and the same applies to members of the pyrimidine series such as cytidine and uracil at concentrations of 2×10^{-3} M.

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[29] Poor solubility limited the study of some of these derivatives as PDEase inhibitors and negated the possibility of their use in adenyl cyclase studies.

FIGURE 20

Effects of [Protein], Caffeine, and Epinephrine on Phosphodiesterase

Time-course of 3', 5'-AMP hydrolysis in the presence of caffeine at 3×10^{-2} M (O——O) and in its absence (□——□); also in the presence of epinephrine 5×10^{-5} M without caffeine and at [protein] = 1 mg/ml (▲——▲) or at [protein] = 0.25 mg/ml (●——●), or [protein] = 0.12 mg/ml (■——■). Reagents: 3', 5'-AMP 5×10^{-5} M (for 1 mg protein/ml) or 3', 5'-AMP 6×10^{-5} M (for 0.12 mg protein/ml); $MgSO_4$ 3.6×10^{-3} M, glygly 0.06 M, pH 7.5; incubations at 25°.

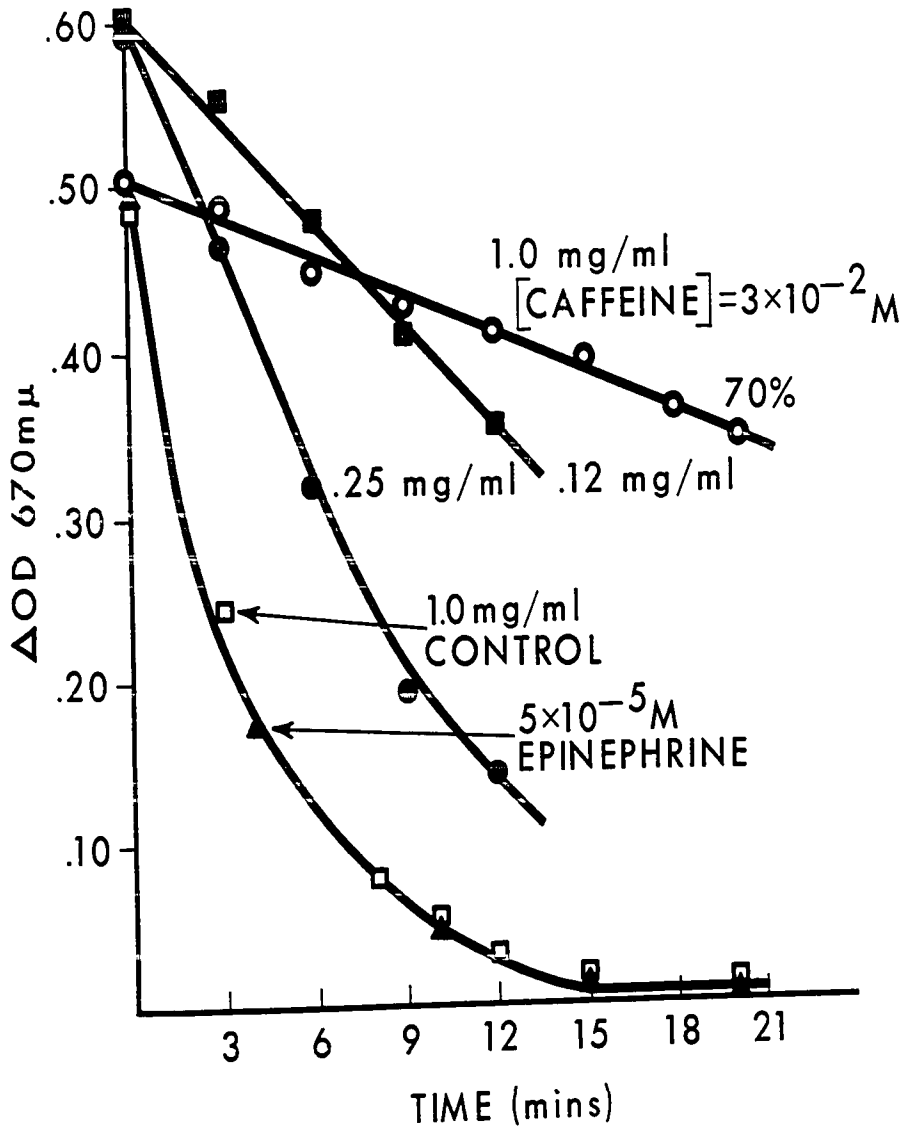

.60
.50
.40
.30
.20
.10
ΔOD 670mμ
1.0 mg/ml
[CAFFEINE]=3×10⁻²M
70%
.25 mg/ml
.12 mg/ml
1.0mg/ml
CONTROL
5×10⁻⁵M
EPINEPHRINE
3
6
9
12
15
18
21
TIME (mins)

FIGURE 21

Substrate Saturation Curve for Phosphodiesterase

Reagents: $MgSO_4$ 3.6×10^{-3} M, glygly 0.06 M, pH 7.5, [protein] = 0.12 mg/ml; assay after 6 minutes incubation at 25°.

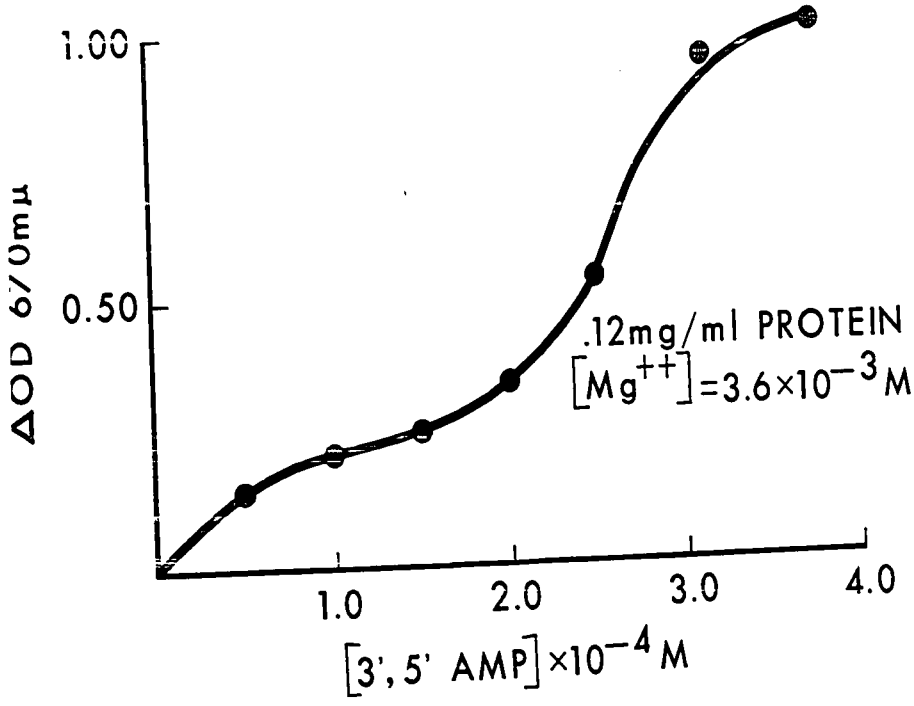

1.00
0.50
ΔOD 670mμ
.12mg/ml PROTEIN
[Mg++]=3.6×10−3M
1.0
2.0
3.0
4.0
[3', 5' AMP]×10−4M

FIGURE 22

Phosphodiesterase Inhibition by Purines

Relative potencies of adenine (△——△), theophylline (○——○), caffeine (▲——▲), and 2,6-dibromopurine (□——□) and 2,6-dichloropurine (■——■) as inhibitors of 3',5'-AMP hydrolysis at 5×10^{-5} M and at [protein] = 1 mg/ml. Reagents: $MgSO_4$ 3.6×10^{-3} M, glygly 0.06 M, pH 7.5. Incubation for 12 minutes at 25°.

