The University of Manitoba

THE SYNTHESIS AND FATE OF CYCLIC 3',5'-ADENOSINE MONOPHOSPHATE IN STREPTOCOCCUS SALIVARIUS

by

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Dedicated to

VIMLA, CHANDRA KANTA,

MANJU LATA and DEEPAK

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ABSTRACT

The synthesis of cyclic 3',5'-adenosine monophosphate (cyclic AMP) by adenyl cyclase present in the soluble fraction of Streptococcus salivarius has been demonstrated. The enzyme was purified from early stationary phase cells of the organism by differential centrifugation, DNase treatment, ammonium sulfate fractionation and gel filteration on Sephadex G-200. The enzyme was repeatedly recovered from the Sephadex G-200 column in three distinct peaks, each devoid of ATPase, GTPase and pyrophosphatase. The enzymes (I, II, III) in each peak, however, contained a small amount (2.1 - 6.7%) of guanyl cyclase activity. Enzyme III was the most homogeneous, containing only two faint contaminating bands on disc gel electrophoresis.

The enzyme was Mg^{2+} or Mn^{2+}-dependent, was stabilized by cysteine (5 and 10 mM) and was activated by NaF. While the pH optimum for enzymes II and III was 8.0, that for enzyme I was pH 7.5. The K_m values for enzymes I and II were both 1 mM for ATP, and were unaffected by 20 mM NaF, the optimal concentration for activation of the enzyme. The K_m value for enzyme III, however, was increased from 0.7 to 3.3 mM in the presence of NaF. In all cases, the V_{max} for all three enzyme preparations was increased in assays with NaF by as much as 5-fold (e.g., II and III). Hill plots for all three enzyme preparations gave slope values (n) of 1 indicating only one substrate-binding site on the enzyme; the slopes were unaffected by NaF. Sulfhydryl inhibitors, N-ethylmaleimide, and p-hydroxymercuribenzoate were almost completely inhibitory at 1 mM suggesting the involvement of -SH groups in catalysis. Cysteine (1 mM) limited inhibition by these reagents significantly at inhibitor concentrations below 0.1 mM.

ATP synthesis from cyclic AMP and pyrophosphate by adenyl cyclase was favored with K values for ATP formation ranging from 43 to 64. NaF inhibited the reverse reaction with the reduction in reaction rate most severe with enzyme III (73%). The average ΔF^{o} for the synthesis of ATP was -2.48 Kcal per mole.

The activity of adenyl cyclase III was further examined in the presence and absence of inorganic phosphate (P_i) and pyrophosphate (PP_i), nucleotides and glycolytic intermediates. The enzyme was inhibited by P_i, ADP and all the triphosphates and monophosphates of guanosine, uridine, inosine and cytidine; inhibition was completely competitive. The inhibition in the presence of PP_i was partially competitive, while AMP produced inhibition of the mixed type i.e., partially competitive and completely non-competitive. PP_i was the most potent inhibitor (K_i = 0.23 mM) followed by ADP (K_i = 0.43 mM), GTP (K_i = 0.52 mM) and UTP (K_i = 0.60 mM). Severe inhibition of the enzyme was also observed in the presence of the diphosphate and sugar nucleotides of the above bases at concentrations between 0.1 and 5 mM, when tested at one substrate concentration (ATP = 0.6 mM). The respective cyclic 3',5'-nucleoside monophosphates were, however, only slightly inhibitory (maximum 11%).

While the nucleotides were generally inhibitory, the activity of the enzyme was variable in the presence of various glycolytic intermediates. Weak activation of adenyl cyclase activity was observed with glucose-6-P, glucose-1-P, 2-P-glycerate and pyruvate. 2-P-glycerate required the lowest concentration for half maximal activation (K_a = 0.13 mM) followed by glucose-1-P (0.24 mM), glucose-6-P (0.55 mM) and pyruvate (1.12 mM). These compounds increased the V_{max} of the enzyme without affecting the K_m for

ATP. Fructose-6-P, fructose-1,6-P_2, glyceraldehyde-3-P and P-enolpyruvate inhibited or activated the enzyme depending on the concentration of the compound used. Both the apparent K_m for ATP, as well as the V_{max}, were altered by fructose-6-P and fructose-1,6-P_2. However, activating concentrations of glyceraldehyde-3-P and P-enolpyruvate increased the V_{max} without affecting the apparent K_m, whereas inhibiting concentrations decreased the V_{max} and increased the K_m for the substrate.

In an initial study on the fate of cellular cyclic AMP in non-proliferating cells of S. salivarius, the cyclic AMP content was shown to increase and decrease rapidly during the fermentation of glucose, fructose, mannose and raffinose. An extensive search with crude cell-free extracts and toluene-treated cells of S. salivarius, under a variety of conditions, failed to detect activity for cyclic nucleotide phosphodiesterase. This evidence indicated that loss of cyclic AMP from cells was not due to the degradation of this nucleotide. The cyclic AMP content of both the cells and the extracellular medium was then assayed with growing and non-proliferating cells of S. salivarius in order to determine the fate of this cyclic nucleotide. With cells growing in glucose-tryptone-yeast extract broth, the cyclic AMP content of both the cells and the exogenous medium did not change during growth. The addition of 12 mM NaF to such cells in the mid-exponential phase stimulated the synthesis and excretion of cyclic AMP into the medium without altering the content of this nucleotide in the cells. On the other hand, cyclic AMP in non-proliferating cells of S. salivarius metabolizing fructose, increased throughout the period of fructose utilization, but was rapidly excreted into the medium just after the depletion of this carbon source.

Conversely, cells metabolizing glucose showed a rapid increase in cellular cyclic AMP only after the complete utilization of this sugar. However, this cellular cyclic AMP was excreted into the medium within two minutes of the depletion of the carbon source.

Experiments were also carried out to determine the function of cyclic AMP in S. salivarius. Glycogen phosphorylase activity was inhibited by preincubating the crude extracts with ATP and Mg^{2+} and this inhibition was further augmented by the presence of cyclic AMP. The preincubation of crude extracts with cyclic AMP alone had no effect on enzyme activity. Cyclic AMP activated (2-fold) partially purified glycogen phosphorylase in the absence of AMP, but was completely ineffective in the presence of AMP. Cyclic AMP activated phosphofructokinase (2 - 6 fold) and slightly inhibited (3 - 15%) the P-enolpyruvate phosphotransferase glucose transport system in S. salivarius. These results, coupled with the effects of various cellular components on the activity of purified adenyl cyclase, indicate that cyclic AMP may play an important role in the control of glycolysis.

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ABBREVIATIONS

Cyclic AMP	cyclic 3',5'-adenosine monophosphate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid
ΔF^o	standard free energy
IPTG	isopropyl-β-D-thio-galactoside
K	equilibrium constant
K_a	activation constant
K_i	inhibition constant
K_m	Michaelis-Menten constant
nm	specific wavelength
NEM	N-ethylmaleimide
PEP-PTS	P-enolpyruvate phosphotransferase system
pHMB	p-hydroxymercuribenzoate
P_i	inorganic phosphate
PP_i	inorganic pyrophosphate
POPOP	p-bis [2-(5-phenyloxazolyl)] benzene 2,5-diphenyloxazole
Tris	Tris-(hydroxymethyl) aminomethane
TYE	tryptone-yeast extract

CHAPTER 1

INTRODUCTION

Cyclic 3',5'-adenosine monophosphate (cyclic AMP) plays a central role in cell regulation and hormone action in mammalian tissues. Various metabolic processes such as glycogen synthesis and degradation, steroidogenesis, lipolysis, gluconeogenesis, and other processes are known to be regulated by low concentrations of this cyclic nucleotide (1-6). Although less is known about the function of cyclic AMP in bacteria, the work of the Pastan-Perlman group has established its role in the regulation of inducible enzyme synthesis in Escherichia coli (7,8). Current evidence suggests that cyclic AMP, in the presence of a cyclic AMP-receptor (CR) protein and RNA polymerase, acts at the promotor site of the corresponding operon to stimulate the synthesis of such enzymes.

The intracellular concentration of cyclic AMP is regulated by the activity of two enzymes: adenyl cyclase and cyclic nucleotide phosphodiesterase. Adenyl cyclase, catalyzes the formation of cyclic AMP and pyrophosphate from adenosine triphosphate (ATP) (equation 1), while cyclic

$$\text{ATP} \longrightarrow \text{cyclic AMP} + PP_i \qquad (1)$$

nucleotide phosphodiesterase, on the other hand, hydrolyzes cyclic AMP with the formation of AMP (equation 2).

$$\text{Cyclic AMP} \longrightarrow \text{AMP} \qquad (2)$$

During previous studies on glycogen metabolism by the oral microbe, Streptococcus salivarius, the presence of cyclic AMP in intact cells of this organism was observed. Since little information was available on the synthesis and degradation of cyclic AMP in bacteria, a

study was undertaken to determine the factors influencing the cellular concentration of this nucleotide. It was hoped that this study would shed some light on the function of cyclic AMP in the oral streptococci and, in particular, whether this nucleotide might be involved in the control of glycogen metabolism in bacteria as it is in mammalian systems. To this end, the research described in this thesis has been concerned with (a) the purification and properties of adenyl cyclase from S. salivarius (Chapter 4) and (b) the fate of cyclic AMP in cells of this organism (Chapter 5). The latter chapter also contains some preliminary data on the possible function of cyclic AMP in this organism.

CHAPTER 2

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I. DISCOVERY OF CYCLIC AMP

Cyclic 3',5'-adenosine monophosphate was discovered simultaneously and independently by two groups of investigators in 1957. Sutherland and his group (9), while studying the effect of epinephrine and other sympathomimetic amines on phosphorylase activity in hepatic tissue, found a heat-stable, dialyzable factor which was responsible for the increased formation of active phosphorylase in cell-free homogenates. This compound was purified, crystallized and was found to be very stable chemically since no loss of activity was observed on boiling under slightly acidic or alkaline conditions for 30 min (10,11). At the same time, Cook, Lipkin and Markham (12,13), who were investigating the hydrolysis of ATP in the presence of barium hydroxide, found in addition to the major products AMP and inorganic pyrophosphate, several unknown products, one being later identified as cyclic AMP. Both groups observed that the compound consisted of adenine, ribose and phosphate in a ratio of 1:1:1 and contained no monoesterified phosphate. Initially, this compound was thought to be a dinucleotide (12), but later studies (14) showed that it was actually a mononucleotide of adenylic acid with the phosphate esterified to the 3- and 5- positions of the ribose moiety (Fig. 2.1).

Following the discovery of cyclic AMP, Sutherland and his group began the search for the enzymes involved in its synthesis and degradation. Initially, Rall and Sutherland (15) using tissue homogenates, showed that cyclic AMP was synthesized by particulate fractions (1200 g pellet) in the presence of Mg^{2+} ions and ATP. Under their assay conditions, the formation of cyclic AMP was augmented by NaF, caffeine, epinephrine

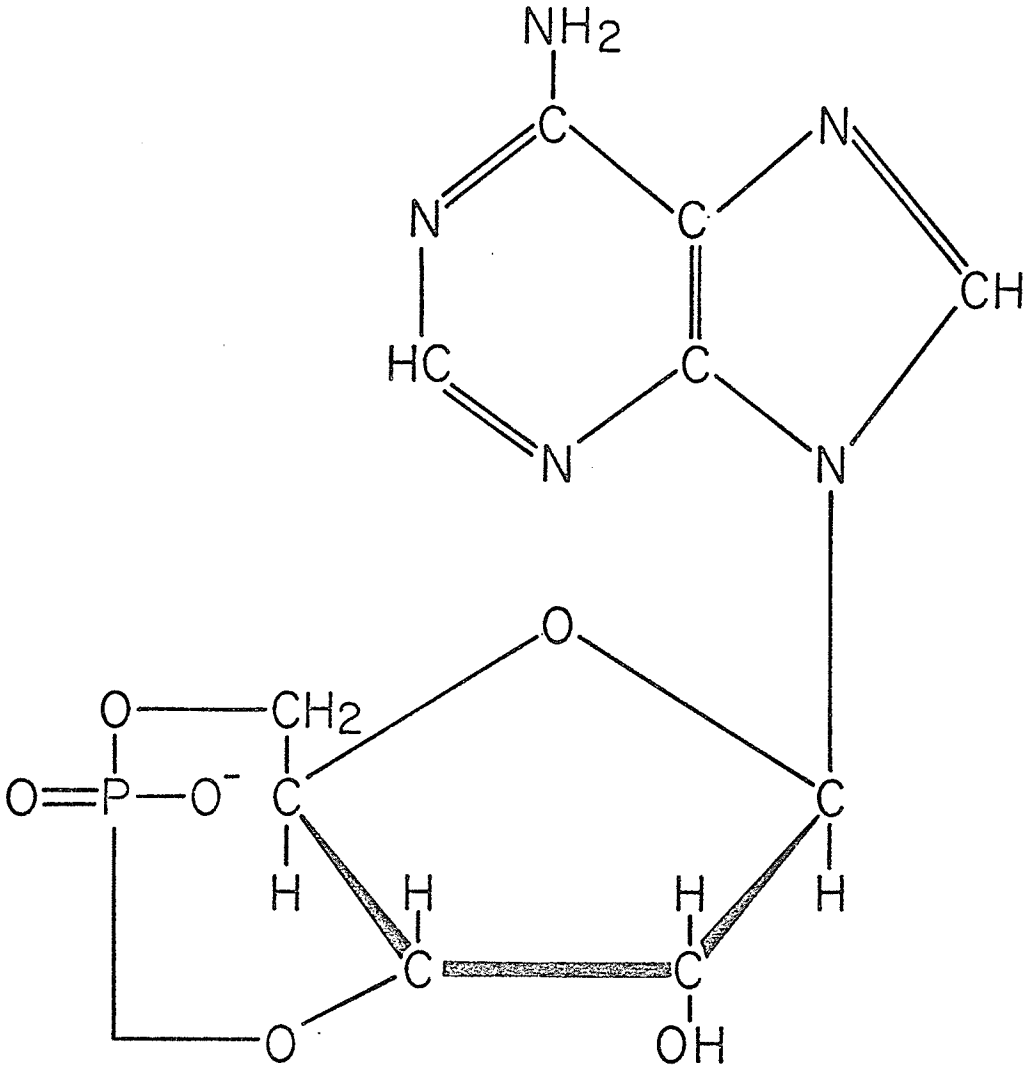


Fig. 2.1. Structure of cyclic AMP.

and glucagon. The same authors later showed (16) that the precursor of the esterified phosphate group of cyclic AMP was the α-phosphate of ATP, inorganic pyrophosphate being the other product of the reaction (equation 1). The enzyme catalyzing this reaction was called adenyl cyclase (17). At the same time, Butcher and Sutherland (18) purified (80-fold) an enzyme from beef heart which was capable of degrading cyclic AMP. The enzyme, named cyclic 3',5'-nucleotide phosphodiesterase, was very specific for the 3',5'-cyclic nucleoside monophosphates and required Mg^{2+} ions for activity. The enzyme was activated by imidazole and was inhibited competitively by the methylxanthines, theophylline being more potent than caffeine or theobromine.

II. BIOLOGICAL ROLE OF CYCLIC AMP

The metabolic regulation of the diverse organs and tissues in mammalian systems is controlled by the nervous and circulatory systems. In the latter system, hormones (first messengers) are secreted from the site of synthesis and transported in the blood to interact with specific target cells (19). Research in the last decade has shown that the regulation of cellular metabolism by many hormones is mediated by cyclic AMP (1). Thus, hormone molecules interact in target tissue with the adenyl cyclase system of the effector cell membrane stimulating the synthesis of cyclic AMP within the cell. Cyclic AMP, in turn, acts as a "second messenger" by producing various physiological responses (see Table 5.1 of Ref 1).

Although cyclic AMP is involved in the action of a variety of

hormones, this review will deal only with its involvement in glycogen metabolism and with its effect on the various glycolytic enzymes. The latter part of this review will be completely devoted to the function of cyclic AMP in unicellular organisms. The function of cyclic AMP, as well as the regulation of cyclic AMP levels in various systems, has been reviewed recently by Robison, Butcher and Sutherland (1,2), Sutherland, Robison and Butcher (3), Hardman, Robison and Sutherland (4), Jost and Rickenberg (5), and by Pastan and Perlman (7).

A. Cyclic AMP and glycogen metabolism

The synthesis of glycogen in mammalian tissue from glucose-1-P is catalyzed by UDPG-pyrophosphorylase(EC 2.7.7.9) and glycogen transferase (synthetase) (EC 2.4.1.11), while the degradation of this polymer to glucose-1-P involves the activity of glycogen phosphorylase (EC 2.4.1.1). Extensive research (6) has demonstrated that the cellular content of glycogen is primarily controlled by regulating the activity of glycogen transferase and glycogen phosphorylase. The following discussion will be concerned with the involvement of cyclic AMP in the regulation of these enzymes.

a) Glycogen phosphorylase. In 1943 Cori and Green (20) purified and crystallized two forms of glycogen phosphorylase from rabbit muscle: form 'a', which was 60 to 70% active in the absence of adenosine monophosphate (AMP), and form 'b', which was inactive without added AMP. In the presence of AMP, both forms of the enzyme had the same activity. Furthermore, they found that muscle extracts possessed a prosthetic-group-removing (PR) enzyme which could be separated from phosphorylase

activity by isoelectric precipitation at pH 5.9. In phosphorylase preparations containing the PR enzyme, phosphorylase was found mainly in the inactive 'b' form. Further study showed that phosphorylase 'a' was converted to the 'b' form when incubated in the presence of the PR enzyme. The effect of the PR enzyme, in essence, was to convert glycogen phosphorylase from AMP-independent activity to AMP-dependent activity.

Cori and Cori (21) subsequently purified the PR enzyme and showed that it had low activity in the absence of cysteine; in the presence of cysteine the enzyme was activated 2 - 3 times by Mg^{2+} ions (0.5 mM). In this study, the PR enzyme was shown to remove a prosthetic group from phosphorylase 'a' which contained organic phosphate. The amount of phosphate removed was proportional to the amount of phosphorylase 'a' converted to 'b' form. In 1955, Krebs and Fischer (22) showed that resting muscle contained phosphorylase predominantly (68 - 76%) in the 'b' form. Furthermore, the conversion of phosphorylase 'b' to phosphorylase 'a' could be accomplished in cell-free extracts in the presence of Mn^{2+} ions, ATP and a protein fraction from the muscle extract (23).

During this period, Sutherland and Cori (24,25) observed that epinephrine and other sympathomimetic amines caused an increase in the rate of glycogenolysis (i.e., the degradation of glycogen) in liver slices. They also reported that the change in catalytic rates of the enzymes involved in glycogenolysis following treatment was in the order, glucose-6-phosphatase>phosphoglucomutase>>phosphorylase. However, since their preparations contained high activities for phosphoglucomutase and glucose-6-phosphatase, Sutherland and Cori concluded that the observed increase in the rate of glycogenolysis was due to the increased activity of

phosphorylase. Furthermore, the above glycogenolytic agents were also shown to cause a rapid and selective increase in the activity of phosphorylase 'a' in liver homogenates and in intact animals without affecting the activity of phosphoglucomutase and glucose-6-phosphatase. On the basis of these findings, they concluded that the cellular content of the active 'a' and the inactive 'b' forms of phosphorylase was influenced by the sympathomimetic amines.

In 1957 Rall, Sutherland and Berthet (9), while studying the effect of epinephrine or glucagon on the formation of active phosphorylase (form 'a') from inactive 'dephosphophosphorylase' (form 'b') in cell-free homogenates of dog and cat liver, observed the formation of an active factor in particulate fractions in the presence of these hormones. This active factor was able to mimic the effect of the hormones by stimulating the conversion of the inactive ('b') to active ('a') form of the enzyme. The factor also stimulated the formation of active phosphorylase in supernatant fractions obtained from cell homogenates which were not affected by the hormones themselves. This factor was the heat stable, dialyzable compound later identified as cyclic AMP.

During this period, Krebs and Fischer (26) recognized that the phosphorylase interconversions in muscle required an enzymatically catalyzed phosphorylation and dephosphorylation of the enzyme. A similar conversion of phosphorylase 'b' to 'a' in liver was reported by Sutherland and Wosilait (27). The latter authors (28) also observed that a phosphate group was incorporated into the enzyme during the reactivation of phosphorylase 'b' and this incorporation was greatly increased by epinephrine and glucagon. They purified (45-fold) the enzyme involved in this

activation (dephosphophosphorylase kinase) and showed that the presence of ATP and Mg^{2+} were necessary for its activating effect (28). Furthermore, they purified another enzyme which removed the phosphate group from the active phosphorylase 'a' thus converting it to the inactive 'b' form (29). This latter enzyme, therefore, catalyzed the same reaction as Cori's PR enzyme (20) and was called phosphorylase phosphatase (EC 3.1.3.17).

From these studies it became evident that the presence of glycogen phosphorylase in the active "a" (phosphorylated) or inactive "b" (dephosphorylated) form was regulated by the activity of phosphorylase phosphatase (equation 3) and phosphorylase 'b' kinase (dephosphophosphorylase kinase) (equation 4).

$$\text{Phosphorylase 'a' (Active, phosphorylated)} \xrightarrow{\text{Phosphorylase phosphatase}} \text{Phosphorylase 'b' (Inactive, dephosphorylated)} \qquad (3)$$

$$\text{Phosphorylase 'b' (Inactive, dephosphorylated)} \xrightarrow{\text{Phosphorylase 'b' kinase}} \text{Phosphorylase 'a' (Active, phosphorylated)} \qquad (4)$$

Danforth, Helmreich and Cori (30) concluded from kinetic data that the increased amount of phosphorylase activity observed following epinephrine administration or muscle contraction was due to the increased activity of phosphorylase 'b' kinase (EC 2.7.1.38) rather than to the inhibition of phosphorylase phosphatase. While studying the effect of epinephrine and electrical stimulation on rat and frog skeletal muscle,

Posner, Stern and Krebs (31) reported a parallel increase in phosphorylase 'a' and phosphorylase 'b' kinase activity. The addition of epinephrine also increased the level of cyclic AMP in a fashion similar to the above two enzymes. However, electrical stimulation had no effect on the levels of cyclic AMP. From these findings the authors concluded that the epinephrine-stimulated increase in glycogenolytic activity was regulated through cyclic AMP and the activity of phosphorylase 'b' kinase.

More recent studies have also shown that phosphorylase phosphatase is important in the regulation of phosphorylase activity (32-36). The preincubation of adrenal cortex phosphorylase phosphatase with ATP and Mg^{2+} resulted in an increase in enzyme activity (32). The activated enzyme could be inactivated by further preincubating the phosphorylase phosphatase with ATP in the absence of Mg^{2+}. Such an inactive enzyme could then be reactivated in the presence of ATP + Mg^{2+}, or at a slower rate, with Mg^{2+} alone. Cyclic AMP, in the presence of ATP and Mg^{2+}, converted the active form of phosphorylase phosphatase to a less active form. This cyclic AMP effect was not reversible unless the enzyme was further inactivated with ATP. Similar results, obtained with liver (36) and skeletal muscle (33-35), supported the view that the control of phosphorylase phosphatase activity was important in the regulation of phosphorylase interconversions.

Krebs and his associates (37-39) studied the properties and regulation of phosphorylase 'b' kinase isolated from rabbit skeletal muscle and rabbit heart. The enzyme was inactive at pH 7.0 but could be activated by preincubating the inactive enzyme with ATP and Mg^{2+}; this rate of activation was further increased in the presence of cyclic AMP. The

activation by cyclic AMP compared to that caused by ATP alone, was half maximum at a concentration 0.5 - 1.0 x 10^{-7} M. Cyclic AMP alone, i.e., in the absence of ATP and Mg^{2+}, had no effect on the phosphorylase 'b' kinase activation reaction (40).

The results obtained by Krebs et al (40) and DeLange et al (41), that the activation of the phosphorylase 'b' kinase by cyclic AMP was not proportional to the phosphorylation of the enzyme with ^{32}P-ATP in the presence of Mg^{2+} ions, suggested that cyclic AMP was not directly involved in the activation reaction. These findings, along with evidence that cyclic AMP did not bind to purified phosphorylase 'b' kinase, supported the possibility that cyclic AMP acted through the stimulation of another enzyme involved in this system. Such an enzyme was finally isolated in 1968 by Walsh, Perkins and Krebs (42) who named the enzyme: phosphorylase kinase kinase, now more commonly called, protein kinase. This enzyme was the final link between cyclic AMP and the phosphorylase system. This complicated cascading system is depicted in Fig. 2.2.

b) Glycogen transferase. Villar-Palasi and Larner (43,44), while studying the effect of insulin on glycogen metabolism, found that the activity of glycogen transferase, when assayed without glucose-6-P, was increased in rat diaphragms after insulin treatment. However, when glucose-6-P was added in the assay the activity of this enzyme in the extracts prepared from control diaphragms increased to the same level as that of insulin-treated diaphragms assayed in the presence of glucose-6-P. The increased activity of glycogen transferase in the presence of insulin was not related to the increased content of glucose-6-P (45) or to any other soluble factor (44). To explain these results, Larner and his associates

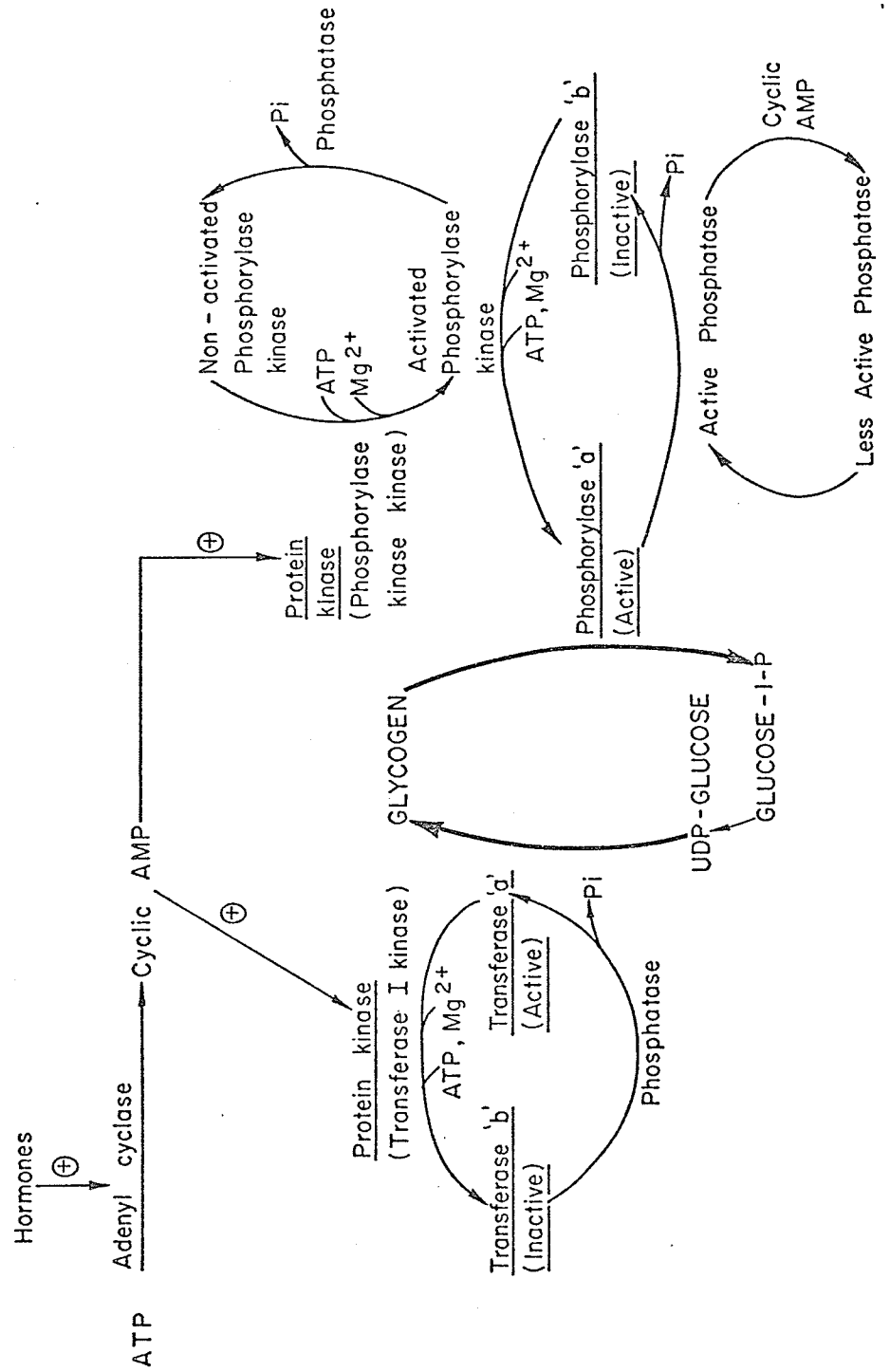
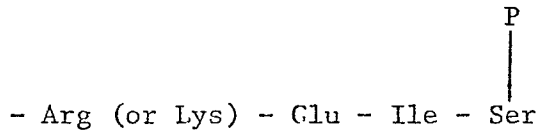


Fig. 2.2. Regulation of glycogen phosphorylase and glycogen transferase by cyclic AMP.

(43-45) postulated that the enzyme might exist in two forms and that insulin might act to regulate their interconversion. The existence of two such forms in muscle was later confirmed following their partial purification and characterization by Rosell-Perez, Villar-Palasi and Larner (46). One form was dependent on glucose-6-P (inactive D form) while the other was independent of glucose-6-P (active I form). Later, Friedman and Larner (47,48) purified (150-200 fold) this enzyme from rat skeletal muscle and showed that these two forms were interconvertible. The D form was converted to the I form by a dephosphorylation reaction, while the reverse reaction (I to D) was an ATP- and Mg^{2+} -dependent phosphorylation of the enzyme. Furthermore, by using ^{32}P labelled ATP, they observed that the I to D conversion involved the transfer of $\gamma^{32}P$- from ATP to the enzyme. The dephosphorylation of the enzyme, converting it from the D to I form, could be accomplished by incubating the ^{32}P- labelled enzyme with crude muscle extract. A direct relationship was observed between the D to I enzyme conversion (51%) and the release of ^{32}P (48%).

Larner and Sanger (49), by using radiochemical techniques, showed that one serine amino acid at the hexapeptide of glycogen transferase was phosphorylated during the I to D conversion; the sequence of amino acids around this serine residue was as follows:



A similar amino acid sequence has been found in phosphorylase 'a' (50,51)

suggesting that these two proteins have similar phosphorylated sites.

Various groups (52-55) have studied the D to I conversion in detail and have reported that this conversion is catalyzed by a cyclic AMP-activated kinase (transferase I kinase). In their study of the conversion, Huijing and Larner (52,53) found that ATP and Mg^{2+} increased the activity of transferase I kinase and that the activation by Mg^{2+} was sigmoidal indicating an allosteric site for Mg^{2+} on the enzyme. These authors further observed that cyclic AMP increased the affinity of the allosteric site for Mg^{2+} and suggested this mode of activation by this nucleotide. In the presence of cyclic AMP, the K_m values for ATP and Mg^{2+} were decreased 4 and 50-fold, respectively.

Since the interconversions of the two forms of muscle glycogen transferase by a phosphorylation-dephosphorylation reaction sequence were analogous to the interconversions of the two forms of glycogen phosphorylase, it was of interest to know whether a single kinase would catalyze the phosphorylation of these two enzymes. Although the possibility that phosphorylase 'b' kinase and transferase I kinase were the same enzyme has been generally discounted (56-58), transferase I kinase activity was observed with the protein kinase isolated from skeletal muscle (59). The protein kinase which catalyzes the activation of phosphorylase 'b' kinase in rabbit skeletal muscle was also capable of catalyzing the conversion of transferase I to transferase D. This transferase I kinase activity was essentially dependent on the presence of cyclic AMP. The K_a value for cyclic AMP (7×10^{-8}M) was 60 - 100 fold lower than the K_a values for the other cyclic 3',5'-nucleotides. However, the maximum activation for all of the cyclic 3',5'-nucleotides studied varied only about 20%. A similar

enzyme, transferase I kinase, has also been isolated from rabbit heart (60). This enzyme preparation was free of glycogen transferase activity and was activated by cyclic AMP (K_a = 5 x 10^{-8}M) to the same extent as of rabbit skeletal muscle (61). The control of glycogen transferase activity by cyclic AMP is depicted in Fig. 2.2.

B. Cyclic AMP and glycolytic enzymes

a) Phosphofructokinase. Phosphofructokinase (EC 2.7.1.11), which catalyzes the formation of fructose-1,6-P_2 from fructose-6-P in the presence of ATP, has been implicated as one of the major regulatory enzymes in the control of glycolysis (62-64). Enzyme activity is regulated mainly by oscillating concentrations of ATP, fructose-6-P, cyclic AMP, ADP, AMP and P_i (65). The activation of phosphofructokinase activity by cyclic AMP was first reported with the enzyme isolated from guinea pig heart (65) and mouse brain (66). The enzyme was strongly inhibited by ATP in the presence of subsaturating concentrations of fructose-6-P. Under such conditions, cyclic AMP caused a marked increase in enzyme activity and ATP was partially competitive with respect to cyclic AMP. Cyclic AMP also caused a decrease in the K_m for fructose-6-P. Cyclic AMP had a similar effect on the phosphofructokinase from rabbit liver (67), from spermatozoa of the rhesus monkey (68) and from the liver fluke, Fasciola hepatica (69,70). In the latter case, however, Mansour suggested that since 5-hydroxytryptamine (serotonin) stimulated glycolysis (71), as well as adenyl cyclase activity in homogenates of Fasciola hepatica (72,73), the serotonin effect on glycolysis was mediated through cyclic AMP. A similar suggestion was made by Bell, Brooker and Harding (74) for the effect of

ACTH on glycolysis in the rat adrenal gland. These authors measured the levels of the glycolytic intermediates and several nucleotides, including cyclic AMP, after the intravenous administration of ACTH. They found that a decrease in the concentration of glucose-1-P, glucose-6-P and fructose-6-P, and a simultaneous increase in levels of fructose-1,6-P_2 and glyceraldehyde-3-P were concomitant with an increase in the content of cyclic AMP which occurred 20 sec after the administration of ACTH. On the basis of this data, they suggested that ACTH stimulated glycolysis by the cyclic AMP-mediated activation of phosphofructokinase.

Kemp (75) reported that cyclic AMP, as well as AMP and P_i, decreased the affinity of rabbit muscle phosphofructokinase for Mg-ATP, whereas citrate had the opposite effect. However, cyclic AMP had no effect on the ATP-inhibited phosphofructokinases isolated from yeast (76,77) and E. coli (78). With these latter enzymes, the affinity for fructose-6-P and Mg^{2+} was increased with increasing concentrations of fructose-6-P, Mg^{2+} or AMP and was decreased with increasing concentrations of ATP. ATP inhibition could be reduced by AMP.

Cyclic AMP also had another effect on the activity of phosphofructokinase from liver fluke and guinea pig heart, in addition to its reversal of ATP inhibition (69,70,79,80). In liver fluke, cyclic AMP was shown to be involved in the activation of an inactive form of phosphofructokinase (69,70). This activation could be observed by incubating the enzyme in the presence of ADP, Mg^{2+}, P_i (or sulfate anions or hexose phosphates) and cylic AMP. ADP could be replaced by nucleoside triphosphates such as ATP. The activation was shown to involve subunit aggregation since the sedimentation coefficient ($S_{20,w}$) of the enzyme increased from 5.5 S

to 12.8 S after the activation process. The activation could be completely reversed by dialysis. In guinea pig heart, phosphofructokinase could be activated by incubating the enzyme with ATP (or ADP), a hexose phosphate, such as fructose-6-P, cyclic AMP and an unidentified, partially heat-stable, dialyzable factor (79). The presence of a nucleotide and a hexose phosphate in combination were much more effective in reactivating the enzyme than either the nucleotides or hexose phosphates alone.

In yeast, two forms of phosphofructokinase were isolated, one inhibited by ATP and another insensitive to ATP (81). The ATP-sensitive form could be converted to the ATP-insensitive form by incubating the enzyme with Mg^{2+}, ATP, cyclic AMP, NaF and a desensitizing protein. Cyclic AMP had no direct effect on the enzyme activity. Dialysis of ATP-insensitive enzyme resulted in its conversion to the ATP-sensitive form. Recently, Afting et al (82) and Atzpodien et al (83) reported additional findings on the conversion of the ATP-sensitive to ATP-insensitive form with highly purified yeast phosphofructokinase. Their studies showed that the interconversion was stimulated by ADP in the presence of fructose-6-P, NH_4^+, Mg^{2+} and NaF, and was inhibited by ATP. They further showed that the desensitizing protein did not, in fact, modify phosphofructokinase itself, but rather altered the composition of the incubation medium to permit the transformation of the ATP-sensitive form to the ATP-insensitive form by converting the negative effector, ATP, to positive effector, ADP. Cyclic AMP or AMP were not required for the transformation process.

In order to further delineate the effect of cyclic AMP on the regulation of phosphofructokinase activity, the binding of cyclic AMP and other nucleotides to this enzyme from skeletal muscle was studied by Kemp

and Krebs (84). The dissociation constants for ADP, AMP and cyclic AMP were 0.5, 1.8 and 0.6 μM, respectively. The dissociation constant for cyclic AMP was decreased more than 2-fold in the presence of fructose-6-P and fructose-1,6-P_2. The binding of each of the above nucleotides was competitively inhibited by the other nucleotide suggesting that they might bind to the same site on the enzyme. This data is in agreement with the kinetic data reported by Lowry and Passonneau (85) with sheep brain phosphofructokinase. These workers suggested the presence of at least seven substrate, inhibitor and deinhibitor sites for the enzyme. They showed that AMP, cyclic AMP and ADP bind at the same site and indicated that this site was probably one of the three sites involved in the ATP inhibition of the enzyme.

b) Other enzymes. Fructose-1,6-diphosphatase (EC 3.1.3.11), which catalyzes the dephosphorylation of fructose-1,6-P_2 to fructose-6-P, has also been shown to play an important role in the regulation of glycolysis. This enzyme from crude kidney extracts was inactivated by ATP and Mg^{2+} and the rate of this inactivation was increased by the presence of cyclic AMP (86). The inactivation of the enzyme in kidney slices by ephinephrine was also observed. During this inactivation process, ^{32}P from $AT^{32}P$ was incorporated into the enzyme protein with a concomitant decrease in enzyme activity; the inactivated enzyme could be reactivated upon incubation with crude kidney extracts.

Yeast glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12) undergoes a time-dependent inactivation by the dissociation of its tetrameric subunits into monomers at $0^{o}C$ in the presence of ATP (87). This inactivating dissociation of the enzyme by ATP was prevented by cyclic AMP and several

other metabolites. The effectiveness of cyclic AMP was greater than that of NAD, AMP, P_i, ADP and glyceraldehyde-3-P. The dissociation (inactivation) of the enzyme was completely reversed by warming to 17°C and it was greatly stimulated by ATP and by 10% sucrose. The effect of cyclic AMP on the reverse reaction was not examined in this study. Cyclic AMP was a competitive inhibitor with respect to NAD with a K_i value of 0.11 mM compared to a K_m of 0.18 mM for NAD itself (62). Other adenine-containing compounds were also competitive inhibitors of NAD, but the K_i values (1.1 - 10.2 mM) were much higher than that for cyclic AMP. The inactivation of the enzyme by chymotrypsin in the presence of ATP was also prevented by cyclic AMP (88).

Cyclic AMP was also shown to stimulate the activity of partially purified pyruvate kinase (EC 2.7.1.40) from loach embryos (89). The enzyme was activated by cyclic AMP and fructose-1,6-P_2, and the activation was further augmented (2-3 fold) by the addition of both compounds together. ATP, on the other hand, did not cause any change in pyruvate kinase activity, but blocked the activating effect of fructose-1,6-P_2. The activating effect of fructose-1,6-P_2 could be restored in the presence of ATP by the addition of cyclic AMP to the reaction medium.

III. CYCLIC AMP AND BACTERIAL SYSTEMS

This part of the review will be divided into three main sections: (A) the discovery of cyclic AMP in bacteria, (B) the microbial functions of cyclic AMP and (C) the enzymes involved in the metabolism of cyclic AMP in bacterial systems.

A. Discovery of cyclic AMP in bacteria

Although the presence of cyclic AMP in animal system was first reported in 1957 (9,13), the occurrence of this nucleotide in bacterial systems was not demonstrated until 1963. In that year, Okabayashi and coworkers (90-92) observed considerable quantities of an unknown adenine ribonucleotide in cultures of Brevibacterium liquefaciens grown in a casamino acid medium. The substitution of DL-alanine for the casamino acids and the presence of a carbon source, such as mannose or glucose, accelerated the excretion of this nucleotide into the medium. The nucleotide was subsequently isolated and identified as cyclic AMP (90).

B. Microbial functions of cyclic AMP

In 1965, Makman and Sutherland (93) observed the presence of cyclic AMP in the medium and in cells of Crooke's strain of Escherichia coli. They showed that the content of cyclic AMP in resting cells increased when incubated in phosphate buffer (pH 7.0) but was extruded into the medium when glucose was added to the cell suspension. In another experiment with resting cells incubated in a glucose-containing phosphate buffer, an abrupt rise in the cellular content of cyclic AMP was observed coincident with the complete utilization of glucose. A similar variation in the cellular cyclic AMP concentration was also observed with other sugars, as well as, with acetate and succinate.

The repression of inducible enzyme synthesis in the presence of glucose and certain other carbon sources has been observed by many workers (94-96) and has been variously called the "glucose effect", "metabolic repression" or "catabolite repression". In 1968, Pastan and Perlman (97)

postulated that the repression of enzyme synthesis by these carbon sources might be due to the lowering of the cellular cyclic AMP content by these compounds. This was confirmed when they showed that cyclic AMP actually increased the differential rate of synthesis of two inducible enzymes, β-galactosidase and tryptophanase, in cells of E. coli grown with glycerol in the presence of the inducer, isopropyl-β-D-thio-galactoside (IPTG); the cells had been made permeable to cyclic AMP with Tris and EDTA. By studying the incorporation of L-leucine-^{14}C it was shown that activation was not due to a generalized activation of protein synthesis. The cyclic AMP effect was specific for the catabolite repressed enzymes since the synthesis of another inducible enzyme, alkaline phosphatase, which is not subject to catabolite repression by glucose, was not affected by cyclic AMP. Furthermore, repression of this enzyme by P_i was not overcome by cyclic AMP.

In addition to its effect on permanent catabolite repression, cyclic AMP also restored the synthesis of β-galactosidase and tryptophanase in glycerol-grown, induced cells of E. coli when their synthesis was repressed by the addition of glucose or pyruvate i.e., transient catabolite repression (97). Glucose-initiated transient repression of β-galactosidase and tryptophanase synthesis in glycerol-grown cells of E. coli has also occurred in the presence of AMP (98). Such repression was also overcome by cyclic AMP. Recently, Aboud and Burger (99) showed that ATP reduced the differential rate of β-galactosidase synthesis in permeable, induced cells of E. coli. This ATP effect was also reversed by cyclic AMP.

The ability of cyclic AMP to overcome both permanent and transient catabolite repression of inducible enzyme synthesis in E. coli has also been reported by other investigators (100-103). The initial conclusions

from these various studies suggested that cyclic AMP acted in the synthesis of these inducible enzymes at the level of messenger RNA (mRNA) synthesis or 'transcription'.

a) β-galactosidase and the lac operon

(i) Intact cells. Subsequent to the discovery of the cyclic AMP effect on the synthesis of β-galactosidase in 1968 (97), considerable effort has been expended to determine how cyclic AMP acts to regulate the synthesis of the mRNA for the lac operon. This operon (Fig. 2.3(A)) contains three structural genes, z, y, and a, which code for the synthesis of β-galactosidase, galactoside permease and transacetylase, respectively. The operon also contains three regulatory genes, i, p and o, which code for the regulator (repressor) gene, the promotor gene, and the operator gene, respectively (104-106).

Since cyclic AMP was shown to increase specifically the differential rate of β-galactosidase synthesis, it was only logical that the effect of this nucleotide would be further examined in a number of mutants of the lac operon. Perlman and Pastan (107) demonstrated that cyclic AMP overcame the transient repression of β-galactosidase in strains of E. coli which produce this enzyme constitutively through mutations in the regulator ($3300(i^-)$) and operator (O^c_{67}) genes (for a list of mutants studied - see Table 2.1). Cyclic AMP also overcame the repression in strain C-600, which has normal regulatory genes but is deficient in the permease (y) gene, and in the mutant, LA-12 G, which is resistant to catabolite (permanent) repression. These authors further showed that glucose prevented the accumulation of β-galactosidase-specific mRNA induced by IPTG in cells of strain C-600 treated with chloramphenicol. The addition of cyclic AMP

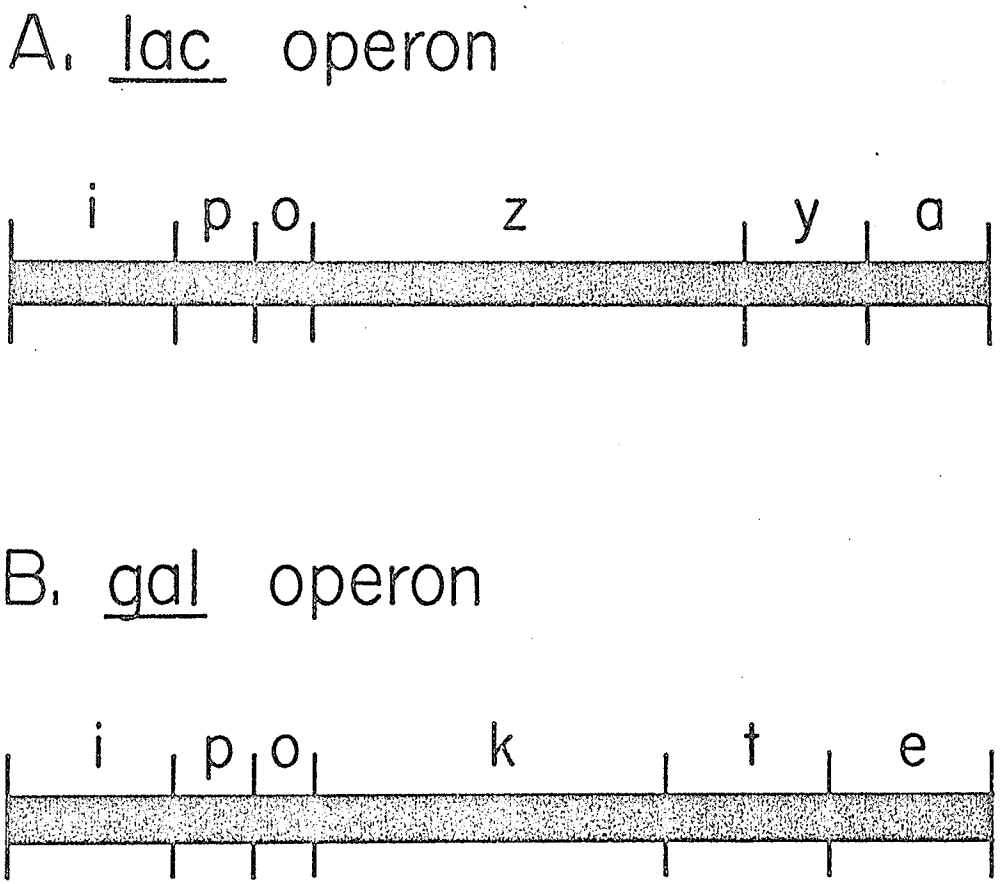


Fig. 2.3. A. The genetic sequence of the lac operon in E. coli. Three regulatory genes i, p and o code for the repressor, the promotor and the operator. The other three structural genes z, y and a contain the sequence information for β-galatosidase, galactoside permease and transacetylase.