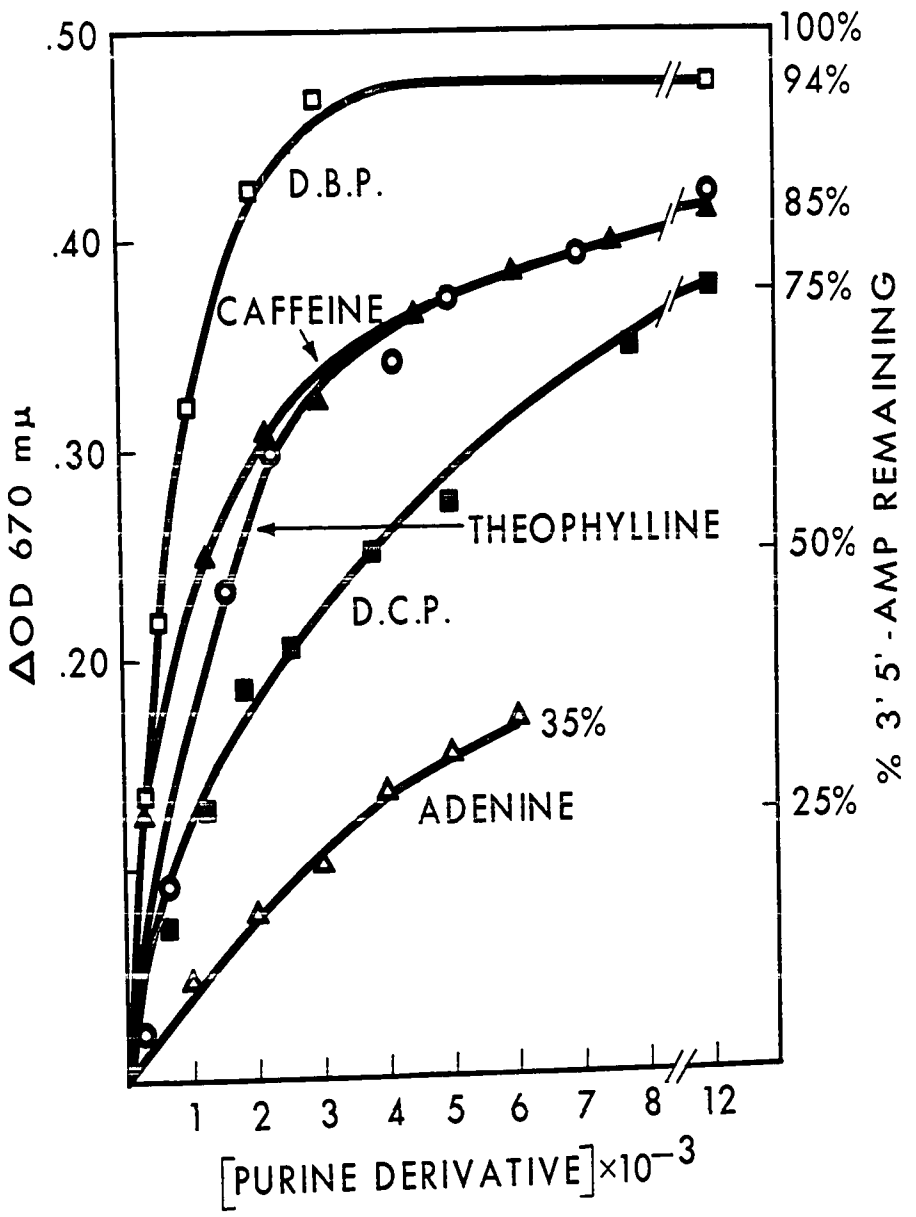
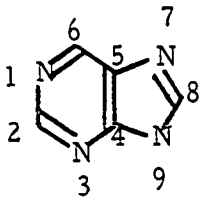

.50
.40
.30
.20
ΔOD 670 mμ
100%
94%
85%
75%
50%
25%
% 3' 5'-AMP REMAINING
D.B.P.
CAFFEINE
THEOPHYLLINE
D.C.P.
35%
ADENINE
1 2 3 4 5 6 7 8 12
[PURINE DERIVATIVE]×10⁻³

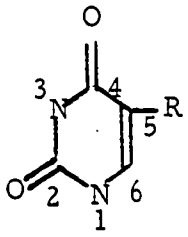
Table XV

Relative Inhibitory Effects of Purine Derivatives, Xanthines and Pyrimidines on PDEase **

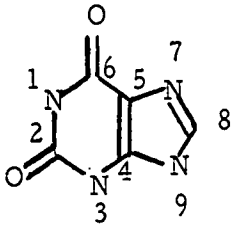
Purine



Pyrimidine



Xanthine (2, 6-dioxypurine)



R = CH_3 Thymine

R = H Uracil

No.	Conc.	Reagent	% Inhibition ($\frac{Vi}{Vc}$ x 100)
1.	-	Control	0%
2.	10^{-2} M	Caffeine (1, 3, 7-trimethylxanthine)	75%
3.	$5x10^{-2}$ M	Caffeine	92%
4.	10^{-2} M	Theophylline (1, 3-dimethylxanthine)	75%
5.	$2.5x10^{-3}$ M	2, 6-Dimercaptopurine	6.7%
6.	$2.5x10^{-3}$ M	2-Amino-6-methylmercaptopurine	10%
7.	$1.35x10^{-3}$ M	2-Amino-6-chloropurine	34%
8.	10^{-3} M	Kinetin (N^6-furfuryladenine)	7.0%
9.	10^{-2} M	2, 6-Dichloropurine (DCP)	72%
10.	10^{-2} M	2, 6-Dibromopurine	95%

.....ctd.

** All reactions were conducted under identical conditions as those for Fig. 22

Table XV. (ctd.)

No.	Conc.	Reagent	% Inhibition
11.	$2x10^{-3}$ M	2-Aminopurine	0%
12.	$5x10^{-4}$ M	2, 8-Dichloroadenine	0%
13.	$5x10^{-4}$ M	2, 4-Dichloro-1-naphthol	2%
14.	10^{-2} M	6-Chloropurine	58%
15.	10^{-2} M	6-Bromopurine	58%
16.	$1.5x10^{-3}$ M	6-Iodopurine	41%
17.	10^{-2} M	2, 6-Diaminopurine	20%
18.	$6x10^{-3}$ M	Adenine (6-aminopurine)	35%
19.	$1.2x10^{-4}$ M	Xanthine	3%
20.	$2x10^{-3}$ M	Cytosine (2-oxy-4-aminopyrimidine)	1%
21.	$2x10^{-3}$ M	Uracil	0%

Other reagents tested on adenyl cyclase gave no significant effect on phosphodiesterase as indicated in Table XVI. Only at very high concentrations, well above those required for the optimal cyclase stimulation, did any significant PDEase inhibition take place.

Table XVI

Effects of Other Reagents on PDEase **

No.	Conc.	Reagents	% Inhibition
1.	10^{-5}M, 10^{-4}M	Epinephrine	0%
2.	10^{-4} M	Butoxamine	0%
3.	$2x10^{-5}$ M	PG_2 *	0%
4.	$2x10^{-4}$ M	DTT *	9%
5.	10^{-2} M	NaF	5%
6.	$5x10^{-2}$ M	NaF	35%
7.	10^{-4} M	Ouabain	7%
8.	10^{-4} M	MSF * (preincubated 10 min. at 25°C)	0%
9.	10^{-4} M	EEDQ * (preincubated 20 min. at 25°C)	0%

* for abbreviations see page xiv

** all reactions were conducted under identical conditions as those for Fig. 22

III. Caffeine as an Inhibitor

A Dixon plot for caffeine inhibition of the "low Km enzyme" (Figure 21) showed the mechanism to be of the competition type (Figure 23), in agreement with earlier observations (10, 33). It was also found that the effectiveness of caffeine as an inhibitor was markedly dependent on protein concentration (from 1.1 mg/ml to 2.2 mg/ml) caused a six-fold increase in caffeine requirement for an equal degree of inhibition (Figure 24). It was evident also that a further reduction of the protein concentration decreased the caffeine requirement for an equal degree of inhibition. Hence the Km value obtained appears to be largely dependent on particulate protein concentration.

IV. Chloroacetyladenine as an Inhibitor

N^6-chloroacetyladenine (CAA) was tested as a PDEase inhibitor as a function of preincubation time in the presence of washed particles. All ingredients except 3',5'-AMP ($MgSO_4$ = 3.6×10^{-3} M, glygly = .06 M, pH 7.5 [CAA] = 10^{-4} M and [proteins] - .12 mg/ml) were preincubated at either 25°C or 0°C and the enzymatic reaction at 25°C was initiated by the addition of 5×10^{-5} M 3',5'-AMP and stopped after 6 minutes. Approximately 50% inhibition of 3',5'-AMP degradation (comparable to the effect of caffeine under the same conditions) was found. However, this inhibition was not a function of preincubation time (up to 15 minutes at 25°C or at 0°C). Longer periods of preincubation decreased the percentage inhibition considerably.

FIGURE 23

Dixon Plot for Caffeine Inhibition of Phosphodiesterase

Reagents: 3', 5'-AMP 5×10^{-5} M (●——●) or 1.2×10^{-4} M (▲——▲), glygly 0.06 M, pH 7.5, $MgSO_4$ 3.6×10^{-4} M, [protein] = 0.12 mg/ml. Incubation for 12 minutes (linear part of initial velocities) at 25°.

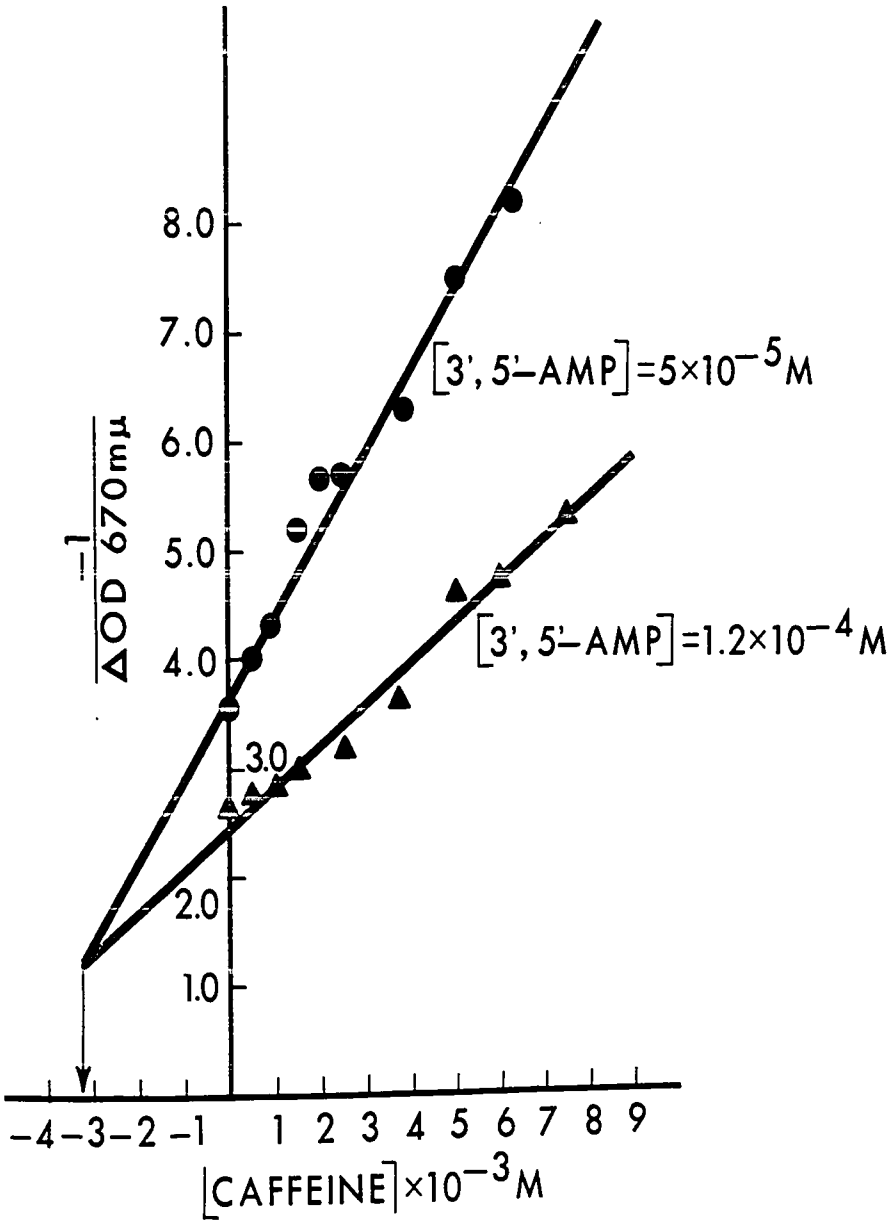
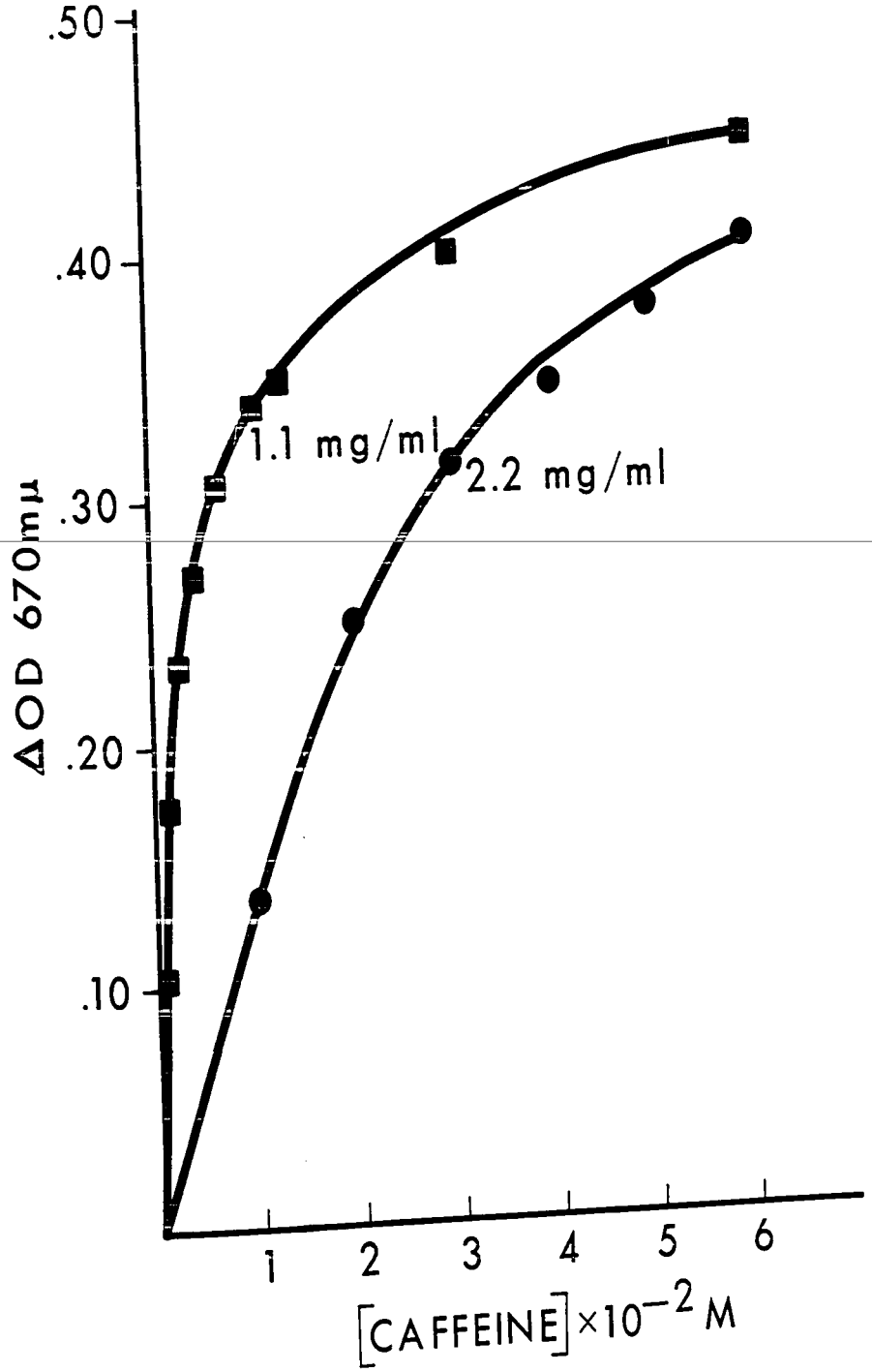

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
$\frac{1}{\Delta OD\ 670m\mu}$
[3', 5'–AMP]=5×10⁻⁵M
[3', 5'–AMP]=1.2×10⁻⁴M
–4 –3 –2 –1 1 2 3 4 5 6 7 8 9
[CAFFEINE]×10⁻³M

FIGURE 24

Caffeine Inhibition of Phosphodiesterase as a Function of Protein Concentration

The inhibition of 3', 5'-AMP-phosphodiesterase is expressed as a function of the concentration of caffeine, using a protein concentration of 2.2 mg/ml (●—●) and 1.1 mg/ml (■—■). Reagents: 3', 5'-AMP 5×10^{-5} M, glygly buffer 0.06 M, pH 7.5, and $MgSO_4$ 3.6×10^{-3} M. Incubations for 20 minutes at 25°.


.50
.40
.30
.20
.10
ΔOD 670mμ
1.1 mg/ml
2.2 mg/ml
1
2
3
4
5
6
[CAFFEINE]×10⁻² M

In addition to a lack of indication of any component of irreversible inhibition, 10^{-4} M CAA was ineffective at larger concentrations of protein (1.0 mg/ml) (see Figure 22). The usable concentration range was limited by the solubility of the inhibitor.

PART "D". STABILITY OF 3',5'-GMP UNDER ASSAY CONDITIONS

The scope of the assay method was evaluated by testing other cyclic nucleotides such as 3',5'-GMP. In contrast to 3',5'-AMP, a high percentage of 3',5'-GMP was found to hydrolyze spontaneously in the absence of any enzymatic preparation (see Table XVII).

The hydrolysis appears to take place during the periodate oxidation period and appears to be rapid initially. Thus the anomalous instability of 3',5'-GMP under these conditions limits the phosphodiesterase assay primarily to the enzymatic hydrolysis of 3',5'-AMP.