B. The genetic sequence of the gal operon in E. coli. Three regulatory genes i, p and o are as above for the lac operon. Three structural genes, k, t and e code for galactokinase, transferase and epimerase.

TABLE 2.1

Mutants of Escherichia coli employed in the study of cyclic AMP function

Mutants	Characteristics	References
I. LAC OPERON		
A. Wild type strains		
Crooke's ATCC 8739		93.113
K12	Intact lac operon	108,131,143
3000		107,108
AB 257	(lac $i^+p^+o^+z^+y^+a^+$)	161,162
B. Regulatory genes		
(i) Regulator (repressor)		
3300, i^-_{522}	Constitutive (i^-)	107
(ii) Operator		
O^c_{67} or O^c	Constitutive (o^-)	107
(iii) Promotor		
L 8	Synthesizes about 5% β-galactosidase compared to 3000 parental strain.	108
64	Revertant of L 8 and synthesizes normal β-galactosidase compared to strain 3000.	108
82	Revertant of L 8 but has second mutation within the promotor locus and synthesizes about 15-20% β-galactosidase compared to strain 3000.	108
N 27	Revertant of L 8 but has second mutation within the promotor locus and synthesizes about 50-70% β-galactosidase compared to strain 3000.	108
C. Structural genes		
(i) Permease		
C 600, 300 U, 200 P	Permease deficient (y^-)	107,130
D. Catabolite repression		
LA-12 G	Resistant to permanent repression but still sensitive to transient repression.	107,161,162
AB 257^{pc-1}	Resistant to both types of catabolite repression.	161,162
E. Additional mutants		
Z $19i^q$	Has regulator (repressor) gene i^q	110
Z $19i^-$	Identical to Z $19i^q$ but does not contain a lac repressor gene.	110
514	Deletion of the entire region occupied by the lac operon and the gene for lac repressor.	110
II. MISCELLANEOUS		
5333	CR $protein^-$	113
5336	Adenyl $cyclase^-$	121
21_i-3	lac i^-z^-, F' lac i^-z^-, CR $Protein^-$	113

Legend: i = repressor gene, p = promotor gene, o = operator gene; z, y and a = structural genes for β-galactosidase, galactoside permease and transacetylase, respectively. CR protein = cyclic AMP receptor protein.

overcame this repression. Furthermore, cyclic AMP failed to stimulate the production of β-galactosidase in induced cells in which mRNA synthesis had been arrested by the addition of proflavine or by the removal of the inducer. On the basis of these results, Pastan and Perlman confirmed that cyclic AMP acted at the gene level (transcription) in the regulation of β-galactosidase synthesis.

In later studies, Pastan and Perlman (108) used a lac promotor mutant, L8, which synthesizes low amounts of β-galactosidase (5%) and which shows normal permanent repression of β-galactosidase synthesis in the presence of glucose. The addition of low concentrations (1 mM) cyclic AMP to this promotor defective mutant did not overcome catabolite repression while with its parental strain (K 12 3000) and revertants (64, N27 and 82), cyclic AMP was capable of overcoming transient repression, but not the permanent repression. This study suggested that transient repression of the lac operon by glucose was mediated by the lac promotor and that cyclic AMP stimulated the synthesis of β-galactosidase through its direct or indirect action on the lac promotor. Perlman, DeCrombrugghe and Pastan (109) later showed that permanent repression could also be overcome by higher concentrations of cyclic AMP (5 mM) than that required to reverse the observed transient repression (1 mM).

(ii) Cell-free systems. Probably the most convincing evidence for the stimulatory effect of cyclic AMP on the DNA-directed synthesis of β-galactosidase was demonstrated with a cell-free system by Chambers and Zubay (110). The system required only a crude extract of E. coli (prepared from three different strains, Z $19i^q$, Z $19i^-$ and 514), DNA containing the lac genes, four ribonucleotides (ATP, GTP, CTP, UTP),

20 amino acids, Mg acetate, and various other salts and cofactors. Cyclic AMP in such a cell-free system increased the yield of β-galactosidase from 8 to 30 times. To further prove that cyclic AMP acted at the level of lac DNA transcription, Varmus, Perlman and Pastan (111) employed DNA-RNA hybridization techniques to measure lac mRNA. This assay involved pulse labelling the lac mRNA with ^{3}H-uridine and comparing the ability of this RNA to hybridize with DNA isolated from the hybrid transducing phage (λh80dlac) in which some of the phage DNA has been replaced by lac operon DNA. DNA from the parent phage, λh80, was used as a control. The difference in hybridization of the lac mRNA with these two DNA species was taken as a measure of the amount of lac mRNA synthesized by the culture. These authors found that both cyclic AMP and glucose altered the rate of mRNA production in direct proportion to their effects on β-galactosidase synthesis. Furthermore, the increase in lac mRNA synthesis was not secondary to protein synthesis since cyclic AMP was still able to regulate the synthesis of lac mRNA in cells where protein synthesis was inhibited by chloramphenicol. In addition, they showed that the repressive effect of glucose was not due to its influence on the rate of lac mRNA degradation. These results were confirmed by DeCrombrugghe et al (112) with crude extracts of E. coli using the same DNA-RNA hybridization techniques.

(iii) Cyclic AMP-receptor protein. In addition to the lac promotor mutants, another group of mutants were discovered which were also unresponsive to cyclic AMP. These latter mutants (21_i-3, and 5333) either were unable to synthesize any of the lac operon enzymes or were much less effective in this regard since they were unable to ferment lactose, galactose, maltose and arabinose on tetrazolium agar (113). The addition

of cyclic AMP did not alter this fermentation defect. The wild-type parental strain (ATCC 8739) was found to contain a cyclic AMP-receptor (CR) protein with a dissociation constant (K_d) of 1-2 x 10^{-6}M. On the other hand, of the two defective strains, 5333 had negligible quantities of the CR protein while 21_i-3 possessed a CR protein with decreased affinity for cyclic AMP (K_d = 2 x 10^{-5}M). These mutants were able to function normally when the CR protein from the wild-type strain (8739) was added to extracts of these organisms.

The purification of this CR protein was reported recently by Anderson et al (114). The purified protein had an average molecular weight of 45,000 with a sedimentation constant ($S_{20,w}$) of 3.5 and was composed of two identical subunits (MW = 22,500). Each native dimer possessed 4 free sulfhydryl groups.

A similar protein to the CR protein was isolated by Zubay, Schwartz and Beckwith (115) and called the "catabolite-gene-activator protein", abbreviated as CAP (115-117) or as CGA protein (118). E. coli extracts containing CAP were shown to stimulate the level of β-galactosidase synthesis in extracts from CAP^- mutants to half that observed in CA^+ extracts; both cyclic AMP and CAP were necessary for stimulation. Since the physical and chemical properties of CAP were similar to that of the CR protein isolated by Emmer et al (113) and by Anderson et al (114), it is likely that these proteins are the same.

As was the case for the CR protein, Eron and Block (117) showed that the initiation of lac transcription at the lac promotor required the presence of this "catabolite-gene-activator protein" (CAP) in addition to cyclic AMP and RNA polymerase. The lac repressor was thought to control transcription by preventing the binding of RNA polymerase and/or CAP to the

lac promotor. Their results also showed that the lac promotor was composed of two binding sites, one for the RNA polymerase holoenzyme and the other for CAP. CAP (or CGA protein) was shown to have substantial affinity for DNA, affinity which was greatly strengthened by cyclic AMP and strongly inhibited by cyclic GMP (118). The opposing effects of these two cyclic compounds on the binding of DNA and CAP is in parallel to their effects on the expression of the lac operon. Thus, it is possible that CAP activates the lac operon by binding to DNA under the influence of cyclic AMP, a process which would be inhibited by cyclic GMP (119,120).

(iv) Adenyl cyclase negative mutant. During the course of their studies, Perlman and Pastan (121) isolated a mutant (5336) deficient in adenyl cyclase activity and which was unable to ferment a variety of sugars. Cyclic AMP could, however, restore the normal utilization of these sugars if added to the cells. The synthesis of lac mRNA was not observed in cells of this mutant until they were supplied with exogenous cyclic AMP (119).

(v) P-enolpyruvate phosphotransferase mutant. The P-enolpyruvate phosphotransferase system (PEP-PTS), which participates in the accumulation and phosphorylation of a number of sugars in bacterial cells (122,123) is catalyzed by two enzymes: Enzyme I catalyzes the transfer of phosphate from P-enolpyruvate to a small, histidine-containing, heat-stable protein, HPr (equation 5). Enzyme II, which is membrane-bound, catalyzes the

$$\text{P-enolpyruvate} + \text{HPr} \xrightarrow{\text{Enzyme I}} \text{P-HPr} + \text{Pyruvate} \qquad (5)$$

$$\text{P-HPr} + \text{Sugar} \xrightarrow{\text{Enzyme II}} \text{Sugar-P} + \text{HPr} \qquad (6)$$

subsequent transfer of the phosphate from P-HPr to the sugar being transported (equation 6). Unlike Enzyme I, a family of Enzyme II's exist each being specific for an individual sugar or group of sugars (124).

In order to determine whether the repression of β-galactosidase synthesis by glucose and other sugars was, in fact, dependent upon the phosphorylation of the added sugar, Pastan and Perlman (125) studied the effects of cyclic AMP in mutants deficient in one of the proteins comprising this transport system. They showed that β-galactosidase synthesis was repressed in the strains which were deficient in Enzyme I or in HPr. These strains were comparatively more sensitive to transient repression by glucose, mannitol and α-methyl glucoside than their parental wild-type strain. This repression was overcome by cyclic AMP. In contrast, β-galactosidase synthesis in a mutant deficient in enzyme II was resistant to repression by glucose and α-methyl glucoside. This indicated that the repression of β-galactosidase synthesis required only the interaction of a sugar with its respective enzyme II and did not require its phosphorylation or subsequent metabolism. These findings also suggested that an intact PEP-PTS may not be required for lactose utilization by E. coli.

(vi) Conclusions of the Pastan-Perlman group. From the studies outlined in the above sections, Pastan-Perlman and their associates (119,126-128) concluded that cyclic AMP acts at the level of lac transcription for the differential synthesis of β-galactosidase in E. coli. The presence of cyclic AMP, CR-protein, lac DNA, RNA polymerase, lac repressor and inducer were the only components required for the regulation of lac mRNA synthesis. Their view of the regulation of the lac operon is depicted diagramatically

in Fig. 2.4.

(vii) Some contrary views. Although the work of Pastan-Perlman group has strongly suggested that cyclic AMP acts at the level of gene transcription for the stimulation of inducible enzyme synthesis, a few studies have suggested that the cyclic AMP effect may be a secondary one. For example, in 1969, Goldenbaum and Dobrogosz (101) found that when glucose was replaced by glucose-6-P, or a combination of glucose plus gluconate as the catabolite repressor in E. coli, repression was reversed only to a limited extent by cyclic AMP. These authors, on the basis of this data, suggested that the ability of cyclic AMP to reverse glucose repression was due to some metabolic interference in the conversion of glucose to glucose-6-P. This was confirmed by Moses and Sharp (129) who showed that cyclic AMP was unable to abolish catabolite repression in E. coli in the presence of a mixture of glucose, glucose-6-P, gluconate and casein hydrolysate. These authors also showed that cyclic AMP stimulated glycolytic activity while having a minor depressing effect on the metabolic flow through the pentose phosphate cycle. The concentration of fructose-1,6-P_2 was greatly affected by the presence of cyclic AMP. Several metabolites such as fructose-1,6-P_2, ribose-5-P, NADP, NADPH and 5-phosphorylribose-1-pyrophosphate, in addition to cyclic AMP, also partially or more effectively relieved glucose repression of β-galactosidase synthesis in EDTA-treated cells. This data was taken as evidence against a direct role for cyclic AMP in overcoming the repression of inducible enzymes.

Evidence has also been presented suggesting that glucose and cyclic AMP do not act on transcription but act at the level of translation. In this regard, Yudkin and Moses (130) observed that glucose repression

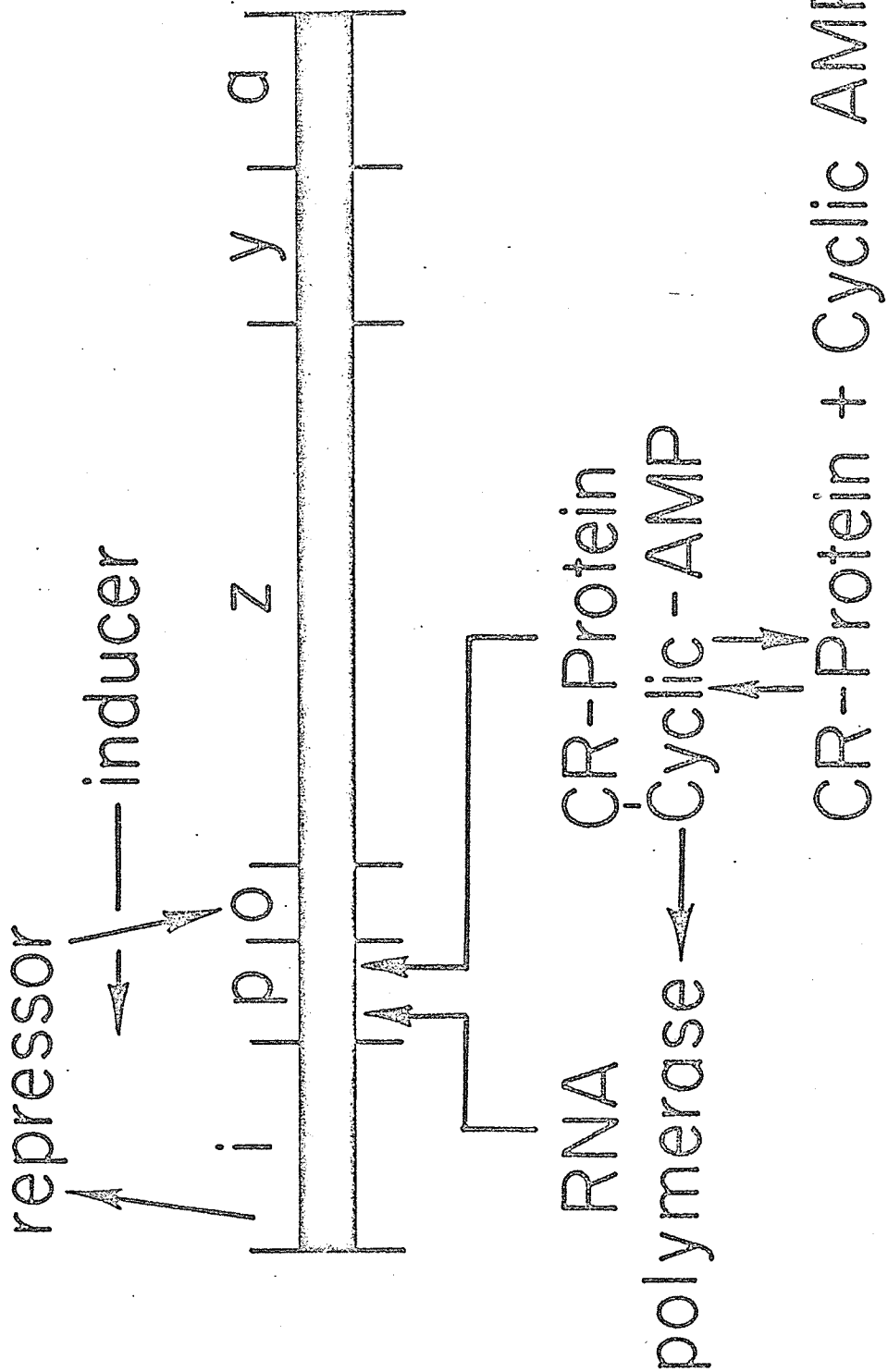


Fig. 2.4. Regulation of lac transcription by cyclic AMP.

occurred in induced E. coli cells even after the transcription of the lac operon was terminated either by the removal of the inducer or by the use of T6 phage known to stop transcription. In E. coli strain 300 U, the glucose effect on translation was sufficient to account for almost all of the catabolite repression observed for β-galactosidase synthesis. In another strain 200 P, however, much more severe repression was observed and it was postulated that glucose may have had an additional effect in this strain. On the basis of these results, Yudkin and Moses (130) suggested that catabolite repression of β-galactosidase synthesis was due to a blockage of translation by glucose. Similar results were reported recently by Aboud and Burger (131) to explain the action of cyclic AMP in reversing glucose repression in induced cells of E. coli K12. Cyclic AMP abolished glucose repression when transcription was blocked by proflavine or by removing inducer. These authors suggested that cyclic AMP reversed catabolite repression by releasing the glucose interference of the translational process. According to these workers the effect of cyclic AMP on lac mRNA synthesis was a secondary effect.

b) Galactokinase and the gal operon. In order to further delineate the function of cyclic AMP in the regulation of other inducible enzymes in E. coli,Miller et al (132) demonstrated that, like lac mRNA, cyclic AMP stimulated the synthesis of gal mRNA in wild-type strain 3000. The stimulation of galactokinase synthesis by cyclic AMP was also reported by Tao and Schweiger (133) and by DeCrombrugghe et al (134). The gal operon in E. coli contains 3 regulatory genes, i, p and o, similar to lac operon, and 3 structural genes, k, t and e coding for the synthesis of galactokinase, transferase and epimerase, respectively (135) (Fig. 2.3(B)).

The adenyl cyclase negative mutant, 5336 (Table 2.1), which does not make cyclic AMP, was unable to synthesize gal mRNA and the proteins coded in the gal operon. As was the case for the lac operon, normal enzyme synthesis was restored by the addition of exogenous cyclic AMP (132). Mutant 5333, which lacks the cyclic AMP-receptor (CR) protein, was also unable to synthesize gal mRNA. Thus, gal transcription is probably controlled by cyclic AMP in a manner similar to that for lac-transcription.

The synthesis of galactokinase was also demonstrated "in vitro" with a coupled system for transcription and translation containing bacteriophage gal DNA and a cell-free extract prepared from a gal deletion strain of E. coli (136). The synthesis of galactokinase in such a system was stimulated 2 - 12 fold by cyclic AMP in the presence of the CR-protein and DNA containing the gal operon. Synthesis was completely reversed by cyclic GMP. Furthermore, the synthesis of gal mRNA was increased 15-fold by cyclic AMP in a system employing bacteriophage gal DNA (λp gal 8 DNA), E. coli RNA polymerase and CR-protein (137). In this system, cyclic GMP was an inhibitor of gal mRNA synthesis while 2',3'-cyclic AMP and 5'-AMP had no effect. When cyclic AMP, CR-protein, DNA and RNA polymerase were incubated together for 10 min, a rifampicin (inhibitor of DNA-dependent RNA synthesis)-resistant preinitiation complex was formed which resulted in gal mRNA synthesis upon addition of all four ribonucleoside triphosphates. Cyclic GMP not only prevented the formation of the complex but also caused the dissociation of the complex after it had been formed. This data suggests that cyclic AMP and CR-protein stimulated gal transcription at a step prior to mRNA chain elongation.

c) Other enzymes. In addition to the regulation of β-galactosidase and galactokinase synthesis, cyclic AMP was also found to regulate the synthesis of a number of other inducible enzymes (Table 2.2), some of which have been studied in detail. For example, early results demonstrated that cyclic AMP stimulated the rate of tryptophanase synthesis in Tris-EDTA treated cells of E. coli (97,138). The cyclic AMP effect was very specific since the overall rate of protein and RNA synthesis, as well as the synthesis of tryptophan synthetase, was not affected (138). Cyclic AMP action was thought to act after transcription since this cyclic nucleotide increased enzyme synthesis in induced cells after mRNA synthesis had been arrested by actinomycin D, proflavine or by inducer removal. However, these results may not be valid as Conde, DelCampo and Ramirez (139) found that cyclic AMP inhibits the uptake of proflavine. These authors, therefore, suggested that this drug should not be used to inhibit RNA synthesis in the presence of cyclic AMP unless the particular experimental conditions ensure that such interference is eliminated. Recently, the same authors (140) demonstrated, by the use of rifampicin, that cyclic AMP acts at the level of transcription by stimulating the production of tryptophanase mRNA in E. coli.

Cyclic AMP also increased the rate of synthesis of the biodegradative enzyme, threonine deaminase, in Tris-EDTA treated cells of E. coli (141). As in the previously mentioned cases, the nucleotide was specific for the enzyme and had no effect on the overall rate of protein or RNA synthesis. Cyclic AMP had no effect on enzyme synthesis in cells in which protein synthesis had been inhibited by chloramphenicol. Regulation of enzyme synthesis appeared to be at the transcriptional level since this

TABLE 2.2

List of inducible enzymes in different organisms affected by cyclic AMP.

Name of the organisms	Name of the enzyme	Reference
Escherichia coli	β-galactosidase	97,107
	Tryptophanase	97,138,140
	Galactokinase	132-134
	lac permease	134
	Glycerokinase	134,142
	L-α-glycerophosphate permease	134
	Enzyme II of the fructose PEP phosphotransferase system	134
	L-arabinose permease	134
	D-serine deaminase	134
	Thymidine phosphorylase	134
	Threonine deaminase	141
	Cyclic AMP phosphodiesterase	143
	GMP reductase	144
	Chloramphenicol acetyl transferase	145
Salmonella typhimurium	Galactokinase	134
	L-arabinose isomerase	146
Salmonella typhimurium F-lac	β-galactosidase	134
Serratia marcescens F-lac	β-galactosidase	134
Proteus inconstans P-lac	β-galactosidase	134
Aerobacter aerogenes	β-galactosidase	134

nucleotide failed to stimulate enzyme production in cells in which mRNA synthesis was inhibited by rifampicin or by a change in the components of the medium.

C. Enzymes involved in the metabolism of cyclic AMP in bacterial systems

a) Adenyl cyclase. The formation of cyclic AMP from ATP is catalyzed by adenyl cyclase, pyrophosphate (PP_i) being the other product of the reaction (equation 1). The activity of adenyl cyclase in unicellular organisms was first demonstrated in 1965 when Hirata and Hayaishi (147) reported its presence in the soluble fractions of Brevibacterium liquefaciens. The enzyme was partially purified (100-fold) and was shown to catalyze the formation of equimolar concentrations of cyclic AMP and pyrophosphate from ATP (148). Both ATP and deoxyATP would serve as the substrate for the enzyme (149) and its activity was dependent on Mg^{2+} and an α-ketoacid, such as pyruvate (147,150). The apparent K_m for ATP was 1 mM and this value was not altered by changes in the concentration of pyruvate in the reaction mixture. The enzyme was inhibited by p-chloromercuribenzoate and this inhibition was reversed by β-mercapto-ethanol. Inhibition was also observed with dATP, AMP, dAMP, ADP and GTP; the inhibition by dATP was competitive when ATP was the substrate.