Table XVII

Non-Enzymatic Hydrolysis of 3', 5'-GMP[30]

Cyclic nucleotide	Conc.	Time of periodate oxidation (40°C) (hrs)	% Cyclic nucleotide remaining after oxidation
3', 5'-AMP	5×10^{-5} M	0	100
3', 5'-GMP	5×10^{-5} M	0	100
3', 5'-AMP	5×10^{-5} M	18	100
3', 5'-GMP	5×10^{-5} M	18	19
3', 5'-AMP	5×10^{-5} M	40	100
3', 5'-GMP	5×10^{-5} M	40	16
3', 5'-GMP	2.5×10^{-5} M	40	12

30 The purity of 3', 5'-GMP was kindly verified by A. D'Iorio and D. M. Horwood according to their own assay, based on the thin layer chromatographic procedure of Randerath (250) and using radioactive substrate.

DISCUSSION

PART "A". THE ASSAY

I. Chemical Parameters of the Assay

The convenience and expediency of the new assay can be illustrated by the fact that as many as 36 incubation mixtures could readily be assayed within 2 days. The prescribed conditions for periodate oxidation invariably led to complete degradation of non-cyclic nucleotides. It is not unlikely that over-oxidation occurred (258), but the subsequent treatment with borohydride completely abolished the orcinol sensitivity of the aldehydes and other by-products. Borohydride treatment was also essential to eliminate interference by the transformation products of periodate which were found to oxidize orcinol to substances giving high blanks[31]. However, borohydride converted all of the periodate (10-fold excess) and iodate to iodide which by itself interfered with color development. The mechanism of this interference is unknown. This problem was finally solved by quantitative precipitation of the iodide with excess lead acetate (which by itself does not interfere with the orcinol reaction[32]).

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[31] No other reducing agent was found to replace borohydride. Excess glycol had insufficient reducing power, leaving iodate which interefered in a similar manner as the periodate. Sodium thiosulphite, though capable of reducing oxidized forms of iodide, strongly interfered with the color development.

[32] Soluble silver salts did not substitute for lead acetate, as they did not yield "cleanly precipitating" iodides. Success was attained in stoichiometric reduction of the periodate to iodine with borohydride and extraction of the iodine with chloroform from acidified medium before color development. However this method was inconvenient and subject to "over" or "under" reduction (therefore subsequent incomplete iodine removal). Hence the O.D. readings for standards did not reproduce from day to day.

II. Sensitivity

As can be ascertained on the basis of the standard curve (Figure 4), the limits of sensitivity of the assay are of the order of 10^{-6} M 3',5'-AMP. This falls short of the sensitivity of the coupled assay systems (Table I, page 14-16) and most of the chromatographic assays (Table II, page 19 - 23). However, it is comparable in sensitivity to the widely used spectrophotometric-chromatographic assay of Bradham and Woolley (72) Table II, page 19). Their method, like ours, is used primarily for brain adenyl cyclase and is limited in sensitivity by the extinction coefficient of 3',5'-AMP at 260 mμ, a situation comparable to the limitations imposed by the orcinol generated green chromophore absorbing at 670 mμ.

It should be mentioned that while the sensitivity of any method depends on the blank or control values,it is mainly the reproducibility of the blanks within a set of experiments that is really important. The percentage yield of chromophore will also influence the accuracy. The very low value of the blanks, the very small standard deviations, the high yield of chromophore under carefully controlled conditions, have allowed measurements at low optical density values with precision and reproducibility.

The sensitivity of the orcinol-method is comparable to that of the chromatropic acid (259) and Nash methods (260) (for the colorimetric measurement of formaldehyde, formic acid, glycerol, and dihydroxyacetone). Other quantitative colorimetric assays such as the Molybdate method (245, 90, 91) for inorganic

phosphate, the Ninhydrin method (261, 262) for amino acids or ammonia and the Cysteine method (263) for pentoses work best at higher limiting concentration than 10^{-6} M.

However, the very low specific activity of adenyl cyclase preparation from most tissues, limits the use of the periodate-orcinol method (in its present form) to the measurement of activity in different regions of the brain or in purified preparations [for example, from E. coli (47), F. hepatica (172), thyroid (48) and frog erythrocytes (50)]. What this assay lacks in sensitivity, however, is compensated for by simplicity, expedience and economy.

III. Specificity and Interfering Materials

It is clear that RNA-like and DNA-like structures should be absent from the assay medium. This did not constitute a problem in the case at hand, since they were removed by centrifugation prior to periodate treatment. It was anticipated that the same procedure could be extended to the determination of nucleotide analogs of 3', 5'-AMP. However, 3', 5'-GMP proved unstable, even under mild oxidation conditions[33]; 3', 5'-IMP may be more likely to survive the assay conditions (although this has not been tested experimentally). Other

[33] This apparently intrinsic stability difference between 3', 5'-AMP and 3', 5'-GMP leads to the possibility of assaying for 3', 5'-AMP in the presence of 3', 5'-GMP (present as an effector, or stabilizer) under conditions of complete degradation of the latter without significant loss of the former (see Table XVII).

cyclic nucleotides such as 2', 3'-AMP and analogs are likely to be unstable under these conditions (264). Analogs of 3', 5'-AMP or ATP which are either substituted or devoid of a hydroxyl at the 2' position (for example, diBu3', 5'-AMP and dATP) interfere by virtue of their resistance to periodate oxidation.

The orcinol test suffers serious interference by strong oxidizing and reducing reagents. However, catecholamines, fluoride, Mg^{++}, Ca^{++}, Zn^{++}, methylated xanthines, purines, pyrimidines, and a variety of other common enzyme modifiers in the usual workable range of concentrations did not interfere. On the other hand, high concentrations of Mn^{++} and Co^{++} ($> 2 \times 10^{-4}$ M) caused interference owing to oxidation to the colored Mn^{+4}, Mn^{+7} and Co^{+4} ions by periodate[34].

Similarly, excessive amounts of DTT, epinephrine and other periodate consuming materials interfered. Large excesses of anions forming insoluble lead salts interfered, since they prevented efficient removal of iodide, itself an inhibitor of color development. Compounds of the dibenzo[a,d-cycloheptylamine] type such as imiprimine and desmethylimiprimine [well known antidepressant agents which have recently been implicated as PDEase inhibitors (41)] also interfered owing to their intrinsic ability to form green chromophores in the assay medium. Similarly, the nitrate ion prevented color development, and led to a red complex even in the absence of orcinol.

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[34] The color, per se, was not the interfering factor, but rather the excess consumption of periodate.

IV. Advantages of a Direct Chemical Assay for Adenyl Cyclase Studies

Since as many as five basic classes of assay procedures for adenyl cyclase are available (page 12 , Introduction) and over thirty-five individual modifications (Tables I and II, ref. 51 - 87), one may question the need for research directed at developing still another assay method. However, for in vitro studies, a direct colorimetric assay presents several advantages. Many of the existing methods are not free of technical problems. For example :

1) Coupled assay systems are subject to interference by the extraneous enzymes and proteins that must be added to the medium. Thus, the presence of Ca^{++}, glucagon and other unidentified materials could somehow cause activation of phosphorylase in some systems and mask the intrinsic effects of 3',5'-AMP (78). Also, various enzyme inhibitors could act at other levels than adenyl cyclase. The greater the number of enzyme links in the system, the greater the possibility of such interferences.

2) Some of the earlier coupled systems rely on standard curves that are not linear or, if so, only over a very limited range (see Table I, page 14-16).

3) Quantitative separation systems are not expedient and are subject to overloading and interference by the presence of other labeled metabolites when the 3',5'-AMP is not homogeneous[35]. The use

[35] The use of one or even two-dimensional chromatography with isopropanol:NH_3:H_2O solvent systems and columns, has been suggested as inadequate in separation 3',5'-AMP from other metabolites (75, 78). Although the use of a ^{32}P-ATP as substrate abolishes the problem of interference caused by insufficient separation of nucleosides and hypoxanthine (82), nucleoside phosphates arising from metabolism of ATP [and improperly separated] also present a problem when C^{14} ATP is used (78).

of periodate discriminates quantitatively between 3', 5'-AMP and non-cyclic nucleotides, whereas the so-called selective precipitation of no-cyclic nucleotides by zinc sulphate and barium hydroxide on which some separation assays are based (66, 67, 68, 79) (Table II, page 19 - 23 is not quantitative.

4). In many separation assays the yield of 3', 5'-AMP is far from quantitative, hence the requirement for internal (and external) labeled standards in the system.

5) Cost and technical training are minimal with our colorimetric periodate-orcinol procedure. Hence this assay is useful not only for routine kinetic studies at the research level, but for laboratory instruction to undergraduates since no involved instruments or manipulations of radio chemicals are required. Other types of assays are much more expensive and impractical for teaching purposes.

6). Although the periodate oxidation period (18 hours) is long, most paper and thin layer chromatography systems require twice as much time. However, Dowex ion-exchange columns and coupled systems are considerably more rapid.

7). The direct, chemical cyclase assay method is also readily adaptable to the determination of phosphodiesterase activities under the same conditions for the determination of 3', 5'-AMP synthesis without significant technical alterations[36].

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[36] The thin layer chromatographic technique advocated by Goldberg (80) is another example of such versatility (see Table III, page 30).

V. Potential Modifications of the Assay Procedure

1. Periodate Oxidation

The time period and temperature for periodate oxidation are essential features of the procedure. We attempted, without success, to shorten the time required for periodate oxidation. At a pH value below 6.7 and above 7.7, incomplete oxidation was invariably observed. Also, termination of the enzyme catalyzed reaction either with perchloric, trichloroacetic acid or barium hydroxide was inconvenient. At 30°C, (initial pH of 7.5) the oxidation was only 95% complete after 14 hours. At 40° or 50°C the reaction was consistently complete after 14 hours. [Higher temperatures did not increase the efficiency of the oxidation in agreement with Bobbit (258), and longer times did not alter the blanks]. Other buffers than glycylglycine did not reduce the time of oxidation[37]. The enzymatic reaction could be stopped by the addition of periodate instead of applying heat but higher blanks resulted because of the greater inefficiency in removing the nuclear fraction by centrifugation. (This procedure, however, may be adequate if more highly purified cyclase preparations were to be used).

Caffeine and theophylline appeared to be oxidizable by excess periodate. Oxidation here, however, appeared to be extremely slow compared to that of ATP so that no interference could be noticed[38].

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[37] Phosphate buffer interfered with the iodide precipitation step in the assay by forming insoluble lead phosphate (see page 119).

[38] A brown pigment, partly extractable into butyl acetate appeared as a result of this oxidation but it did not absorb at 670 mμ and thus caused no interference.

DCP and DBP were totally resistant to oxidation. When searching for more efficient PDEase inhibitors in cyclase preparations, the possible susceptibility of the molecule to periodate oxidation at active concentrations must be taken into account.

Ideally, the introduction of an oxidation catalyst to selectively increase the rate of glycol cleavage would overcome this problem and increase the efficiency of the assay. There does not appear to be any suitable substitute for the periodate ion in our assay procedure[39].

2. Improvements in sensitivity

Optical density readings may be improved several-fold through the use of narrower cuvettes and smaller volumes of butyl acetate in the final color extraction. Increase in sensitivity, here, is limited because of the proportional increase in blank readings. Although the assay in its present form appears adequate for routine kinetic studies with brain tissue, modifications leading to improved sensitivity are obviously necessary in order to extend the assay to cyclase fromother tissues.

It should be mentioned here, that the mechanism of color formation in the orcinol test is not well understood even though this test has been in use in research since its discovery by Bial seventy years ago (265, 266). The pigment is thought to consist of a tertiary

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39 Lead tetraacetate which also cleaves glycols is useful in organic solvents but not in aqueous phase.

complex of Fe^{+++} ions, orcinol (5-methylresorcinol) and furfuraldehyde (which is derived from the pentose part by dehydration and oxidation in the acid medium). It might be possible through the use of more highly conjugated analogs of orcinol to increase the sensitivity of the method. Unfortunately preliminary investigations of this possibility led to negative results.

Rogers et al (267) showed that naphtharesorcinol (1,3-dihydroxynaphthalene) can be used for the determination of ribose fluorometrically, a method whose sensitivity is several-fold greater than the usual spectrophotometric orcinol test[40].

Another example of the marked advantage of fluorometric over spectrophotometric assays can be found in the method of Goldberg (80) who could detect 3',5'-AMP at 5×10^{-8} M by fluorometry as compared to a limiting concentration of 10^{-6} M by the spectrophotometric technique of Bradham and Woolley (72) [see pages 19, 21 and 30 , Part "B", Introduction].

Other chemical manipulations may also be possible which may increase the sensitivity of an assay method. If the glycol oxidation could be stopped at the dialdehyde stage, the reactive intermediate could be selectively adsorbed as Schiff bases on an appropriate resin carrying primary amine groups and subsequently

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[40] Presently, this aspect is being investigated by Singahl and Parulekar (268) who are using our periodate-orcinol method for brain cyclase and are interested in the development of a fluorometric modificiation.

eluted by aqueous acid[41]. Fluorescent derivatives of the aldehyde groups could then be used (especially naphthalene derivatives) which could increase sensitivity by an order of magnitude.

3. ATPase and regeneration systems

Throughout our investigations of the kinetics of adenyl cyclase, efforts were made to establish conditions under which interference by ATPase (and PDEase) was minimum (see Figures 5, 6, 11 and 12). Although ATP regeneration systems are generally popular especially in combination with chromatographic methods, they remain objectionable for our purposes for two major reasons (see pages 8, 9, and 25, Introduction):

a) the amount of PEP* (10^{-2} M) required would exhaust the periodate during the oxidation step;

b) regeneration systems suffer the same disadvantages as indirect assay systems; that is, they are subject to interference by potential cyclase modifiers.

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[41] Polymeric derivatives of hydrazine, possessing free amino groups are claimed to react readily with aldehydes; reactions with dialdehydes (such as those derived from glycol cleavage) results in an extremely insoluble cross-linked derivative (269).

* for Abbreviations see page xiv.

Clearly, the use of a specific ATPase inhibitor (not yet known) or the use of an ATP analog which is a good substrate for adenyl cyclase but not susceptible to attack by ATPase are excellent alternative solutions to the problem. We have not had an opportunity to test this intriguing possibility[42].

VI. The Phosphodiesterase Assay

In 3', 5'-AMP phosphodiesterase studies, the sensitivity of the assay represents an improvement over some of the pre-existing assays (Table III, page 28) and appears useful when studying the "low K_m" enzyme (which is present in bovine and rat brains). The advantages of our method over coupled systems already noted in the case of cyclase studies are equally applicable to the PDEase[43]. The application of the periodate-orcinol method is also particularly useful when 5' nucleotidase activity is present

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[42] It has been reported that ATP analogs have been synthesized in which the last (γ) phosphate group is attached to the β phosphate (1) by a methylene bridge (270) and (2) by an amide linkage (271). Neither compound is substrate for ATPase but the latter is a substrate for adenyl cyclase (271). This latter observation offers a potential solution to the ATPase problem.

[43] PDEase preparations are activated by a non-dialyzable factor in snake venom used for the 5' nucleotidase step (272) in the assay by Butcher and Sutherland (10).

[which is generally true for plasma membrane preparations (44)]. The titrimetric PDEaseassays (based on the rate of proton release) may suffer serious interference by the presence of 5' nucleotidase activity in crude preparations. The main disadvantage of the periodate-orcinol method for PDEase studies is that substrate disappearance is being measured rather than product appearance. Hence, it is the initial velocities that are subject to the largest errors.

VII. Other Possible Applications of the Method

When the sensitivity of the method is ultimately improved, the scope of the method would be considerably increased. For instance, routine studies of hormone effects on adenyl cyclase from tissues of lower specific activity than brain could be studied both at the research and undergraduate levels. Drug interactions with either cyclase or PDEase could be inexpensively and expediently studied in the pharmaceutical industry. New drugs could then be routinely screened for their effects at these levels[44]. In clinical medicine, abnormal drug or hormone-induced response of these key enzymes could also be routinely checked.

Further modifications such as partial or complete automation of the procedure for large scale routine applications is a distinct possibility. Hence both in teaching and research, further developments and uses of this assay would promote progress and increase our knowledge in the field of control mechanisms.

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[44] Interest has been expressed by two well known drug companies in the further development of our assay for this purpose.

PART "B". KINETIC BEHAVIOR AND RESPONSE OF ADENYL CYCLASE TO MODIFIERS

The observed apparent K_m for ATP and for Mg^{++} are not exceptional.* The activating effect of fluoride on cyclase is also well-documented (28, 127, 106, 113). Using an ATP-regenerating system, Bär and Hechter (82) showed that the fluoride effect on cyclase from fat cell ghosts is a reflection of an increase in V_{max} for ATP and not of a change in K_m. Our results with cyclase from brain cortex show that both qualitative and quantitative differences in the fluoride effects occur, the enzyme response being determined by $[Mg^{++}]$, by protein concentration, and by the physical history of the enzyme preparation. None of these effects could be accounted for in terms of effects on ATPase or 3',5'-AMP phosphodiesterase. It is interesting that in preparations where little or no increase in V_{max} (by the presence of fluoride ions) was obtained at high $[Mg^{++}]$, a significant increase in V_{max} could be obtained at low $[Mg^{++}]$. Similarly, in preparations where V_{max} effects can be produced by fluoride (at high $[Mg^{++}]$), a still greater V_{max} effect can be demonstrated at low $[Mg^{++}]$.