Greengard, Hayaishi and Colowick (151) subsequently reported that B. liquefaciens enzyme could catalyze the reverse reaction i.e., the formation of ATP from cyclic AMP and inorganic pyrophosphate. The equilibrium constant favored the synthesis of ATP (K = 0.065 in the forward direction). The reversibility of the enzyme was, however, questioned more recently by Cheung and Chiang (152) who were unable to observe ATP formation

with the enzyme isolated from the same organism. This later claim was discounted by Hayaishi, Greengard and Colowick (153) and by Takai et al (154) who commented that since Cheung and Chiang (152) used very little enzyme and a very low starting isotope level (46,000 cpm), the formation of 0.1% ATP under these conditions could have escaped detection.

In 1969, the presence of adenyl cyclase activity in E. coli was simultaneously reported by Brana (155), Ide (156) and Tao and Lipmann (157). The enzyme was either particulate (155,156) or partially particulate (157) and required Mg^{2+} for activity. The enzyme isolated by Tao and Lipmann (157) was inhibited by NaF and by PP_i with the inhibition being particularly severe when both NaF and PP_i were combined in the reaction mixture. The enzyme from E. coli was also inhibited by pyridoxal-P, oxaloacetate, pyruvate, acetyl-P and ribose-5-P (156,158); the inhibition by pyridoxal-P was partially reversed by the addition of lysine (158). Other inhibitors of the enzyme were, ADP, UTP, CTP, and GTP, the latter nucleotide producing competitive inhibition. Other compounds, such as cyclic AMP, cyclic GMP, glucose, acetate, succinate, glucose-1-P, and glucose-6-P had no effect on enzyme activity (158).

Recently, Ide (159) screened 21 strains of bacteria for adenyl cyclase activity and classified them on the basis of their solubility and pyruvate dependence. Pyruvate-activated adenyl cyclase from B. liquefaciens, Micrococcus lysodeikticus, and Arthrobacter globiformis was found in the 30,000 g supernatant while a similar pyruvate-stimulated enzyme from A. citreus and M. flavus was found in the 30,000 g pellet. In Alkalingenes faecalis, Corynebacterium equi, Erwinia carotovora, E. coli, and Nocardia erythropolis, adenyl cyclase activity was found in the

30,000 g pellet, but it was not stimulated by pyruvate. The enzyme from N. erythropolis required Mg^{2+} or Mn^{2+} for activity, Mn^{2+} being more effective than Mg^{2+}. The enzyme was strongly inhibited by GTP, UTP, CTP, ITP, 3'-AMP, oxaloacetate and pyridoxal-P.

b) Phosphodiesterase. Cyclic AMP is degraded to AMP by cyclic nucleotide phosphodiesterase (equation 2). In 1966, Brana and Chytil (160) first reported the presence of this enzyme in the 20,000 g supernatant of E. coli. Later in 1969, Monard, Janecek and Rickenberg (161) prepared this enzyme from a wild type strain of E. coli (AB 257), sensitive to catabolite repression, and from two mutants (AB 257^{pc-1} and LA-12G), resistant to catabolite repression. They found that the degradation of cyclic AMP required three different components in cell extracts: component I (160,000 g pellet), component II (non-dialyzable 160,000 g supernatant) and component III (a dialyzable fraction) (161,162). Mutant AB 257^{pc-1} was defective in component I, whereas the LA-12G mutant was defective in component II. The formation of component I was not significantly affected by the conditions of growth. The concentration of component II was higher in cells grown under conditions of severe catabolite repression than in cells grown under conditions of low catabolite repression.

Cyclic AMP phosphodiesterase in E. coli K12 was induced by cyclic AMP (143). The level of this enzyme was much lower in cells grown in a glucose-containing medium than in a medium with glycerol. However, the addition of cyclic AMP to the glucose medium resulted in an increase of the enzyme to level as high, or higher than that observed with glycerol. The enzyme from E. coli, and from B. liquefaciens, was inhibited by dipicolinic acid, a physiologically important metal-chelating agent in

spore-forming bacteria (163). In some strains of E. coli (B and Crooke's), the activity of this enzyme was either very low or absent, while in other strains the activity of the phosphodiesterase was dependent on the presence of a reducing agent, such as mercaptoethanol and ferrous ions (7).

The activity of cyclic AMP phosphodiesterase was also found in Serratia marcescens, A. citreus, B. linens, Microbacterium flavum, E. carotovora and a number of other organisms (159). The enzyme from S. marcescens was purified more than 1,000-fold from the 30,000 g supernatant fraction and was shown to require Mg^{2+} for activity; Fe^{2+}, Ca^{2+} and Ba^{2+} were stimulatory (164). This enzyme was able to hydrolyze all of the 3',5'-cyclic nucleotides at a considerable rate. The enzyme was inhibited by the 5'-deoxy-5'-(dihydroxyphosphinyl-methyl)-adenosine 3'-cyclic ester, theophylline, proflavine, 8-hydroxyquinoline, ADP, AMP, ATP, GTP and UTP.

Cyclic AMP phosphodiesterase activity has also been observed in Saccharomyces carlsbergensis (165), in the cellular slime mold, Dictyostelium discoideum, (166 ,167) and in the acellular slime mold Physarum polycephalum (168). The enzyme in S. carlsbergensis was inhibited by ATP, inorganic polyphosphate and PP_i, and was activated by Mn^{2+} ions (165). In cultures of D. discoideum, the enzyme was found in the culture medium and Mg^{2+} was required for its activity (167). The K_m for cyclic AMP was 2 mM and its activity was not inhibited by methylxanthines, such as caffeine. The phosphodiesterase activity was also found in the growth medium of P. polycephalum cultures; this enzyme, however, was inhibited by methylxanthines and ATP (168).

c) Protein kinase. The activity of protein kinase has also been observed

in E. coli (169) and in the acellular slime mold, P. polycephalum (170). In E. coli, the phosphorylation of histone by ATP was increased about 3-fold by 0.7 μM cyclic AMP. With the E. coli enzyme, the apparent K_m for cyclic AMP was 0.2 μM. Two different protein kinases were present in P. polycephalum, one being activated by cyclic AMP and another inhibited by this nucleotide (170). In the latter case, the phosphorylation of casein by ATP was completely inhibited by cyclic AMP at concentrations above 1 mM. These two different protein kinases were readily separable, the cyclic AMP-inhibited protein kinase being released readily from the cells by mild homogenization, whereas the cyclic AMP-activated protein kinase was present in extracts only after passage of cells through a French pressure cell.

CHAPTER 3

METHODS AND MATERIALS

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II. PREPARATION OF CELL-FREE EXTRACTS

III. ENZYME ASSAYS

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VI. MATERIALS

I. BACTERIOLOGICAL

The procedure for the maintenance, growth and harvesting of Streptococcus salivarius (ATCC 25975) were carried out as described previously by Hamilton (171). S. salivarius was routinely maintained in glucose-tryptone-yeast extract (glucose-TYE) broth of the following composition: 10 g tryptone, 5 g yeast extract, 3 g K_2HPO_4, 1 g glucose and 1 liter of deionized water. While the daily maintenance cultures were incubated aerobically in 0.1% glucose-TYE broth, all experimental cultures were incubated anaerobically in an atmosphere of 5% CO_2 in nitrogen. For the majority of the experiments reported here, the cells were grown in 0.3% glucose-TYE broth in oculated with an overnight culture (about 8-10 hr) grown in the same broth with 0.1% glucose. Cells at the desired phase of growth were harvested by centrifugation at 15,000 g for 10 min at $4^{o}C$ and washed once in Tris-HCl buffer (50 mM, pH 7.5). The pellet obtained after further centrifugation was suspended in the same buffer at the appropriate cell concentration. The cell concentration was determined by measuring the turbidity of cell suspension in a Klett-Summerson colorimeter containing a red filter (660 nm). A standard curve had been previously established for Klett units vs. mg dry weight of cell material. One mg dry wt of S. salivarius contains 1.27×10^{9} cells.

II. PREPARATION OF CELL-FREE EXTRACTS

The major interest of the research outlined in this thesis was the isolation and characterization of adenyl cyclase from S. salivarius. Since little information was available on the preparation of cell-free

extracts from cell suspensions of the oral streptococci, it was necessary initially to find a relatively efficient method for cell disruption. To this end, a number of methods were tested under various conditions and the relative efficiency of each method measured by determining the protein concentration, and the specific and total activities of adenyl cyclase. As shown in Table 3.1, maximum cell breakage (i.e., maximum cell-free protein) was obtained when cells were disrupted by sonication with a Biosonik Ultrasonic Probe (Bronwill Scientific, Rochester, New York) for 30 min at $0^{o}C$ in the presence of 5 μm glass beads in a ratio of 3:1 (cells to beads). However, the maximum specific activity of the enzyme was found when the cells were disrupted manually by grinding frozen cells (liquid nitrogen) for 10 min with an equal amount of alumina. The maximum total activity was obtained with extracts prepared from cells disrupted in the French Pressure Cell. However, since reproducible results were difficult to obtain with the French press and alumina grinding methods, preliminary experiments employed extracts obtained by Biosonik sonic disruption for 30 min at $0^{o}C$ without glass beads. This method released a reasonable amount of total enzyme activity and the adenyl cyclase activity in the 30,000 g supernatant was about 90% of that in the whole homogenate.

At the later stages of this work, a Branson Sonifier (Heat Systems Ultrasonic Inc; Plainview, New York) was employed for the disruption of cell suspensions. The efficiency of this newer instrument for the preparation of adenyl cyclase-containing extracts was determined in a manner similar to that described in Table 3.1. As shown in Table 3.2, this Sonifier produced extracts with protein concentrations and total

TABLE 3.1

Maximum total and specific activity of adenyl cyclase in extracts of S. salivarius obtained by using different methods of cell breakage.

Methods of cell breakage		Time (min)	Protein conc. (mg/ml)	Maximum specific activity	Maximum total activity
Biosonik	without glass beads	30	1.37[a]	113.1[b]	155.0[c]
	with glass beads[d]	30	2.92	46.0	134.3
Mickle disintegrator	with glass beads[d]	30	1.01	51.7	52.2
Raytheon	without glass beads	30	0.86	114.9	98.8
	with glass beads[d]	15	0.84	106.3	89.3
		30	1.41	68.9	97.1
Liquid nitrogen freezing and grinding with alumina		10	0.29	309.9	89.9
		Number of passes			
French press		4	1.55	122.6	190.0
		6	2.13	73.2	156.0

[a] Obtained in 30,000 g supernatant (15 min) from 20 mg dry weight cells/ml.

[b] Cyclic AMP formed (pmoles/mg protein/min). Adenyl cyclase activity was measured by Assay A.

[c] Cyclic AMP formed (pmoles/20 mg dry wt cells/min).

[d] Cells:5 μm glass beads ratio = 3:1 (v/v).

TABLE 3.2

Maximum total and specific activity of adenyl cyclase in extracts of S. salivarius obtained by sonication in a Branson Sonifier[a].

	Time (min)	Protein conc. (mg/ml)	Maximum specific activity	Maximum total activity
Without glass beads	10	6.60	17.0	112.2
With glass beads	3	3.62	56.7	205.3

[a] All conditions were as for Table 3.1.

adenyl cyclase activity superior to most of the methods reported in Table 3.1, particularly when glass beads were employed. However, despite the improved cell breakage with the Branson Sonifier, the specific activity of adenyl cyclase in the resultant extracts were low compared to the extracts prepared by other cell-disrupting techniques. As our interest at this stage was the purification of adenyl cyclase, the increased total activity obtained with the Branson Sonifier was deemed more useful than higher specific activities with lower yields. Thus, the purification of adenyl cyclase was undertaken with extracts prepared from glucose-grown cells disrupted in the Branson Sonifier with 5 μm glass beads in the ratio of 3:1 (cells to beads).

III. ENZYME ASSAYS

A. Adenyl cyclase

The activity of adenyl cyclase was determined by measuring the formation of radioactive cyclic AMP from labelled ATP. The following three different assay systems were employed to determine adenyl cyclase activity at different stages of the work.

a) Assay A: This system contained 2 mM α-^{32}P-ATP, 2 mM $MgCl_2$, 8 mM theophylline, 20 mM Tris-HCl buffer (pH 7.5), 9 mM NaF, 50 μg albumin and enzyme in a final volume of 60 μl. NaF and theophylline were added to this assay system to inhibit ATPase and cyclic AMP phosphodiesterase, respectively. The other two radioactive substrates (i.e. ^{3}H-ATP and 8-^{14}C-ATP) were also employed with comparable results (Table 3.3). This

TABLE 3.3

The activity of adenyl cyclase in crude extracts of S. salivarius as measured with ^{32}P-, ^{3}H- and ^{14}C-labelled ATP.

Assay system	Number of experiments	Substrate		
		α-^{32}P-ATP	^{3}H-ATP	8-^{14}C-ATP
A (- NaF)	4	15.9[a]	15.5	16.1
A (complete)	3	70.0	68.7	69.8
B (complete)	3	16.7	16.2	15.9

[a] Enzyme units are expressed as pmoles cyclic AMP formed/mg protein/min. All the figures are average of respective number of experiments.

assay system was used in all studies performed with crude extracts.

b) Assay B: This system was similar to Assay A, except that the NaF was replaced by an ATP-regenerating system consisting of 5 mM P-enol-pyruvate and 5 µg pyruvate kinase. The total volume of the reaction mixture was 0.1 ml. This assay was used during the purification of adenyl cyclase and was later replaced by Assay C.

c) Assay C: This assay contained 0.6 mM 8-^{14}C-ATP, 2 mM $MgCl_2$, 1 mM cysteine, 20 mM Tris-HCl buffer (pH 8.0), 50 µg albumin and enzyme in a final volume of 0.1 ml. This system was used in all studies performed with purified adenyl cyclase following the determination of the optimal assay conditions. When the assay was used in kinetic studies, the ATP concentration ranged between 0.16 mM and 20 mM.

In all of the above assays, the reaction mixtures were incubated for 10 min and the reactions terminated by boiling for 3 min under conditions preventing evaporation. These and all subsequent enzymatic assays were carried out at 37°C.

Cyclic AMP was isolated by two methods: (a) Initial assays (with Assay A) employed a method similar to that of Krishna, Weiss and Brodie (172). Prior to boiling, 0.1 ml of unlabelled cyclic AMP (5 mg/ml) or a cyclic ^{3}H-AMP-carrier mixture (12.5 mM cyclic AMP, 40 mM ATP and 0.2 µC cyclic ^{3}H-AMP (173))was added to each assay to be followed by 0.4 ml of water. The complete mixture was then applied to a column (0.5 x 3.0 cm) of Dowex 50-X8 hydrogen form (100-200 mesh) and the nucleotides eluted with water. The first 3.5 ml of effluent, containing most of the ATP and ADP, was discarded, while the next 3 ml of effluent,

containing most of the cyclic AMP (Fig. 3.1), was collected and subjected to $Ba(OH)_2$-$ZnSO_4$ precipitation (172,173). The purified cyclic AMP fraction (3 ml) was counted in 17 ml of Bray's solution (174) in a liquid scintillation spectrometer (Nuclear Chicago Model 720 Series). The counting efficiency of all ^{14}C samples was determined by the channels ratio method; the standard curve is shown in Fig. 3.2.

The cyclic ^{3}H-AMP-carrier mixture was used only in early experiments to calculate cyclic AMP recovery when α-^{32}P-ATP was employed as the substrate. In such cases, ^{3}H and ^{32}P were counted simultaneously in separate channels of the spectrometer and the cyclic AMP recovery calculated by determining the cyclic ^{3}H-AMP present in the samples. In later experiments, cyclic AMP recovery was calculated by measuring the absorbance of the supernatant fluid at 260 nm and by using the molar extinction coefficient for cyclic AMP (14.6×10^3). The recovery of cyclic AMP by both procedures was 40-60%.

(b) In later experiments, the isolation of cyclic AMP was accomplished by the paper chromatographic method of Drummond and Duncan (175) modified for our purposes. In our assay, 25 µl of unlabelled cyclic AMP (5 mg/ml) was added as carrier to each assay tube, containing 0.1 ml of reaction mixture, just prior to boiling. After cooling at room temperature, 75 µl of the clear supernatant from each tube was spotted on Whatman 3 MM paper and the chromatograms developed (ascending) in a solvent system of 95% ethanol-1 M ammonium acetate (75:30) for at least 12 hr at room temperature. A typical chromatogram is shown in Fig. 3.3 along with the R_f values for adenosine, the adenine nucleotides and

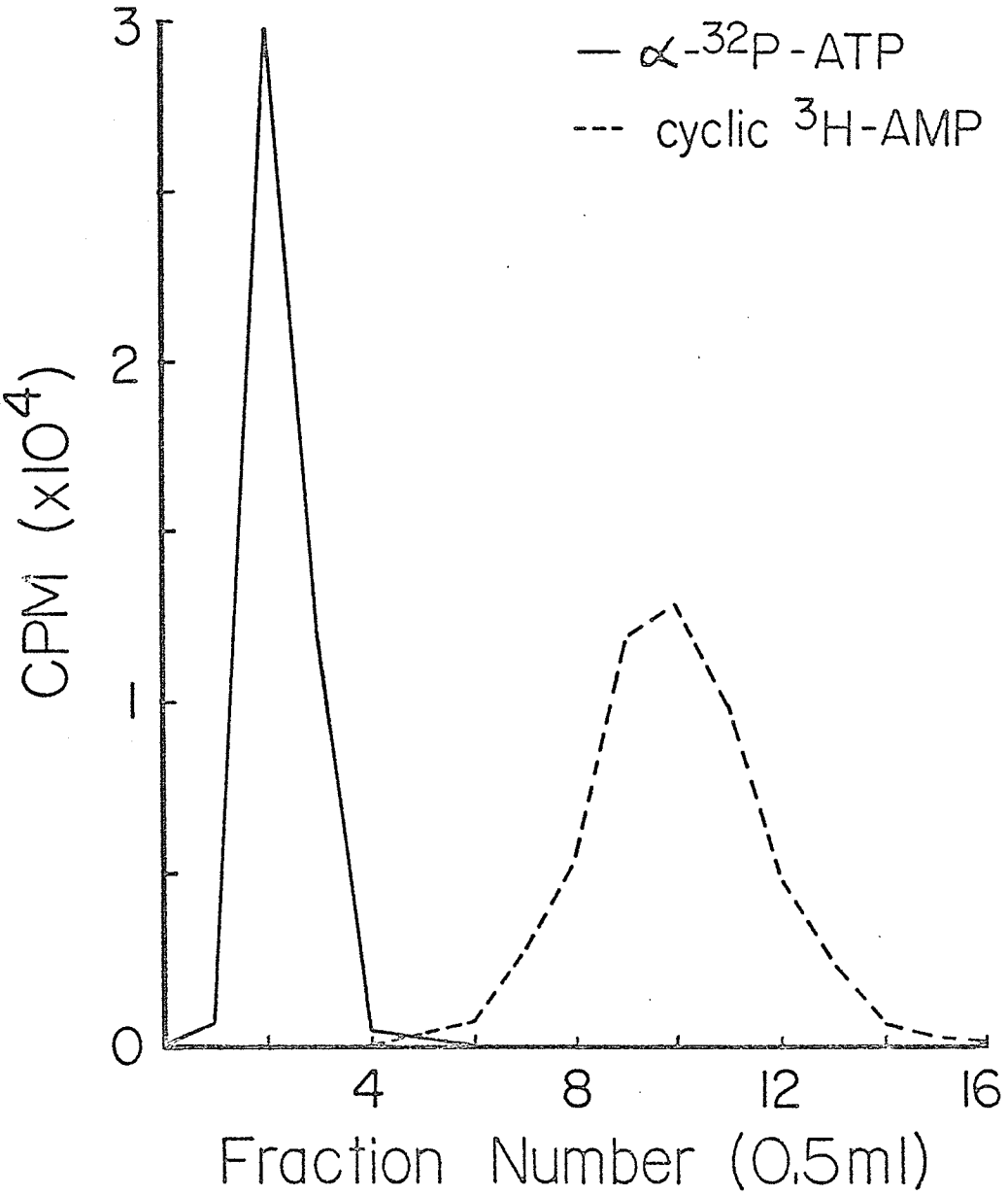

Fig. 3.1. Separation of α-^{32}P-ATP and cyclic ^{3}H-AMP on a column (0.5 x 3.0 cm) of Dowex 50-X 8 hydrogen form (100 - 200 mesh).

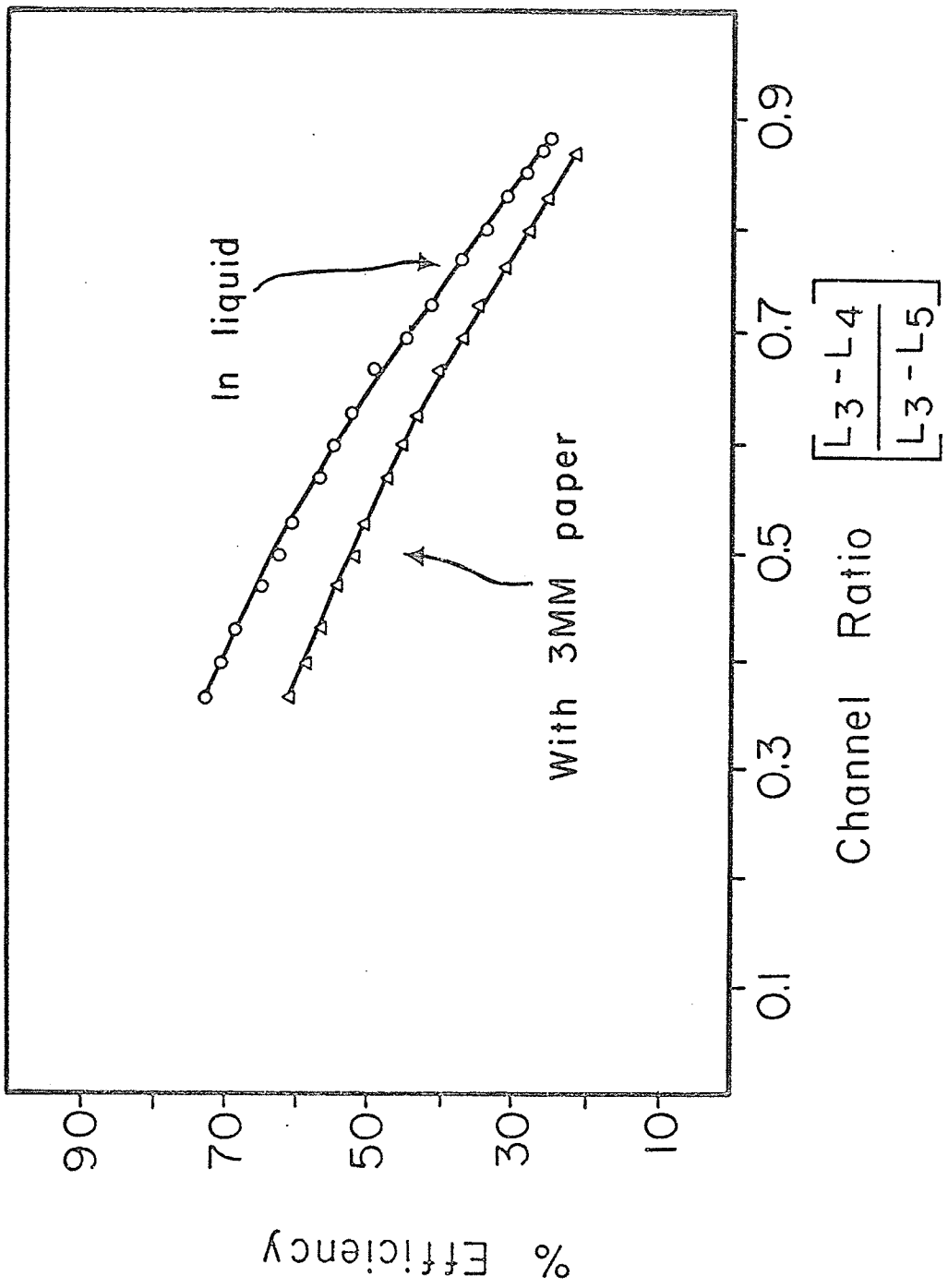

Fig. 3.2. Standard curves permitting the calculation of counting efficiency by the channels ratio method for ^{14}C in Bray's solution with aqueous and paper samples (Whatman 3 MM) in a Nuclear Chicago Spectrometer (Model 720 Series).