It seems likely on that basis that Mg^{++} has a strong influence on the physical state of cyclase and that depending on Mg^{++} concentration different forms of the enzyme may be stablized. Alternately, isoenzymes or different cyclase complexes (which can be discriminated purely on the basis of the type of receptor unit adjoined to a common catalytic unit) (see Part "D" of Introduction) may be present. It seems clear then that the kinetic response of cyclase to fluoride is markedly dependent on the $[Mg^{++}]$.

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*For example the observed apparent Km for ATP on adipose cyclase was 1.2×10^{-4} M (82).

It is known that the physical history of cyclase may determine responses to various modifiers. For instance, cyclase in whole turkey erythrocytes is insensitive to fluoride, but responds when the cells are disrupted (229). In contrast, cell disruption impaired stimulation by epinephrine. Our results show that Mg^{++} is another parameter which has a strong influence on the qualitative and quantitative responses of the enzyme to fluoride. Moreover, fluoride activation is abolished after treatment of the enzyme with Triton X-100. This loss of fluoride sensitivity may be interpreted as a dissociation of a fluoride sensitive subunit from the catalytic subunit (in other words, the fluoride stimulation might be mediated through interaction with "receptor" units). However, Levey (49) succeeded in treating myocardial cyclase with the non-ionic detergent lubrol-PX while retaining fluoride sensitivity. The polydispersed forms of the Triton- or lubrol-treated cyclases may be different and the degree of lipid association with the enzyme may also determine the extent of fluoride activation. (A harsh Triton-X-100 treatment could cnnceivably cause a drastic dissociation of the lipids from the constituents). On the other hand, the loss of epinephrine sensitivity in the absence of a loss of sensitivity to fluoride after lubrol treatment (49) of myocardial cyclase[44] supports the hypothesis that fluoride may act directly at the catalytic unit level. The harsher

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[44] It should be borne in mind when considering the validity of the comparison of detergent action on brain and myocardial homogenates that only the latter appears to be hormone sensitive, hence the physical state of the enzyme before detergent treatment may differ widely.

Triton treatment would merely "damage" further the catalytic unit itself. Ultimately, complete deactivation may be achieved by deoxycholate treatment (6), perhaps by way of a more complete removal of associated lipids. In any event, these detergent effects are generally poorly understood. Our data, however, as will be noted again later, offer some support to the hypothesis that fluoride may act partly at the "receptor" level because the loss of fluoride activity after Triton X-100 treatment may well involved dissociation of the regulatory subunits controlling the cyclase responses.

It may prove interesting to test the possibility that bivalent cations such as Ca^{++}, Mn^{++}, Co^{++} and Mg^{++} may alter the action of detergents on cyclase and on its stability during storage at -20^{o} [45]. It is possible that part of the Ca^{++} effects on cyclase (see below) may be to couple the regulatory with the catalytic units.

As previously reported (6), Zn^{++} ions inhibit cyclase. The chelating agent EGTA whose affinity for Ca^{++} is about 10^6 greater than for Mg^{++} (28, 237) strongly inhibits brain cyclase, and since the inhibition is relieved by Ca^{++}, the conclusion is permissible that this cyclase preparation has requirement for Ca^{++}. Although both Mn^{++}

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[45] In agreement with the observations of Sutherland and co-workers (6) it was found that high concentrations (.06 M) of glycylglycine (pH 7.5) when added directly to particulate preparations provided some protection against deactivation during storage at -20°C. The mechanism of this protection may be due to the presence of as much as 2 to 3 x 10^{-5} M Ca^{++} impurity in .06 M glycylglycine. Glycylglycine buffer, did not however appear to prolong the brief storage life of Triton-treated preparations.

and Co^{++} and Sr^{++} can replace Ca^{++} [the affinity of EGTA for Mn^{++} is only 25 times smaller than for Ca^{++} (73, 237)], we are forced to agree with the conclusion of Bradham et al (73) that the most probable in vivo cofactor of cyclase is Ca^{++}. The actual levels of bivalent ions in tissues and the known implication of Ca^{++} in its coupled translocation with 3', 5'-AMP synthesis (234, 235, 236) tend to support this view. Moreover, the 3', 5'-AMP mediated α-amylase secretion by parathyroid glands (238) as well as the PTH mediated gluconeogenesis in rat kidney cortex (239) are both Ca^{++} dependent, thus indicating that these activities may be mediated by specific Ca^{++} dependent cyclase systems. Significantly, optimal activity is obtained over a rather narrow range of Ca^{++} concentrations (see Figure
It should be pointed out that the reason for greater fluoride activation in the presence of Mn^{++} and Co^{++} rather than Ca^{++} appears to relate to cyclase inhibition at Ca^{++} concentrations exceeding 3×10^{-5} M when fluoride is present. In other words Ca^{++} inhibition would seem to be potentiated by fluoride (Figure 14). This effect was not observed with the other divalent cations [(Mn^{++}, Co^{++} and Sr^{++}] which nevertheless can substitute for Ca^{++} ions in the cyclase preparation (Figures 14 and 15). In fact it is already known that the Mn^{++} ion is able to substitute both for Ca^{++} and Mg^{++} (6, 127) in the same system. However, Ca^{++} is generally antagonistic to Mg^{++} with most ATP requiring enzymes (273, 274). The Ca^{++} ion is unique, and distinguished from substitutes by the biphasic response of the enzyme that it can induce (see Figure 14). Thus it would appear that Ca^{++}

may be an antagonist of Mg^{++} at the active site level, yet would behave as an agonist at some other level. This biphasic response as elicited by Ca^{++} may be relevant to control mechanisms in vivo as suggested by Burkard and Gey (275) so that its substitution by Mn^{++} in vivo would have drastic consequences for the organism.

An intriguing feature of the Ca^{++} dependency of cyclase is its incomplete dependency on the same ion when fluoride is present (see Figures 13 and 14). Indeed, as shown in Figure 17 , the fluoride activated but Ca^{++} independent cyclase component requires a greater fluoride concentration in the absence of Ca^{++} than in its presence for optimum activation. It is this component which appears to be completely eliminated after Triton X-100 treatment, either in the presence or absence of Ca^{++} (Fig. 17).

These results support the concept that cyclase is under the control of discriminator or receptor units (28, 30, 105). Table XVIII was constructed on the basis of our own data which is summarized below. Thus a model, [similar to that formulated by Bar and Hechter (28) and extended by Kuo (30)] which interprets the control of cyclase by distinct "receptor" units, can be represented in tabular form as follows:

Table XVIII

A General Model interpreting observed Properties of Brain Adenyl Cyclase

No.	Receptor	Effector	Property
1.	"Fluoride"	Fluoride ions	Activation by F^- ions optimal at very high $[F^-]$ (1.5×10^{-2} M)
			Optimal F^- effect at low $[Mg^{++}]$ (6×10^{-4} M)
			Does not require Ca^{++}
			Inhibition by a $[Ca^{++}]$ greater than 3×10^{-5} M
			Complete loss of activity by Triton X-100 treatment.
2.	Calcium	Ca^{++} Sr^{++} Mn^{++} Co^{++}	Inhibition by 10^{-2} M NaF [as witnessed by plateau region of F^- variation curve (Ca^{++} present) Figure 17]
			Absolute requirement for $[Ca^{++}]$ (85% loss in activity by EGTA)
			Not as sensitive to inhibition by excess Ca^{++} ions
			Purification of this component of activity by Triton X-100 treatment.

Clearly, a "fluoride" receptor has no meaning in vivo, While fluoride activation may be simply an artifact involving a receptor protein (whose role in vivo may be relevant to the actions of catecholamines) the mechanistic significance should not be underestimated. A similar effect was demonstrated on the epinephrine-sensitive, fluoride-insensitive cyclase of intact turkey erythorcytes (229). The general ease of loss of catecholamine sensitivity in contrast to the retention of the fluoride effects after physical manipulations (such as lubrol treatment of myocardial cyclase) could be explained on the basis of a physical change in the discriminatory units rather than by total disaggregation.

This model (Table XVIII) implies that the fluoride effect is exerted at the receptor unit level rather than at the catalytic unit level and that the triton treatment would simply lead to an uncoupling of the subunits (blocking modulation of the catalytic subunit by the receptor subunit). As was mentioned earlier in the discussion, the mechanism of action of fluoride is still controversial owing to the multitude of interpretations that the data lend themselves to. Nevertheless the model offered in Table XVIII appears to reconcile much puzzling data.[46] As in the model formulated by Bar and Hechter (28)

[46] Similarly Pohl et al (276) show differential behavior of fluoride and glucagon stimulation of cyclase from liver plasma membrane in the presence of digitonin, phospholipase A, Mn^{++}, EGTA and PP_i. The large loss in glucagon stimulation and equally large increase in fluoride stimulation by phospholipase A treatment is consistent with our results and model (Table XVIII). Hence, although the in vivo significance of activation by fluoride ions is doubtful, it serves as a useful tool for studying the action of cyclase.

and extended by Kuo (30) (page 60 of Part "D", Introduction) the data are, at this stage, inadequate to chose between a system where the enzyme would consist of one catalytic unit associated with several (2 or more) receptor units or another system where identical catalytic units would be each associated with distinct regulatory units. Extensive purification of the enzyme will be essential before further progress can be made. Even at that, purification could well be detrimental to a finely structured aggregate of subunits and thus could defeat the purpose; very mild treatments (such as is the case with lubrol-PX) may present a possible solution to this difficult problem. In essence, our results do not contradict and in fact extend the observations of Bradham et al (73, 226) who worked with calf brain homogenates in the presence of sodium fluoride. On the other hand, the reported behavior of fat cells towards Ca^{++} chelation in the presence of catecholamines and glucagon (28, 30) is in direct contrast with our results. (See also, Table VII, page 60 in Part "D" of the Introduction). It should be noted, however, that former investigators of brain cyclase (73) worked with a preparation where a high phosphodiesterase activity was maintained owing to low caffeine concentrations (6.7×10^{-3} M). Moreover, the effect of EGTA in the absence of fluoride was never reported. The brain cortex enzyme appears more sensitive to Ca^{++} inhibition than that from fat cells (127). Our results support the view that the cyclase activity of membranes and Ca^{++} transport may well be linked (234, 235, 236, 277).

The requirement of Ca^{++} ions for optimal activity of a protein is not biochemically exceptional. For muscle contraction (skeletal, smooth and cardiac) Ca^{++} plays a central role (278). The neutralization by Ca^{++} of two strategically localized negative charges furnished by bound nucleotides permits actin filaments to slide over myosin units without resistance (230, 278). Calcium transport through the sarcoplasmic reticular membrane to the sites of action is an active process (278). In canine heart, hormone sensitive adenyl cyclase activity [present in the sarcoplasmic reticulum (278)] was shown to be directly related to this Ca^{++} transport, which serves to increase the sarcotubular calcium pool (236).

The effect of either Ca^{++} or 8M urea on a purified specific protein, "S100" from bovine brain leads to the formation of multiple forms of the protein (280). While only a single form was identified in the absence of Ca^{++} or urea, more unfolded structures were induced by either one of these two effectors. The mechanism may involve the exposure of several previously "buried" amino acid residues. Hence, Ca^{++} has been shown to produce regulatory effects on protein structure and function. However, these effects quoted above were not obtainable by the substitution of other divalent ions for Ca^{++} as in the brain cyclase system. Interestingly, Mg^{++} ions are replaceable by Mn^{++}, Co^{++} and Fe^{++} in mitochondrial Mg^{+}-dependent ATPase systems (273, 281). In contrast, Ca^{++} is antagonistic.

Although tetraphenylboron, a potassium chelating agent, could be shown to inhibit brain cyclase at 10^{-3} M, no requirement for potassium could be demonstrated for this enzyme. Under our assay conditions, we have not been able to demonstrate an effect of epinephrine on cyclase. This result is at variance with an earlier report by Sutherland and his associates (6), but in agreement with those of Burkard and Gey (275). The origin of this discrepancy is unknown. Significant, but not strong catecholamine effects[47] are obtainable with tissue slices from both brain cortex (115) and cerebrum (116). It seems likely that the history of the enzyme (with particular regard to its "in vitro" physical manipulations), special medium effects, or perhaps the release of various lipolytic or proteolytic "scavenger enzymes" during the course of cell disruption could serve as the basis of an explanation. The lack of catecholamine effect in the fluoride sensitive brain homogenates appears similar to the apparent lack of fluoride effects in otherwise catecholamine sensitive tissue slices (115, 227). Parallel results were obtained by Øye et al with turkey erythrocytes (229).

It is worth noting that Pohl and co-workers (283) have also been unable to demonstrate an epinephrine effect on liver plasma membranes. Marinetti et al (77) suggested that this discrepancy with the results of Sutherland and associates (20) may have its origin in

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[47] Evidence has been put forward that epinephrine in promoting release of acetyl choline from motor neurons, would stimulatecyclic AMP mediated neuromuscular transmission (282) and is consistent with the presence of an epinephrine sensitive cyclase in the central nervous system.

the different temperatures of incubation (30° and 37° respectively) utilized by the two groups. It appears unlikely to us that a 7° difference in temperatures could determine an all or nothing effect by epinephrine. On the other hand, we have shown that even protein concentration has a strong influence on the specific activity of cyclase (Figure 5)[48], which suggests that perhaps aggregation either represses catalytic activity or simply serves to bury active sites. It may be then, that the epinephrine control sites, if they exist, may be accessible only when the cells are intact. This may possibly also apply to 5-hydroxytryptamine, prostaglandin, ACTH and glucagon, all of which were also without influence on our brain cyclase preparation. It should be pointed out though that only 5HT and epinephrine had been previously shown to have any effect on brain cyclase even on tissue slices (115).

Surprisingly, however, we observed that certain adrenergic β blockers such as butoxamine may act as weak agonist on a cyclase system which had been stored in the presence of a high DTT concentration (2×10^{-3} M) (Macdonald and Belleau, unpublished observations), a phenomenon which was also recently observed with cyclase from uterus (but without DTT) (157). Yet the same brain cortex preparation was totally unresponsive to epinephrine. These results, although not rationalizable as yet, may have a bearing on the increased stability of cyclase in the presence of DTT. The physical form or state of aggregation may also depend on the arrangement of sulphydryl groups.

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[48] Caffeine inhibition of phosphodiesterase is markedly influenced by protein concentration (Fig. 24). However, enough caffeine is present in this system (Fig. 5) to produce nearly maximal inhibition of phosphodiesterase at all protein concentrations.

In summary, our results, although not amenable to interpretation in concrete molecular terms as yet, tend to confirm the models proposed by Sutherland and his associates (19, 105), Vaughan and Murad (251), Bär and Hechter (28), and Kuo (30), in which cyclase would be under the influence of regulatory subunits or discriminators endowed with tissue dependent specificities.

On the other hand, our data also suggest that cyclase itself may assume different physical forms that need not be under the control of receptor subunits. For example, similarly to our observations, the results of Ray et al (126) show that an optimum protein concentration exists for maximum activity of a liver plasma membrane cyclase preparation, thus also suggesting that an aggregation phenomenon may be involved. However, their optimum protein concentration is less than 50% of the value observed by us, a difference which may be due to the low caffeine concentrations (7×10^{-3} M) which they used.

PART "C". 3',5'-AMP PHOSPHODIESTERASE AND ITS INHIBITORS

The saturation curve for crude phosphodiesterase (Fig. 21) displays an intermediate plateau region which suggests that two or more enzymes may be present, as also appears to be the case in rat brain (86)[49]. This curve is qualitatively completely reproducible at different protein concentrations and with different preparations. Only the inflection point appears to vary slightly from one run to the other. This type of curve would also be obtained with a single enzyme possessing multiple binding sites for 3',5'-AMP according to Teipel and Koshland (257). It seems probable that enzyme oligomers may indeed be involved as could also be the case for the rat brain enzyme (86). Studies with purified phosphodiesterase fractions from bovine brain are in order since the regulatory properties of this enzyme may be relevant to the control of 3',5'-AMP levels in vivo, an aspect which until recently has been largely neglected. Caffeine inhibits the enzyme competitively as previously reported (10, 33). The observed K_i is about 10 times lower than that reported by Cheung for the soluble rat-brain enzyme (33) and 100 times lower than reported by Nair for the dog-heart enzyme (285). It is pertinent to note that caffeine inhibition is a

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[49] More recent studies by the same workers (284) indicate two major and one minor fractions of diesterase activities separable from rat brain by sonication, centrifugation and gel filtration. These diesterase appear, by molecular weight determination, to contain: one, two and eight basic subunits, the most complex form again displaying a bi-phasic saturation curve.

function of protein concentration (Fig. 24), the higher the concentration, the lower the degree of inhibition. It seems possible, particularly in the light of the work of Thompson and Appleman (284), that aggregation of the membrane-bound enzyme is responsible for the differences in sensitivity to caffeine. The aggregation of subunits may be a function of protein concentration (or the concentration of some membrane factor) thus accounting for some masking effects of the caffeine binding sites, or conceivably the substrate binding sites[50]. The structural requirements for inhibition by purines appears quite flexible. The best inhibitor that we have so far encountered is 2,6-dibromopurine, which under conditions of high protein concentration surpasses caffeine in potency by a factor of 2. It is too early, at this time, to draw any definite conclusions regarding the optimum structural requirements for inhibitory activity (see Table xv). Several structural parameters appear necessary for good PDEase inhibitory activity. They can be summarized as follows:

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[50] It was noted that, in spite of the apparent greater inhibitory ability of DBP than caffeine (Fig. 22) at higher protein concentrations (1.0 mg/ml), the K_i for DBP as determined at lower protein concentrations (.12 mg/ml) was very similar to that of caffeine ($\simeq 4 \times 10^{-4}$ M) and also appeared to be competitive (Macdonald and Belleau, unpublished observations). Thus, it is indicated that different forms of the enzyme may display differential affinities towards related molecules.