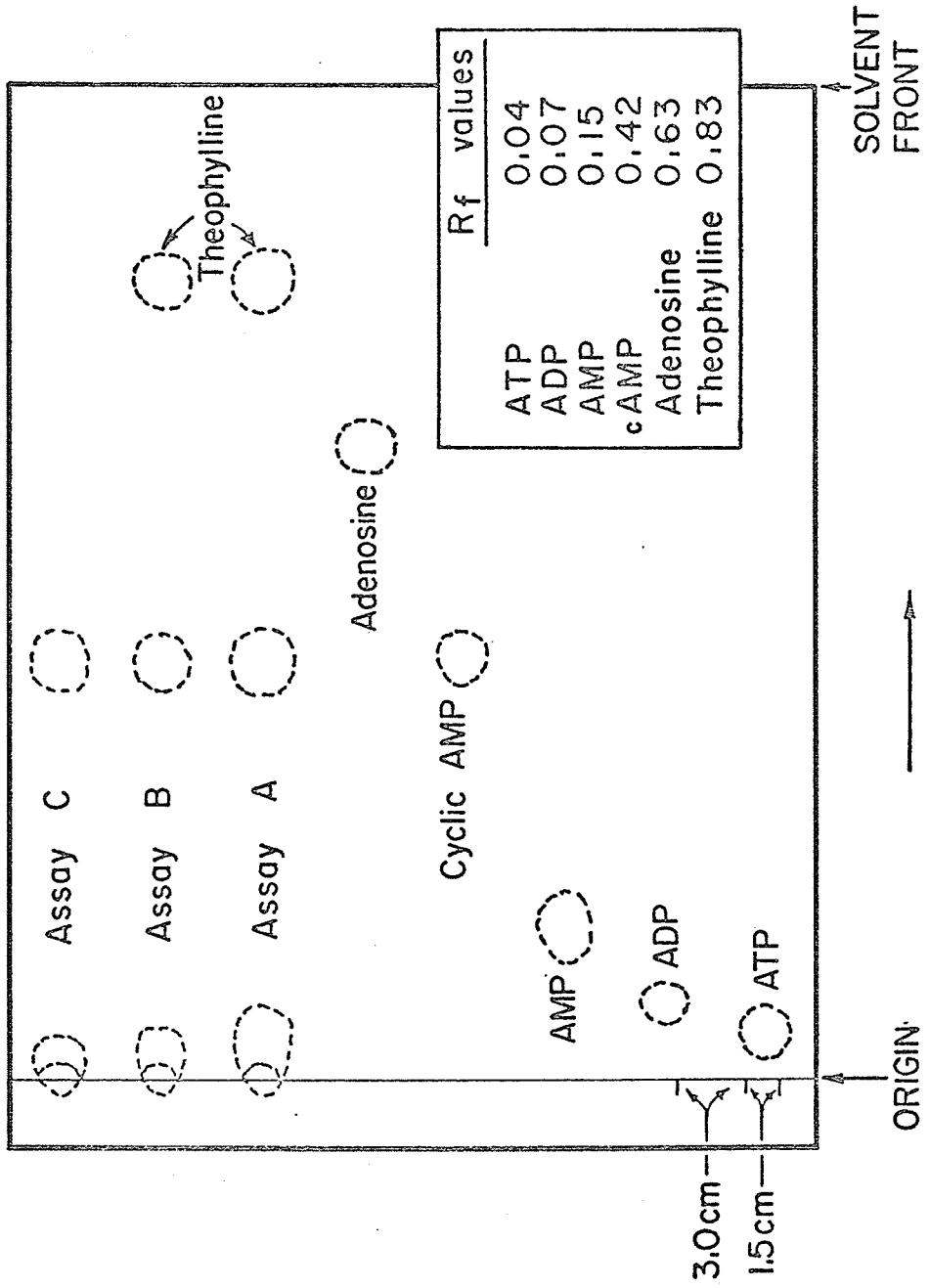

Fig. 3.3. Diagram of a typical chromatogram for the isolation of cyclic AMP formed from ATP by adenyl cyclase in Assay A, B and C. Standards for ATP, ADP, AMP, cyclic AMP and adenosine have been included. The solvent system was 95% ethanol-1 M ammonium acetate (75:30).

theophylline. The spots corresponding to cyclic AMP were located by ultraviolet light, cut out and immersed in 18 ml of Bray's solution followed by liquid scintillation counting. Recovery from the paper was more than 97% with the counting efficiency determined by the channels ratio method from a quenched curve determined specifically with Whatman 3 MM paper (Fig. 3.2). As shown in Table 3.4, the results obtained with the above two methods of isolation were very comparable.

The formation of ATP from cyclic AMP and pyrophosphate by the reversal of the adenyl cyclase reaction was determined with Assay C, except that the ATP was replaced by 2 mM cyclic ^{3}H-AMP and 2 mM sodium pyrophosphate. The ^{3}H-ATP formed in this system was isolated by two methods: (a) by paper chromatography, as previously described, with the ATP spot being counted rather than the spot for cyclic AMP, or (b) by passing the reaction medium through a column (0.5 x 6 cm) of Dowex 50-X8 hydrogen form (100-200 mesh) and eluting with water. In the latter method, the first 0.5 ml was discarded and the next 1 ml, which contained 80-90% of the total ^{3}H-ATP, was collected; 0.8 ml of this latter fraction was counted in 15 ml of Bray's solution. Both methods gave identical results. ADP, AMP and adenosine were not formed in this assay system when tested by paper chromatography employing the $(NH_4)_2SO_4$-sodium acetate-isopropyl alcohol solvent system of Hirata and Hayaishi (148), or a solvent system containing isobutyric acid-NH_4OH (concentrated)-EDTA(0.1 M)-water (66:1:1:32).

Adenyl cyclase activity in the forward or reverse direction is expressed in nmoles of cyclic AMP or ATP formed/mg protein/min, respectively.

TABLE 3.4

The effect of the column and paper chromatographic methods on the isolation of cyclic AMP synthesized by adenyl cyclase in crude extracts of S. salivarius.

Assay system	Number of experiments	Chromatographic method	
		Dowex	Paper
A (- NaF)	2	16.9[a]	16.3
A (complete)	2	83.8	83.9
B (complete)	1	14.5	14.8

[a] All conditions were as for Table 3.3.

B. Guanyl cyclase

The activity of guanyl cyclase was determined by measuring cyclic ^{14}C-GMP formation from ^{14}C-GTP (176-178). The reaction mixture contained 2 mM ^{14}C-GTP, 2 mM $MnCl_2$, 5 mM KCl, 10 mM theophylline, 50 µg albumin, 20 mM Tris-HCl buffer (pH 7.5), 5 mM P-enolpyruvate, 5 µg pyruvate kinase and enzyme in a total volume of 0.1 ml. The incubation and isolation of cyclic GMP was carried out by the paper chromatographic method previously described for cyclic AMP. Enzyme activity is expressed as nmoles of cyclic GMP formed/mg protein/min.

C. Cyclic nucleotide phosphodiesterase

The activity of cyclic nucleotide phosphodiesterase was determined by one of the following three assay procedures.

a) Assay A (P): This procedure was essentially as described by Butcher and Sutherland (18), Rosen (179) and Hirata and Hayaishi (148). In this method, enzyme activity was determined by converting the AMP formed from cyclic AMP by cyclic nucleotide phosphodiesterase to adenosine with snake venom phosphomonoesterase (Naja naja or Crotalus atrox) and assaying the P_i released. The reaction mixture contained 5 mM $MgCl_2$,2 mM cyclic AMP, 50 µg snake venom, 0.1 M Tris-HCl buffer (pH 7.5) and enzyme in a total volume of 0.2 ml. After incubation for 10 min, the reaction was terminated by boiling for 3 min under conditions preventing evaporation. The released P_i was determined either by the method of Chen, Toribara and Warner (180) or by the method of Taussky and Shorr (181), as outlined later in this chapter.

b) Assay B (P): With this assay system, phosphodiesterase activity was determined by measuring the disappearance of the radioactive (^3H) substrate cyclic AMP. The reaction mixture contained 2 mM cyclic ^3H-AMP, 2 mM $MgCl_2$, 2 mM AMP, 50 mM Tris-HCl buffer (pH 7.5) and enzyme in a final volume of 0.1 ml. After incubation for 10 min, the reaction was terminated by the addition of 0.1 ml $ZnSO_4$ (0.25 M) and 0.1 ml of $Ba(OH)_2$ (0.25 M) to remove the reaction product, ^3H-AMP (172,182,183). After 5 min, the samples were centrifuged for 10 min at 3,000 g and 0.1 ml of the clear supernatant was counted in 10 ml of Bray's solution.

c) Assay C (P): This assay procedure was similar to Assay B(P) except that the substrate and product were separated by the paper chromatographic procedure described previously for cyclic AMP (Fig. 3.3). The reaction mixture contained 2 mM cyclic ^3H-AMP, 2 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.5) and enzyme in a final volume of 0.1 ml. Following an incubation period of 10 min, 25 µl of a carrier mixture (cyclic AMP, AMP, adenosine - 5 mg/ml each) was added to each tube and the reaction was terminated by boiling for 3 min. After cooling at room temperature, the samples were centrifuged for 10 min at 3,000 g and 75 µl of the clear supernatant from each tube was spotted on Whatman 3 MM paper and the chromatograms developed as described previously. The spots corresponding to cyclic AMP, AMP and adenosine were located by UV light, cut out and immersed in 18 ml of Bray's solution followed by liquid scintillation counting.

In all cases, the efficiency of the assay procedure was tested with cyclic nucleotide phosphodiesterase from rat brain.

D. ATPase, GTPase and pyrophosphatase

The activities of adenosine triphosphatase (ATPase) (EC 3.6.1.4), guanosine triphosphatase (GTPase) and inorganic pyrophosphatase (EC 3.6.1.1.) were determined by measuring the P_i released from their substrates, i.e., ATP, GTP and sodium pyrophosphate, respectively (148). For ATPase, the reaction mixture contained 5 mM $MgCl_2$, 5 mM ATP, 0.1 M Tris-HCl buffer (pH 7.5) and enzyme in a total volume of 0.5 ml. GTPase and pyrophosphatase were determined in a similar manner, except that 5 mM GTP and 5 mM sodium pyrophosphate were used as the substrate, respectively. In all cases, incubation was for 10 min and the reaction was terminated by boiling. Enzyme activity is expressed as nmoles of P_i released/mg protein/min.

E. Glycogen phosphorylase

Glycogen phosphorylase was assayed by measuring the incorporation of glucose-^{14}C moiety from glucose-^{14}C-1-P into glycogen (184,185). The reaction mixture contained 0.1 M β-glycerophosphate buffer (pH 6.8), 1 mM glucose-^{14}C-1-P, 0.5 mg glycogen (Fisher Scientific Co., Fair Lawn, New Jersey) and enzyme in a final volume of 0.1 ml. Enzyme activity with the Fisher glycogen was same as that obtained with the glycogen isolated from S. salivarius (186). The reaction proceeded for 30 min and was terminated by the addition of 1.5 ml of 75% methanol containing 1% KCl. The precipitated radioactive glycogen was then centrifuged at 3,000 g for 10 min and the subsequent pellet dissolved in 0.2 ml of 0.1 M glycine-NaOH buffer (pH 10.5) containing 5 mM $ZnSO_4$ and 10 µg of

alkaline phosphatase. This mixture was incubated for 20 min and then the glycogen again precipitated by the addition of 1.5 ml of the methanol-KCl solution. The pellet, obtained after centrifugation, was dissolved in 0.2 ml of water, and the precipitation and centrifugation steps repeated. The final pellet was dissolved in 1 ml of water and an aliquot (usually 0.8 ml) added to 10 ml of Bray's solution for liquid scintillation counting. Phosphorylase activity is expressed in nmoles of glucose-^{14}C incorporated into glycogen/mg protein/min.

F. Phosphofructokinase

Phosphofructokinase activity was determined by measuring the fructose-1,6-P_2 formed from fructose-6-P and ATP (65). The reaction mixture contained 50 mM glycylglycine buffer(pH 6.9), 0.5 mM ATP, 1 mM fructose-6-P, 50 mM KCl, 1 mM $MgCl_2$, 10 mM cysteine, 0.1 mg albumin and enzyme in a final volume of 1.0 ml. After an incubation period of 5 min, the reaction was terminated by adding 1 ml of 8% perchloric acid. The samples were then neutralized with 45% KOH and centrifuged at 3,000 g for 30 min to remove the insoluble potassium perchlorate.

The amount of fructose-1,6-P_2 in aliquots of the supernatant fluid was determined by the enzymatic-fluorometric method of Maitra and Estabrook (187). The assay involved the conversion of fructose-1,6-P_2 to glyceraldehyde-3-P, dihydroxyacetone-P and finally to α-glycerophosphate in presence of commercial aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase and NADH. Quantitation was obtained from an internal standard of fructose-1,6-P_2. The decrease in the fluorescence was analyzed in a Farrand Ratio Fluorometer equipped with

a modified Heath model EU-20B Servo recorder (Farrand Optical Co., Mt. Vernon, New York). The standard reaction mixture contained 20 mM triethanolamine buffer (pH 7.6), 20 mM KCl, 2 mM $MgCl_2$, 15 µm NADH, 10 µg α-glycerophosphate dehydrogenase, 10 µg triosephosphate isomerase, 25 µg aldolase and the appropriate amount of sample in a final volume of 1.0 ml. Enzyme activity is expressed as nmoles of fructose-1,6-P_2 formed/mg protein/min.

G. P-enolpyruvate phosphotransferase system (PEP-PTS)

The activity of the PEP-PTS for glucose transport was assayed "in vivo" with toluene-treated cells of S. salivarius according to the method of Gachelin (188). The assay consisted of measuring the ^{14}C-2-deoxyglucose-6-P formed by the toluene-treated cells in the presence of ^{14}C-2-deoxyglucose and P-enolpyruvate. Cells were grown and harvested as previously described and incubated in phosphate buffer (50 mM, pH 7.0) in the presence of toluene (5 µl/ml) with gentle shaking for 30 min. Following this, the cells were incubated at a concentration of 2 mg dry wt/ml in a reaction mixture containing 5 mM 2-deoxyglucose-U-^{14}C and 5 mM P-enolpyruvate in a final volume of 1.0 ml. Following incubation, the ^{14}C-2-deoxyglucose-6-P formed in the reaction was separated from 2-deoxyglucose-U-^{14}C by the method of Romano et al (189). In this method, aliquots of the reaction mixture were applied to columns (0.6 x 3 cm) of Dowex 1-X8, formate form (200-400 mesh) and the substrate 2-deoxyglucose-^{14}C eluted with 3 ml of water. The ^{14}C-2-deoxyglucose-6-P was then eluted with 3.5 ml of 0.5 M ammonium formate in 0.2 M formic acid and collected in liquid scintillation counting vials. After the addition of 16.5 ml of

Bray's solution to each vial, the samples were counted in a liquid scintillation spectrometer.

IV. ANALYTICAL METHODS

A. Protein

Protein was determined by the method of Lowry et al (190). To the samples containing 0-50 µg of protein (in 0.2 ml) was added 1 ml of a copper sulfate reagent (a 49:1 mixture of 2% Na_2CO_3 in 0.1 N NaOH and 0.5% $CuSO_4.5H_2O$ in 1% potassium sodium tartarate). The resultant solutions were mixed vigorously and then allowed to stand at room temperature for 10 min after which 0.1 ml of 1 N phenol reagent was added and mixed instantaneously. After a period of 30 min, the samples were read in a Unicam SP 500 spectrophotometer at a wavelength of 600 nm. Bovine serum albumin was used as the protein standard (Fig. 3.4(A)).

B. Phosphate

The amount of phosphate was determined by two methods. In initial experiments, phosphate was determined by the procedure of Chen, Toribara and Warner (180) (method I), whereas in later stages of the work, phosphate was determined as described by Taussky and Shorr (181) (method II). Method II, although less sensitive than method I, was used in some situations because of its simplicity and quick colour development. However, in experiments requiring greater accuracy, phosphate was determined by method I.

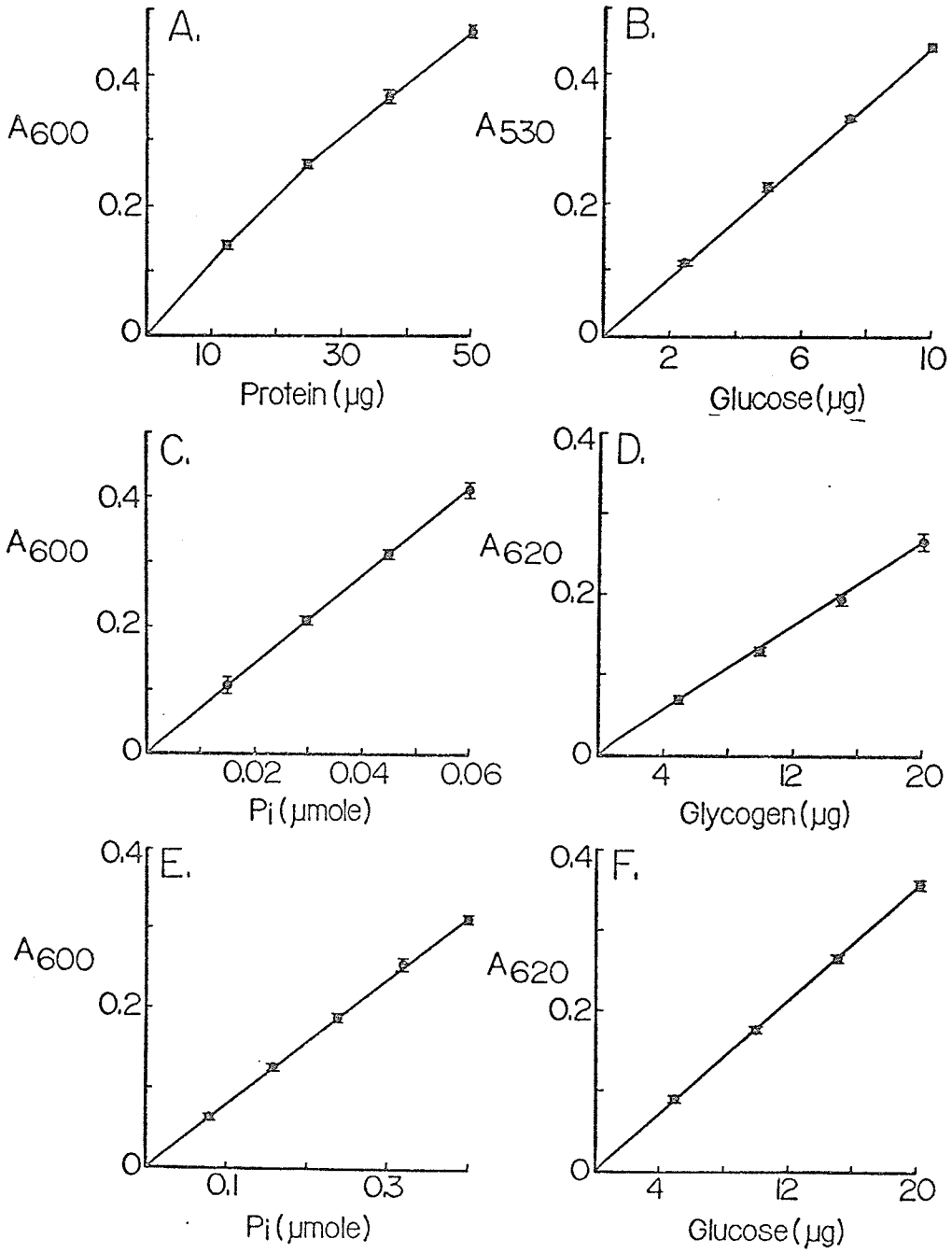

Fig. 3.4. Standard curves for (A) Protein by the method of Lowry et al (190), (B) glucose by the method of Kingsley and Getchell (191), (C) inorganic phosphate (I) by the method of Chen, Toribara and Warner (180), (D) glycogen by the anthrone method (192,193), (E) inorganic phosphate (II) by the method of Taussky and Shorr (181), and (F) glucose by the anthrone method.

a) Method I. To the samples containing 0-0.06 µmole of inorganic phosphate (in 0.5 ml), was added 0.5 ml of a mixture of 0.5% ammonium molybdate-1.2 N H_2SO_4-2% ascorbic acid and the tubes well mixed. The tubes were then covered with parafilm and incubated at 37°C for at least one hr. After cooling to room temperature, the optical density of the samples was determined at 600 nm in a Unicam SP 500 spectrophotometer. A typical standard curve for such a determination is shown in Fig. 3.4(C).

b) Method II. With this procedure, $FeSO_4.7H_2O$ was used in place of ascorbic acid as the reducing agent. To the samples containing 0-0.4 µmole of inorganic phosphate (in a volume of 2 ml with 12% trichloroacetic acid), was added 1 ml of a mixture of 1% ammonium molybdate-1.0 N H_2SO_4-5% $FeSO_4.7H_2O$ and the tubes mixed instantly. After 5 min at room temperature, the optical density of the samples was determined at 600 nm along with the appropriate standard (Fig. 3.4(E)).

C. Glucose assay

Glucose was determined by the glucose oxidase method of Kingsley and Getchell (191). Each assay tube contained 0-10 µg of glucose in 0.2 ml, 0.8 ml of peroxidase buffer reagent and 0.1 ml of glucose oxidase (10 mg/ml). The peroxidase buffer solution contained 0.42 g K_2HPO_4, 0.56 g KH_2PO_4, 2.81 mg peroxidase and 2.81 ml of 1% O-dianisidine (3',3'-dimethoxybenzidine) in a volume of 250 ml. After incubation at 37°C for 1 hr, 0.4 ml of 37.4% H_2SO_4 was added and the tubes were allowed to cool to room temperature. The absorbance of the samples were then read at 530 nm in a spectrophotometer. A typical standard curve is shown in Fig. 3.4(B).

D. Glycogen assay

The concentration of the glycogen used in the glycogen phosphorylase assay was determined by the anthrone method of Morris (192), modified by Sandham and Kleinberg (193). To the assay tube, containing 0-20 µg of glycogen in 0.4 ml, was added 0.8 ml of freshly prepared anthrone reagent (2 g anthrone in 1 litre of 95% H_2SO_4) and the contents of the assay tubes mixed immediately. The assay tubes were then placed in an ice bath until the contents had reached room temperature and then heated in boiling water bath for 6-8 min with continuous shaking. Following this, the tubes were again brought to room temperature in an ice bath and absorbance of the samples determined at 620 nm. The anthrone standard curves for glycogen and for glucose are shown in Fig. 3.4(D) and 3.4(F), respectively.

E. Quantitative analysis for cyclic AMP

In addition to determining adenyl cyclase activity in enzyme preparations of S. salivarius, experiments were also designed to measure the cyclic AMP content in cells and in the medium under a variety of conditions. Initially, the quantitative analysis of cyclic AMP was carried out by the procedure of Brooker, Thomas and Appleman (194,195) (method A), however, this method was replaced in later studies by the more sensitive assay of Gilman (196) (method B).