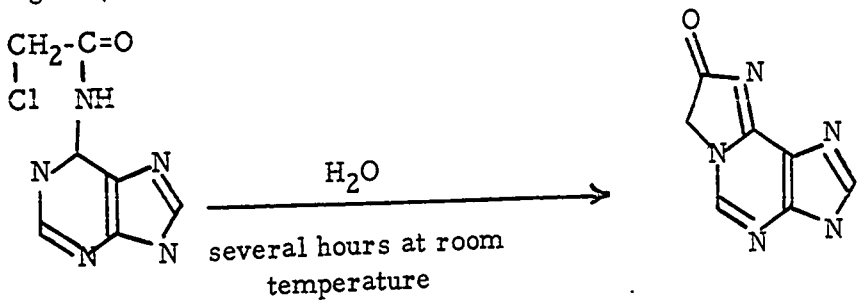
1. The two rings of the aromatic nitrogenous bases are required. (It is not known yet what the effect of enlargement of the five membered ring would be on inhibition). Analogous naphthalene and pyrimidine derivatives are ineffective.

2. Bulky substituents on the six membered ring are favorable. Xanthine itself (which lacks methyl substituents) is a very poor inhibitor. Substitution on the five membered ring at position 7 enhances activity [compare caffeine (1, 3, 7-trimethylxanthine) with theophylline (1, 3-dimethylxanthine)] while substitution at position 8 is not favorable [compare 2, 8-dichloroadenine with 2, 6-dichloropurine and 6-chloropurine].

3. Polarity of the substituents on the six membered ring, particularly at positions 2 and 6 may be of some importance. The keto groups at the equivalent positions in xanthine derivatives appear to participate in the binding process. When the electron withdrawing halogens at positions 2 and 6 of purine derivatives are replaced with groups of lesser electronegativity a large loss in biological activity is observed.

Although the purpose of our investigations on the relative inhibitory abilities of purine and xanthine derivatives on PDEase was originally intended to improve on caffeine in the cyclase system, it became evident that more will have to be known about the caffeine binding sites of PDEase before highly potent inhibitors can be designed. The ineffectiveness of EEDQ and MSF could possibly reflect the absence of both a carboxyl group (255, 256) and a serine hydroxyl group (286, 287) respectively at the active site level. Hence PDEase does not appear to fall into the general classification of a "serine hydrolase".

The fact that adenine will inhibit (albeit weakly) phosphodiesterase suggests that the levels of newly synthesized 3', 5'-AMP may be influenced by feedback inhibition of the enzyme. However, the true significance of adenine inhibition in vivo cannot be assessed at this time. Chloroacetyladenine showed excellent inhibitory activity against PDEase under conditions of low protein concentration. Because its chemical reactivity is similar to that of iodoacetate, it was anticipated that a thiol group on the enzyme surface could suffer specific irreversible alkylation. However, no component of irreversible inhibition in the crude system could be demonstrated for this compound. The apparent loss of inhibitory activity of CAA after prolonged preincubation in glycylglycine buffer (pH 7.5) can be attributed to the facile competing intramolecular cyclization which this compound readily undergoes (288):



Thus it appears that specific irreversible PDEase inhibition must await the development of other potential alkylating agents.

PART "D". SOME OBSERVATIONS CONCERNING THE HYDROLYSIS OF CYCLIC NUCLEOTIDES

The curious anomaly of the instability of 3', 5'-GMP as compared to 3', 5'-AMP under conditions of oxidation and at normal pH values does not appear to be explainable on the basis of the difference in their respective enthalpies of hydrolysis of the 3' bond. [ΔE 3', 5'-AMP = -14,100 cal/mole ; Δ E 3', 5'-GMP = -10,500 cal/mole (17)]. The amino group at position 2 of the guanine moiety of 3', 5'-GMP is in proximity to the phosphate group and thus may catalyze hydrolysis (289). Molecular models support this hypothesis. In addition, the presence of metal ions (4) such as Mg^{++} may be functional in the hydrolytic opening of the six membered ring. This may explain the apparent difference between the two cyclic nucleotides. In other words, special kinetic effects rather than differences in thermodynamic stability could be responsible. The relation of such differences to the specificities of the cyclic nucleotide-dependent histone kinases (178, 221) is conjectural.

It should be mentioned also that the free energy change attending the hydrolysis of the 3' esteratic linkage of 3', 5'-AMP is about -4000 cal/mole greater than for the terminal phosphate of ATP. Therefore, the synthesis of 3', 5'-AMP would appear to require an input of energy. This is at variance with a mechanism involving only the departure of a pyrophosphate moiety as the driving force; in other words, the cyclization process would be energy deficient if a single ATP molecule drives the reaction leading to 3', 5'-AMP (6). The same

is evidently true for 3', 5'-GMP synthesis (223). These considerations illustrate those aspects of cyclic AMP synthesis which are still poorly understood. Obviously, more research with purified systems [such as the one described by Lipmann (47)] will be required in order to clarify the apparent thermodynamic anomaly associated with ATP cyclization to cyclic AMP.

CLAIMS TO ORIGINAL RESEARCH

1. A direct chemical assay for 3', 5'-AMP was developed, based on the susceptibility of noncyclic nucleotides to periodate oxidation and the resistance of 3', 5'-AMP to periodate. Sensitivity, limitations, interfering materials and some advantages of this assay over other methods have been described.

2. The application of the assay for the study of kinetics of bovine brain adenyl cyclase resulted in :
   (a) the determination of optimal conditions of 3', 5'-AMP synthesis, minimizing the effects of the contaminating ATPase and PDEase activities;
   (b) the measurements of kinetics parameters of the substrates, ATP and Mg^{++} ;
   (c) further studies of the properties of adenyl cyclase with regard to its stability and behavior on treatment with Triton X-100, dithiothreitol and other chemical effectors.

3. A calcium dependent component of adenyl cyclase activity was demonstrated by the loss in activity on the addition of EGTA to the reaction mixture and its restoration on replacement of the chelated calcium ions.

4. The relative abilities of Mn^{++}, Co^{++} and Sr^{++} to replace Ca^{++} both in the presence and the absence of fluoride ions and the biphasic nature of the Ca^{++} response which appears to be potentiated by fluoride were demonstrated.

5. Only a partial inhibition of cyclase by EGTA resulted in the presence of fluoride, while nearly complete activity was lost in the absence of fluoride. Hence the presence of a fluoride activated but Ca^{++} independent component of cyclase activity was demonstrated.

6. A model in tabular form for bovine brain adenyl cyclase was formulated in an analogous manner to the one postulated by Bar and Hechter and further amplified by Kuo. This model supports the hypothesis that cyclase is under control of distinct "receptor" units and has rationalized a great deal of data which otherwise appears puzzling and accounts for points 2 - 5 (above).

7. The assay was also applied to the measurement of 3', 5'-AMP phosphodiesterase (PDEase) activities:

   (a) under conditions used for adenyl cyclase studies (high protein concentration and in the presence of a given concentration of PDEase inhibitor) ;

   (b) under conditions of lower protein concentration for the convenient study of the kinetics of PDEase <u>per se</u>.

8. The relative inhibitory strengths of a series of purine, xanthine and pyrimidine derivatives on PDEase were examined. Dibromopurine was shown to be the best inhibitor among the series of compounds studied. Some tentative rules concerning the structure activity relationship of the inhibitor in vitro were proposed.

9. An intermediary plateau phase in the saturation curve of PDEase similar to that analyzed in the model by Teipel and Koshland was obtained. These kinetic results are in agreement with the biphasic saturation curve found by Appleman et al for rat brain PDEase and indicates the probability of the presence of a multisubunit enzyme similar to that occurring in rat brain.

10. Caffeine inhibits bovine brain PDEase competitively in agreement with the mode of action of caffeine on both rat brain and dog heart PDEase.

11. N^6-Chloroacetyladenine (CAA) was synthesized by a modification of an earlier method and obtained in an apparently purer form than previously. CAA was demonstrated to have an inhibitory action on PDEase, at low protein concentration comparable to that of caffeine, but lacked any irreversible component as indicated by the time course curve.

12. In contrast to 3', 5'-AMP, 3', 5'-GMP appeared to be unstable under the assay conditions, approximately 80% hydrolyzing during the 18 hours required for periodate oxidation. The presence of an amino group at the 2 position of the purine moiety of 3', 5'-GMP is in proximity to the phosphate group and thus may catalyze hydrolysis. Molecular models support this hypothesis.

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