In method (A), the cells were separated from the medium by centrifugation at 3,000 g for 5 min at $0^{o}C$. Following this, the cells were extracted at room temperature in one ml of 1 N perchloric acid containing a tracer amount of cyclic ^{3}H-AMP. After a 30 min period,

the samples were neutralized to pH 7.4 - 7.6 with KOH-triethanolamine buffer (185,197) followed by rapid freezing and thawing. The clear supernatant, obtained after removal of the cell debris and precipitated $KClO_4$ by centrifugation, was further fractionated by passage through a column (0.5 x 6 cm) of Dowex 50-X8 hydrogen form (100-200 mesh). The fractions (3.5 - 6.5 ml) containing the cyclic AMP were subjected to $Ba(OH)_2$-$ZnSO_4$ treatment as previously described (172) and the supernatants, obtained after centrifugation, adjusted to pH 8.0 The recovery of the cyclic AMP following this procedure was determined by counting an aliquot of the sample.

An appropriate amount of each sample (maximum 50 µl containing 20 - 320 pmoles of cyclic AMP) was placed in a vial followed by 50 µl of a substrate-mixture consisting of 120 mM Tris-HCl buffer (pH 8.0), 2.5 mM EGTA, 120 mM $MgCl_2$, 0.12 mM 5'-AMP and 0.16 µM cyclic ^{3}H-AMP (about 10,000 cpm). Similarly, 50 µl of this substrate mixture was added to vials containing standard amounts of non-radioactive cyclic AMP (0 - 320 pmoles) in a volume of 50 µl. The reaction was started in all vials by the addition of 50 µl of an enzyme mixture containing rat brain phosphodiesterase (prepared by the procedure of Brooker, Thomas and Appleman (195)), snake venom, Naja naja (1 mg/ml), bovine serum albumin (2 mg/ml), 60 mM Tris-HCl buffer (pH 8.0) and 5 mM 2-mercaptoethanol. Sufficient phosphodiesterase was added to permit the hydrolysis of about 40% of the cyclic ^{3}H-AMP without the addition of the non-radioactive cyclic AMP. The reaction was stopped after a 10 min incubation period by the addition of 0.8 ml of a slurry (50% settled volume) of Dowex 1-X2, chloride form (200-400 mesh) and

equilibrated for another 10 min. After the equilibration period, 10 ml of Bray's solution was added to each vial and the radioactivity counted by the liquid scintillation procedure. A typical standard curve for known amounts of cyclic AMP is shown in Fig. 3.5(A).

In later studies, the sensitive method of Gilman (196) (method B) was employed for the quantitative assay of cyclic AMP. This method is based on binding competition between known amounts labelled cyclic AMP and unknown quantities of unlabelled cyclic AMP for a cyclic AMP-dependent protein kinase. Increased amounts of unlabelled cyclic AMP in the assay mixture produced a linear decrease (on a log scale) in radioactive (^{3}H) cyclic AMP binding to the protein kinase (Fig. 3.5(B)). The protein kinase and protein kinase inhibitor preparations required for this assay were prepared from bovine skeletal muscle as outlined by Gilman (196).

This assay system was employed to measure the cyclic AMP content in cells and in the extracellular medium separately. The cells were separated from the medium by a rapid filtration procedure (<20 sec) using Celite 503 as a filter aid. One ml of a 5% celite suspension was spread evenly on a 0.45 μm Acropor filter (Gelman Instrument Co., Ann Arbor, Michigan) in a Millipore filter holder attached to a suction flask. This amount of celite prevented clogging of the filter by S. salivarius cells and was sufficient to filter up to 10 mg dry weight of cells. After filtering the original volume of cell suspension, the celite-cell layer was washed with 0.5 ml of phosphate buffer (50 mM, pH 7.0) to remove the intercellular volume of medium. The filtrate was collected in a test tube placed inside the suction flask. The cells

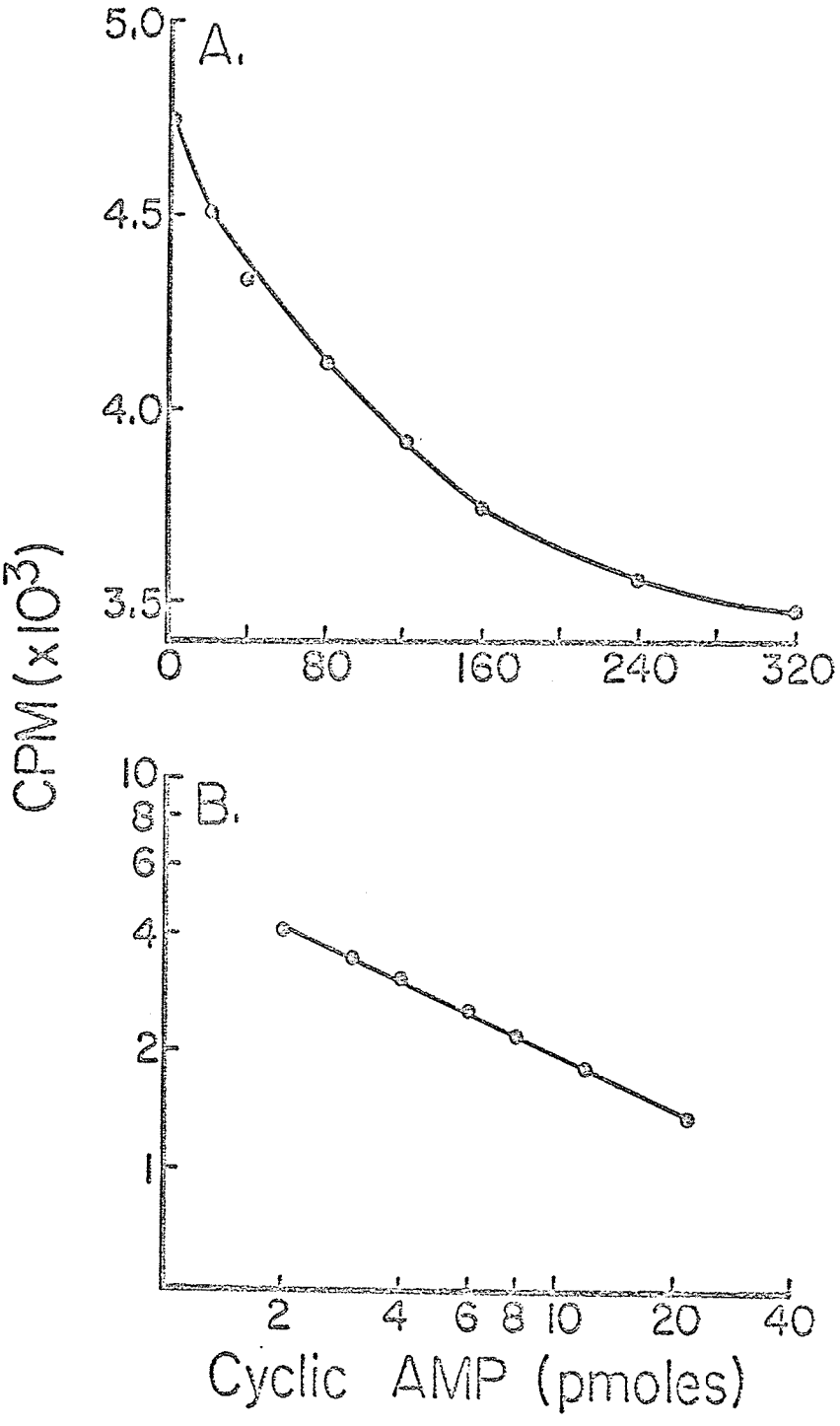

Fig. 3.5. Standard curves for the quantitative determination of cyclic AMP by (A) the method of Brooker, Thomas and Appleman (195), and (B) the method of Gilman (196).

were extracted by adding 0.5 ml phosphate buffer and 0.5 ml of 1 N perchloric acid to the filter-cell-celite fraction in a centrifuge tube; the extraction period was 30 min. This extraction mixture was then centrifuged and the clear supernatant was subjected to $Ba(OH)_2$-$ZnSO_4$ treatment as outlined previously. Following centrifugation, the supernatant was adjusted to pH 4.0 for cyclic AMP analysis. The recovery of cyclic AMP through all these procedures was more than 95%.

The filtrate containing the extracellular cyclic AMP was subjected only to $Ba(OH)_2$-$ZnSO_4$ treatment followed by pH adjustment. In certain cases when the cyclic AMP content of the filtrate was low, the samples were concentrated to dryness by flash evaporation. The dried samples were usually dissolved in one-tenth of the original volume with 50 mM sodium acetate-acetic acid buffer (pH 4.0) before analysis.

The cyclic AMP in both sample types was assayed with a standard binding reaction mixture containing 50 mM sodium acetate-acetic acid buffer (pH 4.0), 20 nM cyclic ^{3}H-AMP (15,000 - 20,000 cpm) sufficient protein kinase (binding protein) to bind 30-40% of the nucleotide, enough protein kinase inhibitor to give maximal activation of the nucleotide binding to protein kinase, and standard or sample cyclic AMP (unlabelled and in the range of 1-20 pmoles) in a final volume of 0.1 ml. Reactions were always started by the addition of the protein kinase and terminated after a 60 min incubation period at 4°C by the addition of 1 ml of cold phosphate buffer (pH 6.0). The samples were then filtered through a 0.22 μm cellulose-ester filter (Millipore GSWP) previously rinsed with the same buffer. The filters, now containing the cyclic AMP-protein kinase complex, were washed with approximately 10 ml of the

phosphate buffer and then placed in a counting vial with 3 ml of methyl cellosolve. The vials were then shaken until the filters were dissolved and 10 ml of a scintillation mixture (4 g PPO, 50 mg POPOP in 1 litre of toluene) subsequently added to each vial for liquid scintillation counting. A typical standard curve for known amounts of cyclic AMP is shown in Fig. 3.5(B).

F. Disc gel electrophoresis

Disc gel electrophoresis was carried out by the method of Ornstein (198) and Davis (199), as modified by Kapitany and Zebrowski (200). Acrylamide gels (7%) were used and stained, just after the electrophoresis, with 0.5% Amido Schwartz solution for one hr and were then destained electrophoretically.

IV. DATA PROCESSING

All of the results presented in this thesis are the average of at least duplicate determinations. All kinetic data in double reciprocal plots of 1/v and 1/s (201), in Dixon plots (202) of 1/v against inhibitor concentration, in Hill plots (203), and in slope and intercept replots (204,205), were subjected to linear regression and correlation analysis with a program for the Olivetti-Underwood Programma 101. Correlation coefficients were never less than 0.96. Basic statistical calculations, such as estimation of standard errors, calculations of analysis of variance and student's t-test, were also carried out with suitable programs.

V. MATERIALS

All radioactive compounds were purchased from either International Chemical and Nuclear Corporation (City of Industry, California) of New England Nuclear Corporation (Boston, Massachusetts), and were purified, when necessary, by suitable chromatographic methods. All nucleotides, glycolytic intermediates and commercial enzymes used in this study were purchased either from Boehringer Mannheim Corporation (New York, N.Y.), or from Sigma Chemical Co. (St. Louis, Missouri). Cyclic AMP and Aquacide I were purchased from Calbiochem (La Jolla, California). RNase, DNase and snake venom (*Naja naja* or *Crotalus atrox*) were obtained from Sigma Chemical Co. All other chemicals were of reagent grade.

CHAPTER 4

PURIFICATION AND PROPERTIES OF ADENYL CYCLASE

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- B. Subcellular distribution of enzyme activity
- C. Optimal conditions for enzyme assay
- D. Effect of NaF
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VI. DISCUSSION

I. INTRODUCTION

Adenyl cyclase catalyzes the formation of cyclic AMP and inorganic pyrophosphate from ATP (equation 1). The enzyme is widely distributed in animals (17,175,183,206-234), higher plants (235,236) and in bacteria (148,155-159,237). In mammalian systems, evidence suggests that the enzyme is particulate, being found in cell membranes (209,211-218), in mitochondria (209-211,217,225) and in the nuclear fraction (233,234).

Adenyl cyclase activity in bacterial systems has been found both in particulate (155-159) and in soluble fractions (148,157-159). The enzyme from the soluble fraction of Brevibacterium liquefaciens was shown to require both Mg^{2+} and pyruvate for activity (148). In E. coli, Mg^{2+}-dependent adenyl cyclase activity was either particulate (155,156) or partially particulate, being easily solubilized by buffer extraction (157,158). The E. coli enzyme was inhibited by NaF and inorganic pyrophosphate (157), as well as by ADP, UTP, CTP, GTP, oxaloacetate, pyruvate and pyridoxal-P (158). Similarly, the enzyme in B. liquefaciens was inhibited by AMP, dAMP, ADP, dATP and GTP (148).

More recently, Ide (159) demonstrated adenyl cyclase activity in 21 strains of bacteria and classified the enzyme from these strains on the basis of their solubility and activation by pyruvate. In this study, the enzyme from Nocardia erythropolis was studied in detail and, like the enzyme from E. coli, was strongly inhibited by various nucleoside triphosphates, oxaloacetate and pyridoxal-P, but was unaffected by NaF.

In the present study, the properties of adenyl cyclase in crude extracts of S. salivarius are outlined as well as the protocol for the purification of the enzyme from the soluble fraction. The purified enzyme

was then subjected to kinetic analysis followed by a detailed study of the effects of various cellular constituents, such as nucleotides and glycolytic intermediates, on the activity of the enzyme.

II. CRUDE EXTRACT STUDIES

Unless otherwise indicated, all studies with crude extracts of S. salivarius were performed on the 30,000 g supernatant fraction obtained following disruption of washed, glucose-grown cells in a Biosonik sonic oscillator for 30 min at 0°C. Assay A was used to measure adenyl cyclase activity in these crude preparations.

A. Enzyme activity during growth

As preliminary step to enzyme purification, the activity of adenyl cyclase was assayed in crude extracts prepared from cells obtained at different phases of growth. Maximum adenyl cyclase activity was observed in cells harvested in the late exponential or early stationary phase of the growth when the exogenous carbon source (glucose) was almost depleted (Fig. 4.1). In this thesis, all studies on adenyl cyclase were, therefore, performed with enzyme preparations obtained from cells in the early stationary growth phase.

B. Subcellular distribution of enzyme activity

In order to determine whether adenyl cyclase activity in S. salivarius was associated with particulate or soluble fractions, a crude extract, prepared from sonically disrupted cells, was fractionated by differential centrifugation. The extract was centrifuged in a stepwise

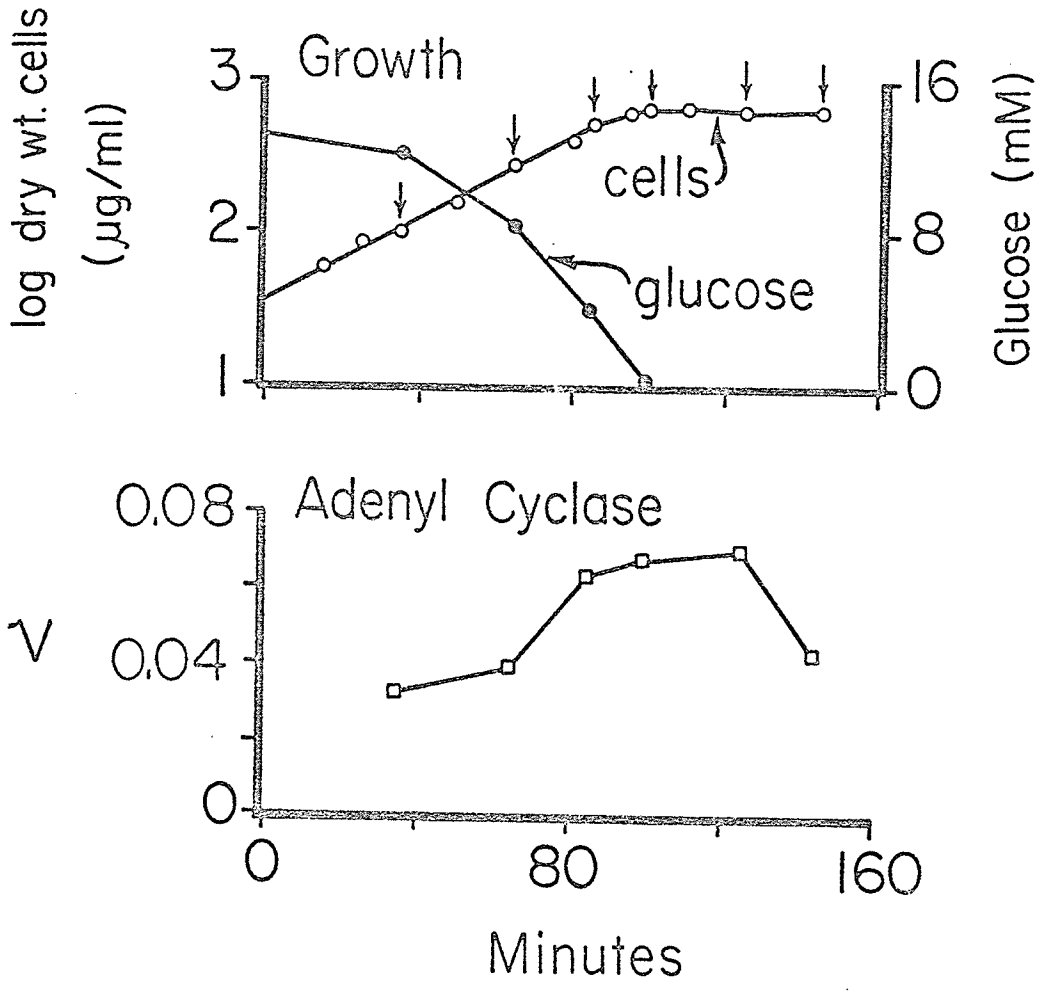


Fig. 4.1. Adenyl cyclase activity in crude extracts prepared from cells of S. salivarius harvested at different phases of growth. Enzyme activity was determined on 30,000 g supernatant (15 min) with assay A. Enzyme activity (v) is expressed as nmoles of cyclic AMP formed/mg protein/min.

fashion at 10,000, 30,000 and 50,000 g for 15 min each and at 100,000 g for 1 hour at 0°C. The maximum specific enzyme activity was observed in the pellet obtained between 30,000 and 50,000 g, although the total activity was maximum in the 100,000 g supernatant (Fig. 4.2). Enzyme activity in the whole homogenate (i.e., sonically disrupted cell suspension before centrifugation) was very low and the total enzyme activity obtained in the different fractions was higher than that observed with total whole homogenate. This was probably due to the presence of interfering material in the whole homogenate. As will be shown later, a similar increase in total enzyme activity was observed during ammonium sulfate fractionation of the enzyme.

As shown above, the major portion of the adenyl cyclase activity was associated with the soluble fraction of the cell and for this reason initial studies with crude extracts employed the 30,000 g supernatant fraction.

C. Optimal conditions for enzyme assay

Prior to enzyme purification, the optimal conditions for adenyl cyclase activity were determined with respect to time of incubation, protein concentration and pH of the reaction mixture. Employing Assay A, cyclic AMP formation was shown to be almost linear for 10 min with crude extracts of S. salivarius (Fig. 4.3(A)). Adenyl cyclase activity was also proportional to protein concentration with a distinct change in slope beyond 0.2 mg protein (Fig. 4.3(B)). This latter effect was probably due to the presence of increasing concentrations of ATPase in the crude extract. The amount of this substrate-degrading enzyme at high protein levels probably exceeded the effective concentration of NaF in the assay mixture

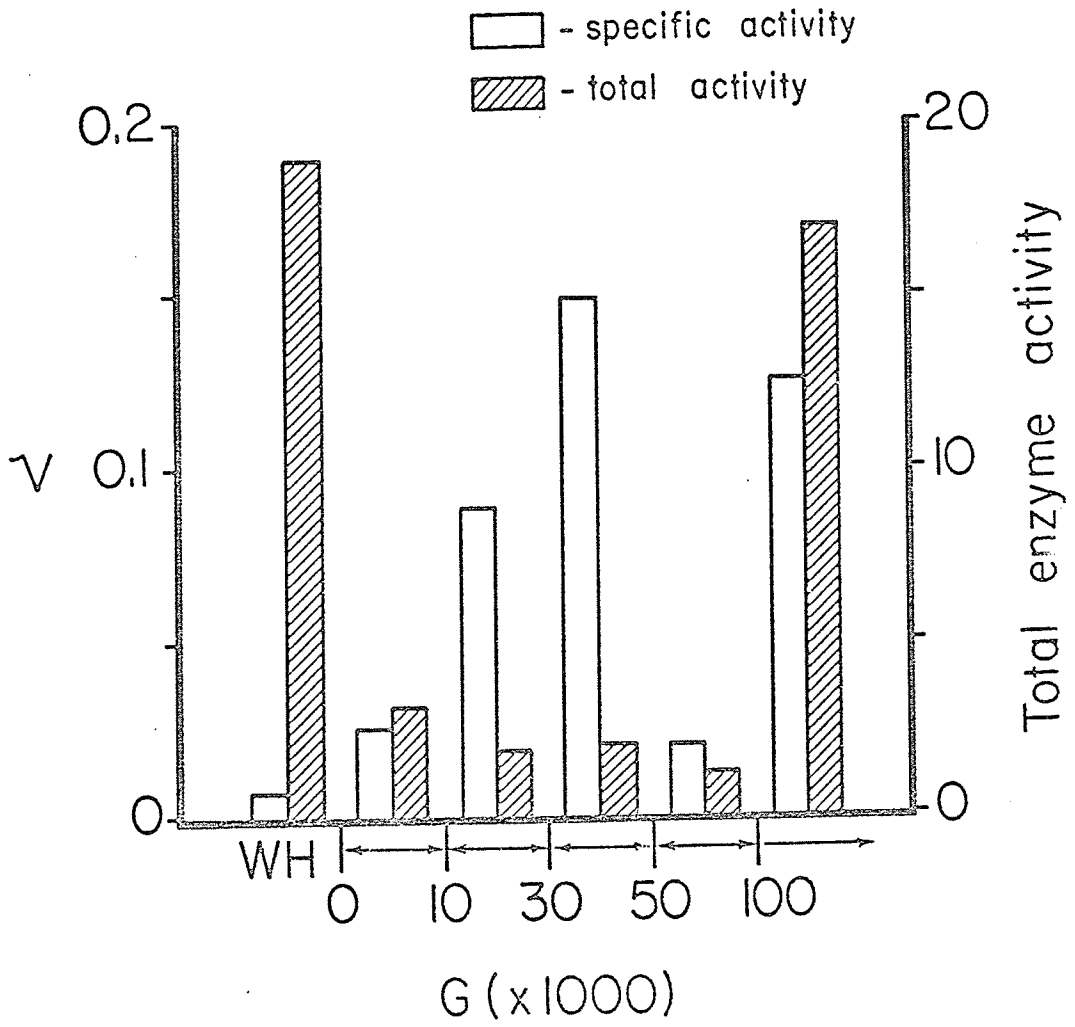


Fig. 4.2. Adenyl cyclase activity in the whole homogenate (WH) of S. salivarius and in various fractions obtained by differential centrifugation. The crude extract was prepared from 200 mg dry wt cells and the total enzyme activity calculated from the protein content of those cells. Assay conditions as for Fig. 4.1.

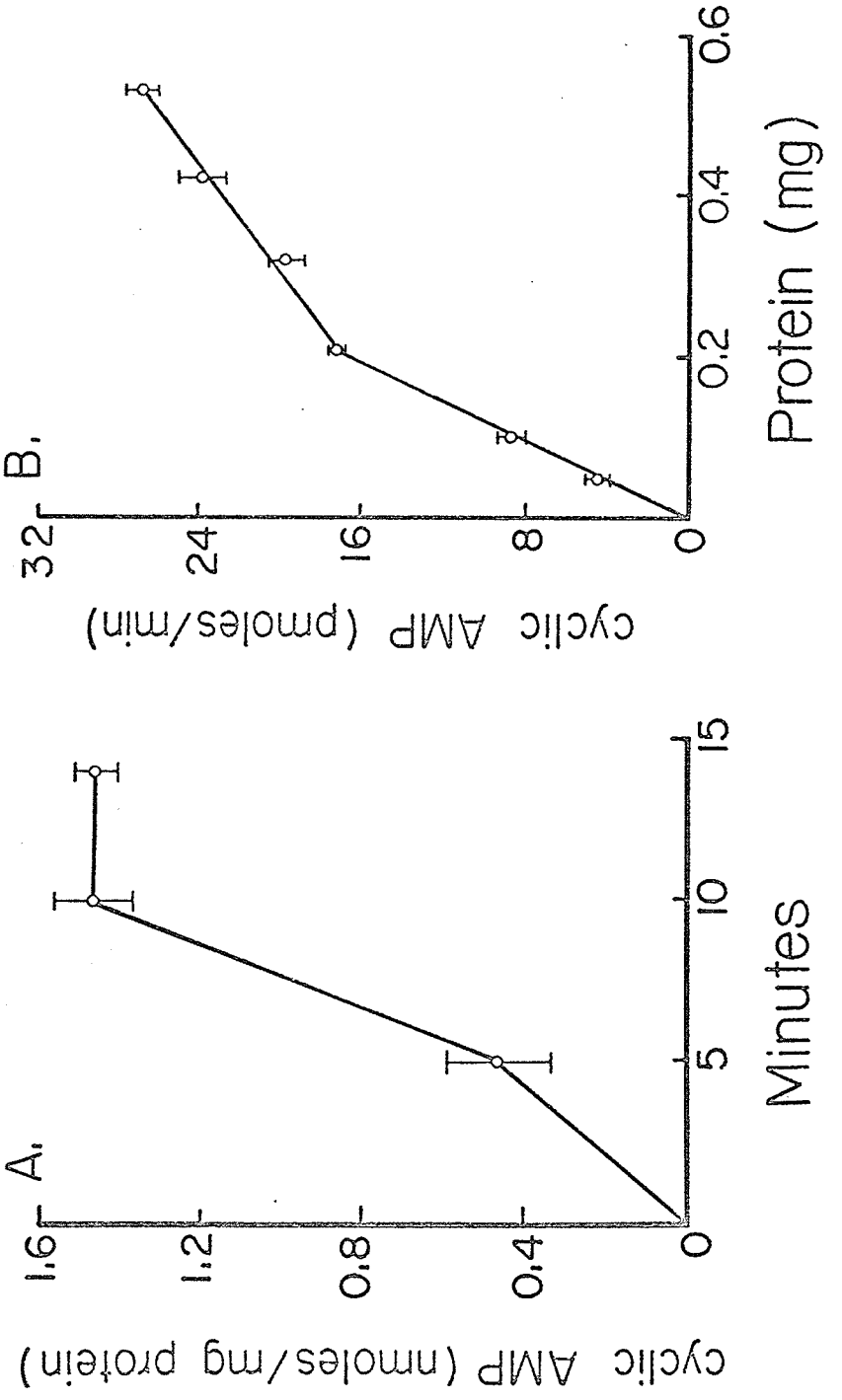


Fig. 4.3. Effect of time of incubation (A) and protein concentration (B) on the activity of adenyl cyclase in crude extracts of S. salivarius.

(see D below). Although the presence of cyclic nucleotide phosphodiesterase in the extracts could have accounted for this effect, subsequent study demonstrated that this enzyme is absent in S. salivarius (Chapter 5). Based on these findings, all assays with crude extracts were carried out with less than 0.2 mg protein for an incubation period of 10 min.

Attempts to find the optimum pH for adenyl cyclase activity were carried out at pH values ranging from 4 to 11. Surprisingly, no single pH optimum was observed for the enzyme in crude extracts since the activity increased and decreased at three different pH values, 5, 8, 10, as shown in Table 4.1. These results could have been caused either by the presence of interfering enzyme(s) in the crude extract, as mentioned above, or by the presence of more than one enzyme or isozyme, each enzyme having a different pH optimum. Since reasonably good activity was obtained between pH 7 and pH 8, all assays with crude extracts used Tris-HCl buffer at pH 7.5.

D. Effect of NaF

NaF is an activator of adenyl cyclase in mammalian systems (17,175,238-244). This compound also activated adenyl cyclase activity in crude extracts of S. salivarius with the maximum stimulation of the enzyme being observed with 20 mM NaF (Fig. 4.4). The maximum activation observed with this compound, however, varied from experiment to experiment normally ranging from 2.5 - 9-fold. This variation may have been due to different amounts of ATPase activity in the different crude extract preparations. ATPase activity in such preparations was inhibited 30% by 10 mM NaF. As will be shown later, NaF also had a direct activating effect on S. salivarius adenyl cyclase.

TABLE 4.1

Effect of pH on the activity of adenyl cyclase in the crude extracts of *S. salivarius*.

pH	Enzyme activity
4	16.0[a]
5	42.9
6	11.2
7	31.7
8	39.2
9	21.2
10	30.4
11	23.0

[a] Adenyl cyclase activity was determined with Assay A. The buffers used were sodium citrate-citric acid (pH 4-6), Tris-HCl (pH 7-9), Glycine-NaOH (pH 9-11). Enzyme activity is expressed as pmoles of cyclic AMP formed/mg protein/min.

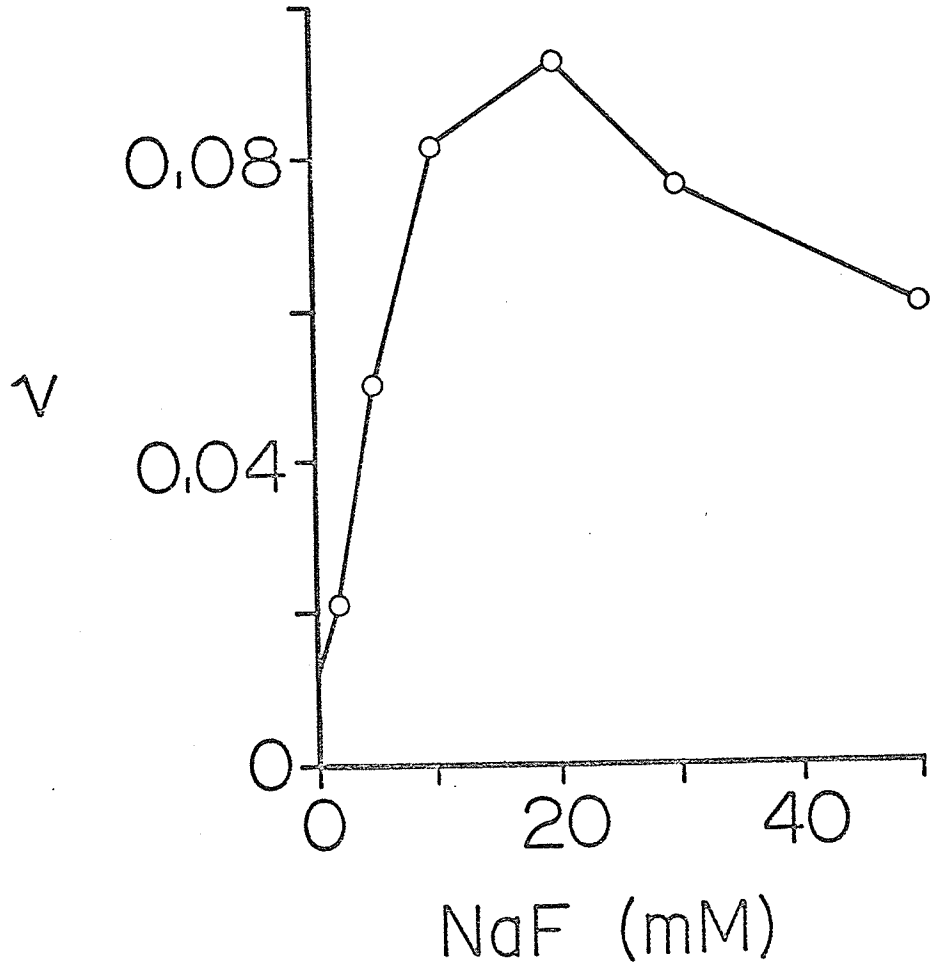


Fig. 4.4. Effect of NaF on the activity of S. salivarius adenyl cyclase in crude extracts. Assay A was used except that the NaF concentration was varied as indicated. Enzyme units (v) as in Fig. 4.1.

E. Enzyme stability

Adenyl cyclase activity in cell-free extracts of S. salivarius prepared by Biosonik sonic disruption was unstable, losing all of its activity in about 40 days even after storage at -70°C (Fig. 4.5). Attempts were made to stabilize enzyme activity by adding, to extract preparations stored at -70°C, various reducing agents (10 mM) such as, mercaptoethanol, mercaptoacetic acid, cysteine, dithiothreitol and glutathione. The agents, however, failed to protect enzyme activity regardless of whether the extracts were kept frozen until use, or frozen and thawed at 0°C at different time intervals (Table 4.2). In fact, adenyl cyclase activity in fresh cell-free preparations was inhibited initially by all of the above reducing compounds, except mercaptoethanol and mercaptoacetic acid. Despite this fact, however, the activity of the preparations treated with mercaptoethanol and mercaptoacetic acid decreased at the rate of about 3% a day, while the control extract decreased at a slightly faster rate (4%). While the adenyl cyclase activity decreased at a slower rate (1-2% a day) with dithiothreitol and cysteine-treated extract preparations, the initial activity with these compounds was only 53% and 59% of the control activity in fresh preparations, respectively. The initial activity of adenyl cyclase in the presence of 10 mM glutathione was only 64% that of the control activity.

Subsequent partial purification of the enzyme (60-fold) through DNase treatment, ammonium sulfate fractionation and gel filtration on Sephadex G-50 produced preparations which were very stable, losing less than 1% activity in 38 days when stored at -70°C (Table 4.3). Lyophilization of this partially purified enzyme caused an initial loss in activity as well as decreased stability at -70°C. The results obtained with crude

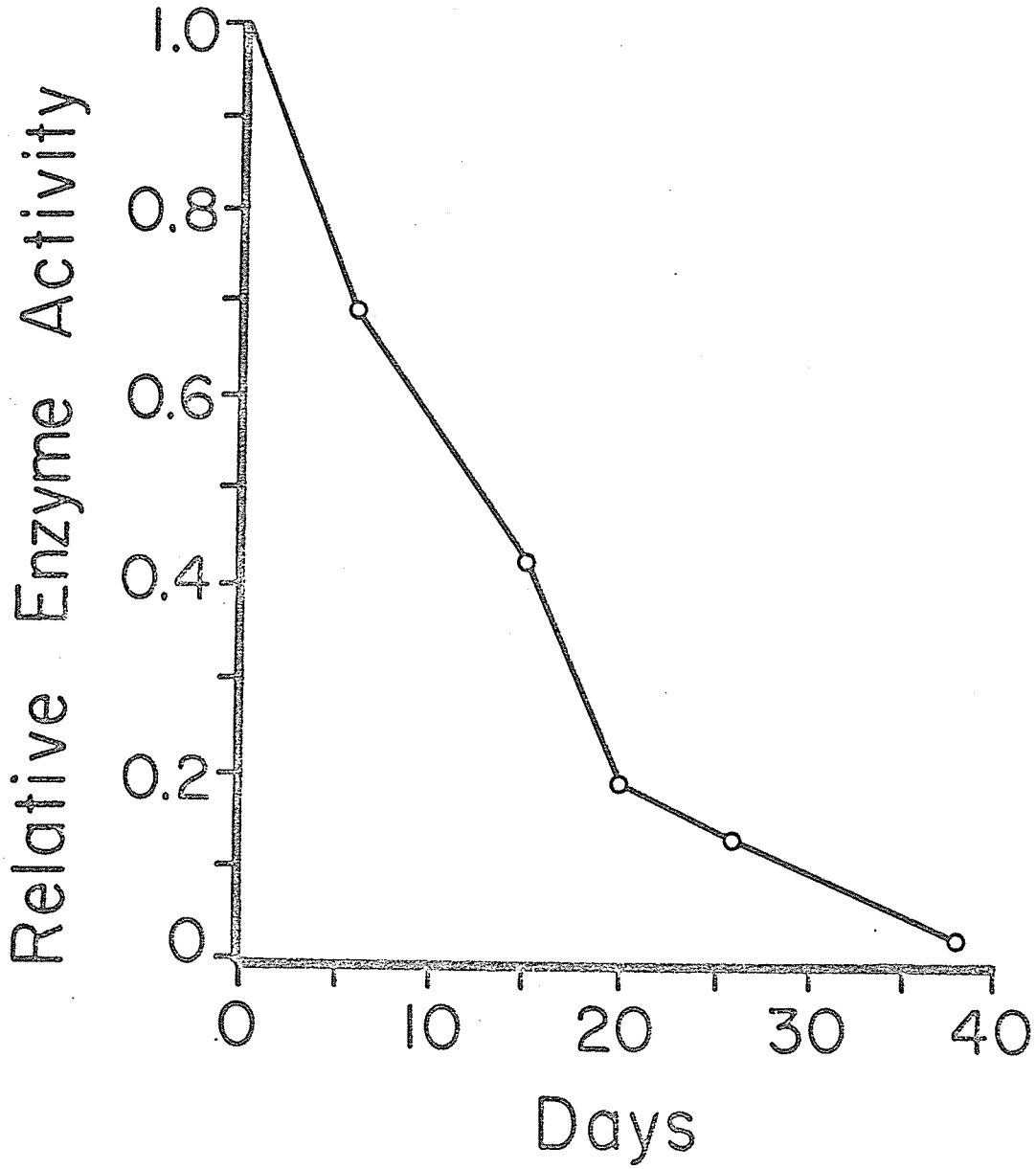


Fig. 4.5. Stability of adenyl cyclase activity in crude extracts of S. salivarius during storage at -70°C.

TABLE 4.2

Stability of adenyl cyclase in crude cell-free preparations of S. salivarius stored in presence and absence of various reducing agents at -70°C.

Reducing agents[a]	Enzyme activity		Percent loss activity
	Initial	After 20 days	
None	91.0[b]	17.0	81.3
Glutathione	58.0	15.0	74.1
Mercaptoacetic acid	95.0	29.0	69.5
Mercaptoethanol	87.0	41.0	52.9
Cysteine	54.0	35.0	35.2
Dithiothreitol	48.0	32.0	33.3

[a] Final concentration was 10 mM.

[b] Enzyme activity is expressed as pmoles of cyclic AMP formed/mg protein/min.

TABLE 4.3

Comparative stability of adenyl cyclase in crude extract and partially purified (60-fold) preparations of S. salivarius.

	Enzyme activity		Percent loss activity
	Initial	After 38 days	
Crude			
Control	91[a]	2	98
+ Mercaptoethanol (10 mM)	87	9	90
Purified			
Lyophilized	3740	1270	66
Frozen (-70°C)	5820	5810	<1

[a] Enzyme activity is expressed as pmoles of cyclic AMP formed/mg protein/min.

extracts, stored with and without mercaptoethanol, are also shown in this table for comparison.

III. PURIFICATION OF ADENYL CYCLASE

A. Crude homogenate

For the purpose of enzyme purification, adenyl cyclase was always prepared from early stationary phase cells of S. salivarius (Fig. 4.1). The cells were disrupted with a Branson Sonifier in the presence of 5 μm glass beads for 3 min at 0°C; the ratio of cells to glass beads was 3 to 1. The supernatant obtained after centrifugation of these sonically disrupted cells at 2,000 g was considered the crude homogenate. Since the crude enzyme was unstable even in the presence of reducing agents (Table 4.2) but was stable in more purified form (Table 4.3), purification procedures were initiated immediately after cell breakage in the absence of a reducing agent. Assay B, which contained an ATP-regenerating system, was used to measure adenyl cyclase activity at all steps of the purification process except with the enzyme preparations obtained after gel filtration on Sephadex G-200. These latter samples were assayed with Assay C, which contained no ATP-regenerating system, since it was subsequently shown that these fractions were devoid of ATPase activity.

B. Centrifugation at 100,000 g and DNase treatment

Further centrifugations of the crude enzyme at 100,000 g for 90 min resulted in the removal of 40% of the total protein with retention

of approximately 80% of the adenyl cyclase activity in the supernatant (Table 4.4). Incubation of the supernatant with DNase (10 µg/ml) and 2 mM $MgCl_2$ for 30 min at room temperature resulted in increased specific activity upon dialysis. The dialyzed, DNase-treated preparations had increased enzyme activity when compared to that of the non-dialyzed samples indicating that nucleotides lost during dialysis probably inhibited the enzyme. Furthermore, RNase or protamine sulfate treatment of the 100,000 g supernatant always resulted in loss of enzyme activity even after dialysis. Therefore, only DNA was removed at this stage.

C. Ammonium sulfate fractionation

After DNase treatment, the extract was brought to 45% saturation with ammonium sulfate and the pellet, obtained after centrifugation at 20,000 g for 10 min, was discarded. The pellet, which was obtained after further precipitation to 80% saturation and which contained most of the enzyme, was retained and dissolved in Tris-HCl buffer. A typical profile of the adenyl cyclase activity in the various ammonium sulfate fractions is shown in Fig. 4.6. The enzyme at this stage was stable for at least two weeks at -70°C. Surprisingly, when the adenyl cyclase activity of this preparation was assayed after dialysis a 6-fold increase in total activity was observed in spite of a 40% reduction in total protein (Table 4.4). The same results were obtained on 4 different occasions. Tao and Lipmann (157) reported similar results while purifying adenyl cyclase from E. coli, indicating the presence of inhibitors or interfering proteins in extract preparations of both E. coli and S. salivarius.

TABLE 4.4

Purification of adenyl cyclase from S. salivarius[a].

Step	Protein (mg)	Specific activity ($X10^{-2}$)	Fold purification	Total activity
1. Crude homogenate	492	1.2[b]	1	5.9
2. 100,000 g supernatant	288	1.6	1.3	4.6
3. DNase treatment	268	2.2	1.8	5.9
4. Ammonium sulfate (45-80%)	157	23.2	19.3	36.4
5. Sephadex G-200 eluate				
Peak I	0.60	319	267	1.9
Peak II	1.02	1166	975	11.9
Peak III	0.33	3857	3217	12.7

[a] Enzyme activity in all of the above fractions except those from Sephadex G-200 column chromatography was determined after overnight dialysis against 50 mM Tris-HCl buffer (pH 7.5).

[b] nmoles cyclic AMP formed/mg protein/min.

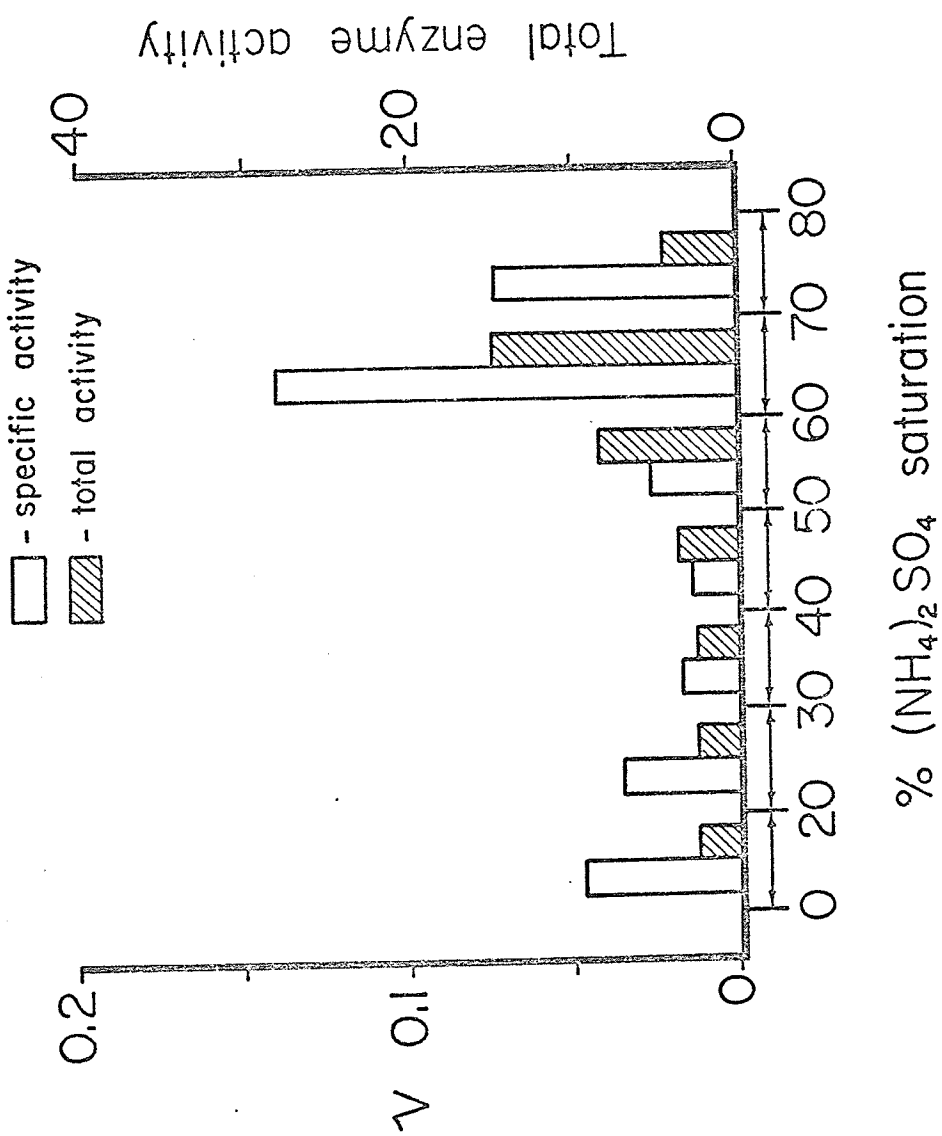


Fig. 4.6. Ammonium sulfate fractionation of S. salivarius adenyl cyclase activity following 100,000 g centrifugation and DNase treatment. Enzyme activity was determined with Assay B (containing an ATP-regenerating system) after overnight dialysis of the samples against Tris-HCl buffer (50 mM, pH 7.5).

D. Gel filtration

Five ml (approximately 80 mg protein) of the 45 - 80% ammonium sulfate fraction was applied to a column (5.0 x 85 cm) of Sephadex G-200 which had been previously equilibrated with Tris-HCl buffer (50 mM, pH 7.5). The column was run in the ascending direction at a flow rate of 12 ml per hour with the same buffer. The enzyme was recovered in three distinct peaks (I, II and III) in low protein fractions (Fig. 4.7). The fractions in each peak were pooled, concentrated with Aquacide, and stored separately in small aliquots at -70°C after overnight dialysis. High enzyme specific activities were always obtained in each peak when small amounts of protein in small volumes were applied to the column. Higher protein concentrations, on the other hand, resulted in a 2 - 3-fold decrease in the specific activity.

Further purification of enzyme III by ion exchange chromatography on DEAE-cellulose or CM-cellulose always resulted in inactivation of the enzyme. Therefore, all further studies were carried out on the enzymes obtained after Sephadex G-200 gel filtration.

IV. PROPERTIES OF PURIFIED ADENYL CYCLASE

A. Interfering enzymes

The activity of all interfering enzymes, i.e., ATPase, GTPase, inorganic pyrophosphatase and guanyl cyclase was determined at all steps. As shown in Table 4.5, adenyl cyclase activity in the three peaks obtained from gel filtration on Sephadex G-200 was free of all of these enzymes, except guanyl cyclase. The specific activity of this enzyme in the three

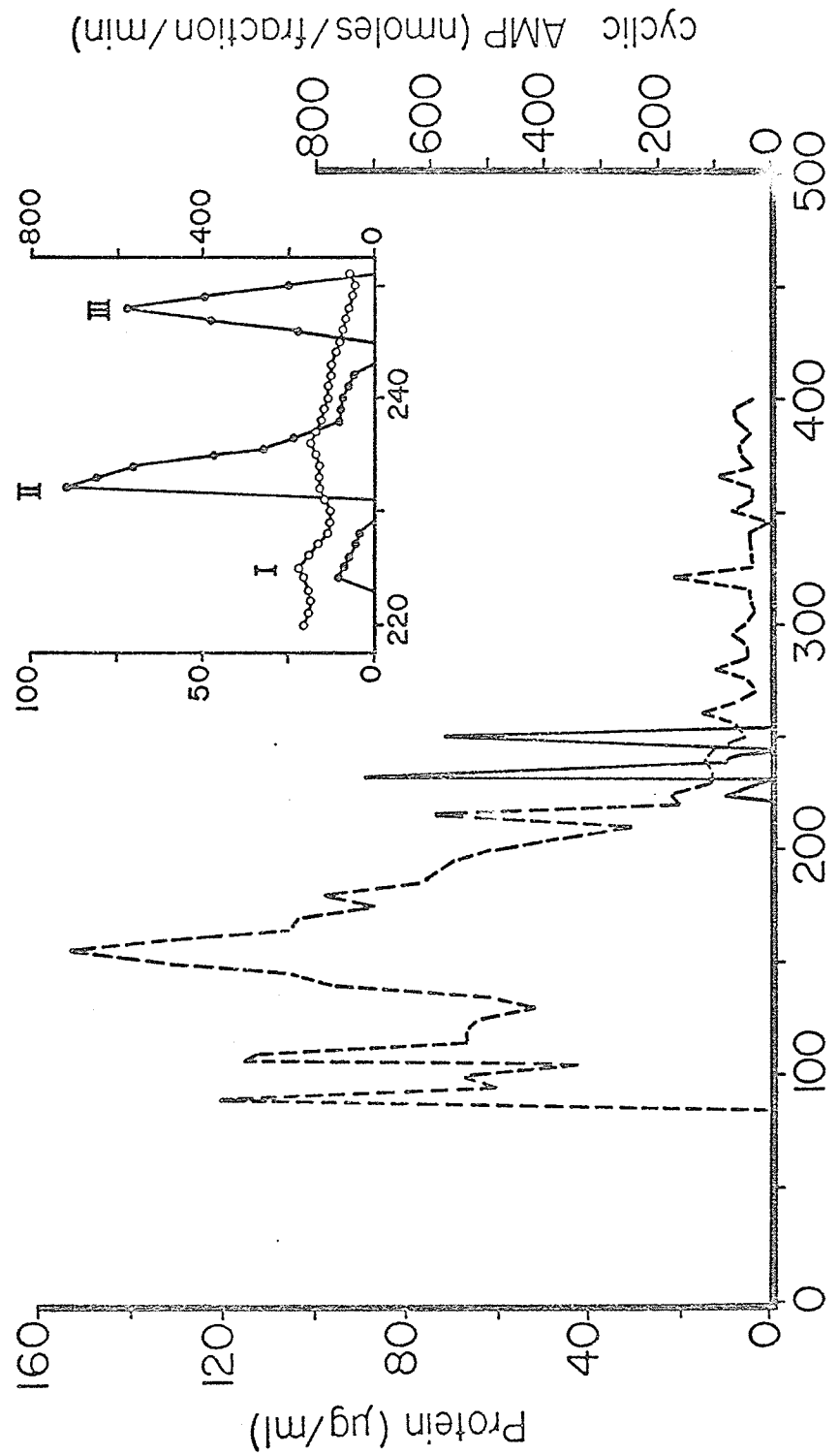


Fig. 4.7. Profile of gel filtration of S. salivarius adenyl cyclase on Sephadex G-200. 78 mg of protein was applied to a 5.0 by 85 cm column of Sephadex G-200 and run with 50 mM Tris-HCl (pH 7.5). The flow rate was 12 ml/hour and 120 drop fractions were collected. Inset diagram is an expansion of the area where the enzyme activity is found. Broken lines (- - - -) and open circles (-O-O-) denote protein. Solid lines (———) and closed circles (-●-●-) denote enzyme activity. Salt routinely emerged from the column after fraction 400.

TABLE 4.5

Presence of interfering enzymes at different steps of the purification of S. salivarius adenyl cyclase.

	Step	Percent of initial activity			
		ATPase	GTPase	Inorganic pyrophosphatase	Guanyl cyclase
1.	Crude homogenate	100 (29.0)[a]	100 (14.0)[a]	100 (97.0)[a]	100 (19.0)[b]
2.	100,000 g supernatant	14.6	0	63.7	74.6
3.	DNase treatment	7.2	0	67.6	137.4
4.	Ammonium sulfate (45-80%)	3.5	0	18.1	73.0
5.	Sephadex G-200 eluate				
	Peak I	0	0	0	3.8
	Peak II	0	0	0	6.7
	Peak III	0	0	0	2.1

Values in parentheses indicates actual initial activity

(a) nmoles P_i released/mg protein/min.

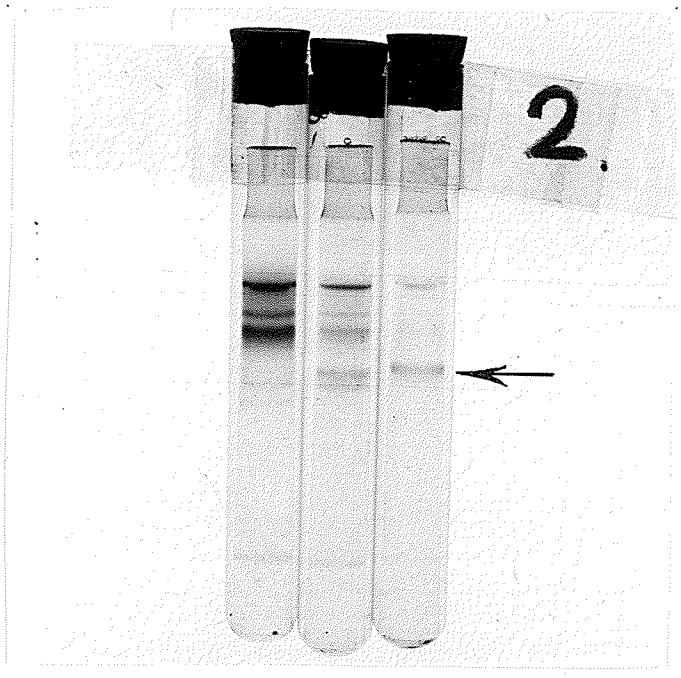
(b) nmoles cyclic 3',5'-GMP formed/mg protein/min.

peaks increased 30-fold compared to its activity in the crude extract, while the total activity ranged from 2.1 - 6.7%. As will be shown later, this activity may have been due either to natural contamination of the preparations with this enzyme or simply represented the non-specific activity of adenyl cyclase. The homogeneity of the protein in the peaks, as tested by disc gel electrophoresis and Amido/Schwartz staining, increased from peak I through peak III (Fig. 4.8). This latter peak was essentially pure, being contaminated with two very light and well-separated bands. While peaks I and II each contained five contaminating bands, peak II had much less protein in these bands than did peak I.

B. Stability

Adenyl cyclase from all three peaks was very unstable at 4°C and lost more than 90% of the original activity in about 2 weeks. Storage at -70°C reduced the rate of loss in activity such that 3 months was required to reach the same level of inactivation. In order to reduce this loss, various reducing agents were tested for their ability to prevent inactivation at -70°C. Of the agents tested, cysteine (5 or 10 mM) was the most effective for all three preparations (Table 4.6). Mercaptoacetic acid was equally effective for the enzyme in peak I, but less satisfactory for the enzyme in peaks II and III. The other reducing agents tested were much less effective. With this in mind, all enzyme preparations were stored with 5 mM cysteine at -70°C. Unlike their effect on crude extract preparations (Table 4.2), these reducing agents did not inhibit the activity of the purified enzyme.

Fig. 4.8. Electrophoretic pattern of the proteins contained in the three different peaks of adenyl cyclase activity obtained after gel filtration on Sephadex G-200.



I II III

TABLE 4.6

Effect of sulfhydryl reagents on the stability of adenyl cyclase in the three enzyme peaks eluting from Sephadex G-200[a].

Additions	Concentration	Percent activity after 3 months		
		Peak I	Peak II	Peak III
	mM			
None	-	8	11	10
Cysteine	1	79	77	71
	5	100	101	101
	10	99	100	102
Mercaptoacetic acid	1	51	53	26
	5	101	75	52
	10	100	86	63
Dithiothreitol	5	15	23	16
	10	26	33	25
Reduced glutathione	5	11	22	15
	10	13	27	20
Mercaptoethanol	5	10	12	10
	10	20	22	29

[a] The enzyme in all three peaks was concentrated with Aquacide after G-200 Sephadex chromatography and stored at -70°C with and without sulfhydryl reagents. Initial activity determined without sulfhydryl reagent was taken as 100%.

C. Optimal conditions for the purified enzyme

The reaction velocity of adenyl cyclase in all three preparations was linear at enzyme concentrations up to at least 1.75 µg protein (Fig. 4.9(A)). At this enzyme level, the rate of cyclic AMP formation was linear for 20 min (Fig. 4.9(B)). Thus, all assays employed less than 1 µg of enzyme for an incubation period of 10 min.

As shown previously (Table 4.1), the optimum pH for the enzyme was difficult to establish with crude extracts probably because NaF and theophylline were present in the assay system (Assay A). However, when the purified enzyme in peaks I, II and III was assayed as a function of pH, much clearer results were observed (Fig.4.10). The activity of enzymes II and III was optimum at pH 8.0, while the optimum for enzyme I was between 7.0 and 7.5. Maximum activity at 8.0 has been reported for frog erythrocyte adenyl cyclase (183), while the optima for E. coli (155-157) and B. liquefaciens (148) was between pH 9 and 10.

The enzyme had an absolute requirement for a divalent ion with either Mg^{2+} or Mn^{2+} salts satisfying this requirement (Table 4.7). No detectable activity was observed with Ca^{2+}, Zn^{2+}, Ba^{2+} and only partial activity with Sn^{2+}. At a fixed concentration of ATP (2 mM), the optimum Mg^{2+} concentration was 2 mM for each of the three enzyme preparations (Fig. 4.11), suggesting that the actual substrate for the enzymes is MgATP. The apparent K_m for Mg^{2+} was 1.12 mM, 0.89 and 0.63 mM for the enzyme in peaks I, II and III, respectively.

D. NaF activation

Like the enzyme in animal tissues (17,175,238-244), the purified adenyl cyclase from S. salivarius was activated by NaF. As indicated

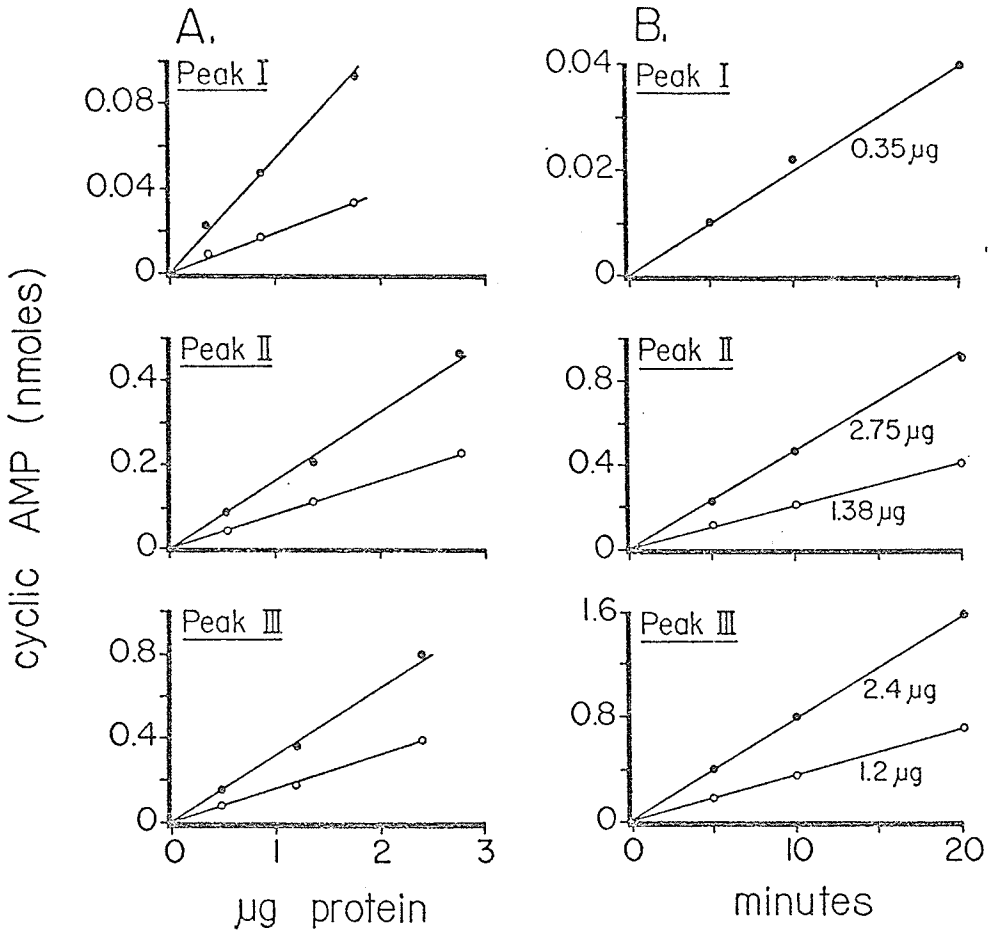


Fig. 4.9. A. Effect of protein concentration on adenyl cyclase activity in peaks I, II and III obtained from Sephadex G-200 column chromatography. Incubation time: (-O-O-)-5 min, (-◎-◎-)-10 min.

B. Effect of incubation time on adenyl cyclase activity in peaks I, II and III. Values indicate the amount of protein used in each reaction mixture.

Assay C was used in all cases.

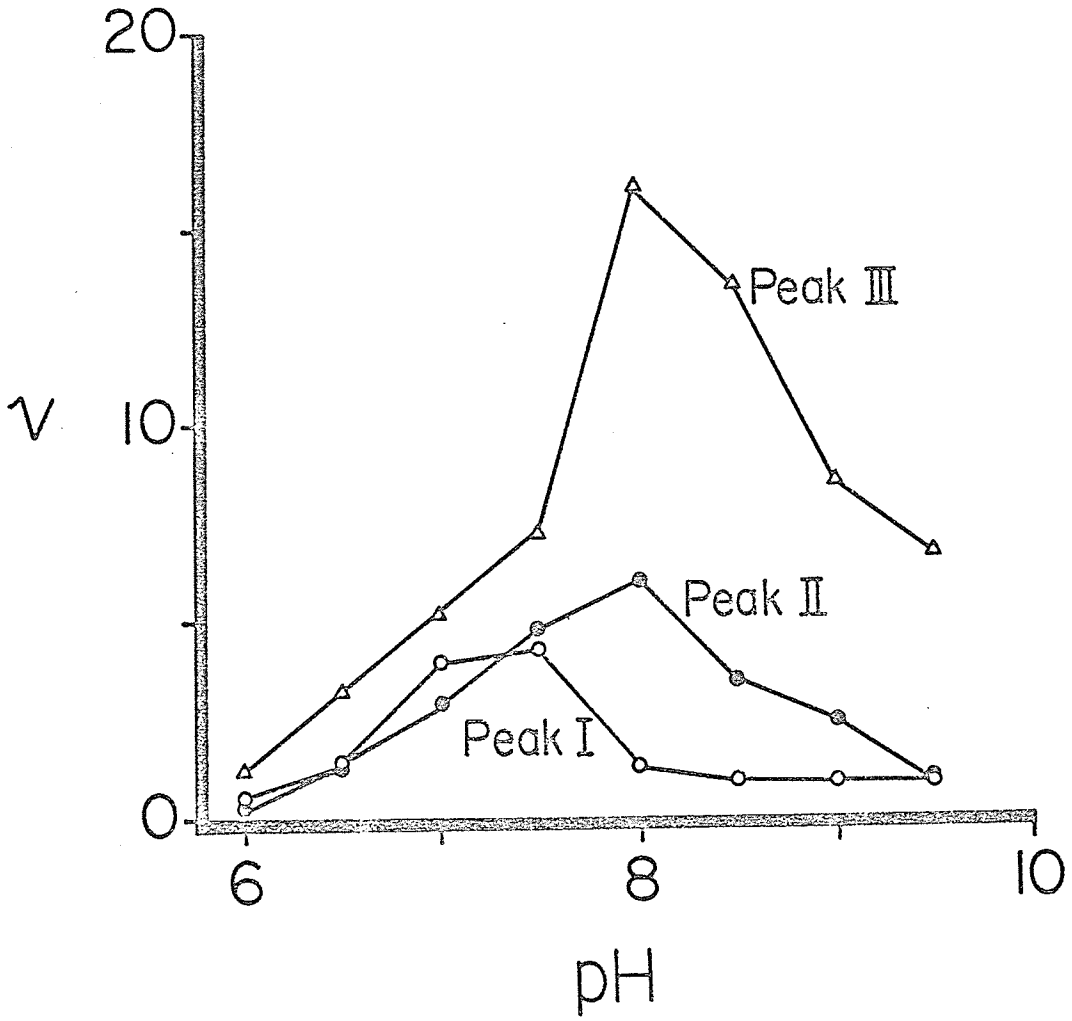


Fig. 4.10. pH optima for the purified adenyl cyclases in the peaks I, II and III following Sephadex G-200 chromatography. All reaction constituents were prepared in buffer at the desired pH. The assays were carried out with Assay C, except that the buffer concentration was increased from 20 mM to 40 mM. Tris-β-glycerophosphate buffer was used between pH 6 and 7, whereas Tris-HCl was used from 7 to 9.5. The activity at pH 7 with both buffers was the same.

TABLE 4.7

Effect of divalent ions (2 mM) on the activity of purified adenyl cyclase from S. salivarius.

Divalent ions	Enzyme activity		
	Peak I	Peak II	Peak III
$MgCl_2$	3.8[a]	8.8	15.8
$MnCl_2$	4.1	9.0	15.9
$ZnSO_4$	0	0	0
$BaCl_2$	0	0	0
$CaCl_2$	0	0	0
$SnCl_2$	0	2.2[b]	7.7[b]

[a] Enzyme activity is expressed as nmoles cyclic AMP formed/mg protein/min.

[b] Precipitate formed during assay.

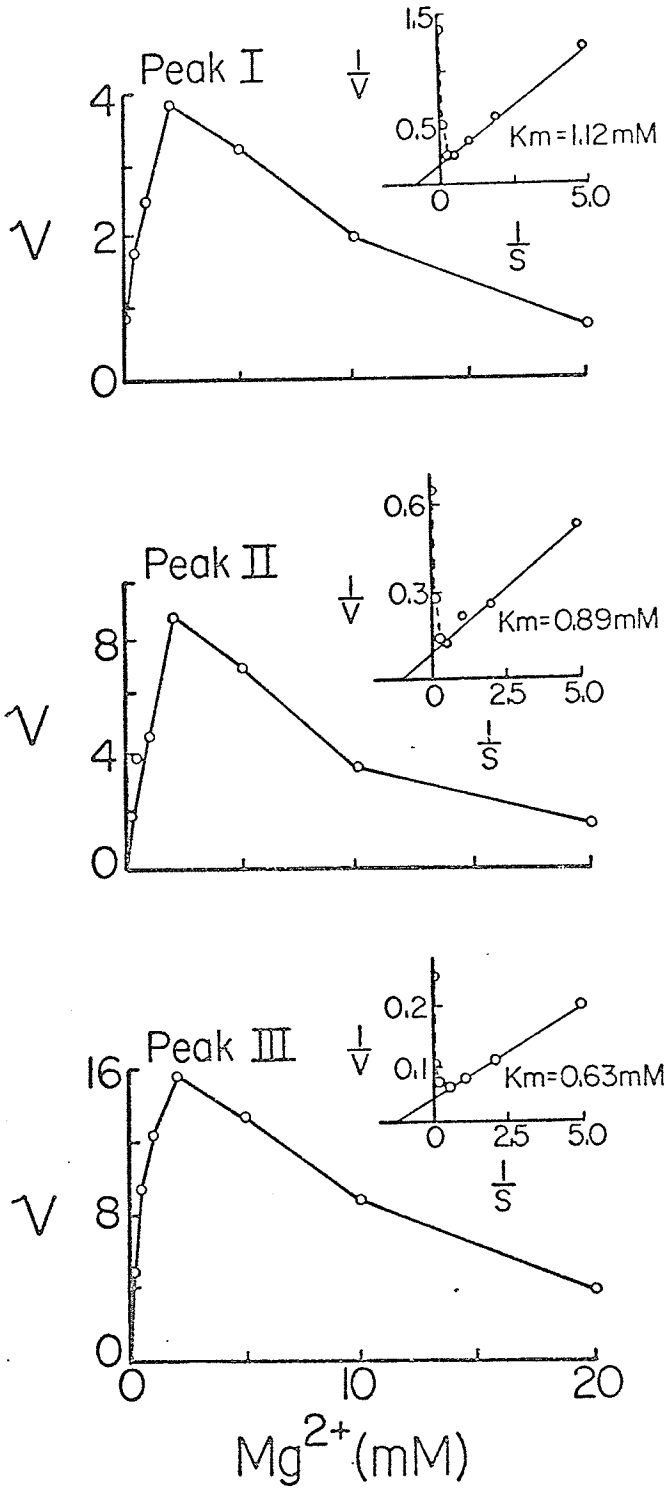


Fig. 4.11. Effect of Mg^{2+} concentration on the activity of the purified adenyl cyclases from S. salivarius. The enzyme activity was determined with Assay C except that the buffer for the peak I assay was at pH 7.5 instead of pH of 8.0. Inset diagrams are the corresponding double reciprocal plots.

previously, NaF was routinely added to Assay A to inhibit ATPase when crude S. salivarius extracts were used. However, when an ATP-regenerating system (P-enolpyruvate-pyruvate kinase) was incorporated into the assay method (Assay B), NaF activation was still observed with crude extracts suggesting that NaF had a direct effect on the enzyme. This activation was confirmed with the purified enzyme in peaks I, II and III, which, as shown in Table 4.5, contained no ATPase activity. Optimum activation of the enzyme in these peaks was obtained at 20 mM NaF (Fig. 4.12). This is the first report of a bacterial adenyl cyclase being activated by NaF. The activation was specific for the fluoride ion since the chloride salts of Na^+, K^+, Ba^{2+} did not activate the enzyme.

E. Effect of ATP on enzyme activity

Increasing the ATP concentration with a fixed amount of enzyme produced hyperbolic rate curves for the enzyme in peaks I, II and III (Fig. 4.13, 4.14 and 4.15, respectively). In all cases, the addition of 20 mM NaF to the assays increased the V_{max} for the enzyme. This increase was particularly great with enzymes II and III, where the V_{max} was increased 5-fold in the presence of NaF (see summary, Table 4.8).

The apparent K_m values for enzymes I and II, on the other hand, were similar (1 mM) and unaffected by NaF. The value for enzyme III, however, was somewhat lower (0.70 mM) in the absence of NaF, but increased 5-fold (3.30 mM) in the presence of this compound (Table 4.8). The K_m for Mg^{2+} at a fixed concentration of ATP was very similar to the K_m values for ATP further indicating that the true substrate for these enzymes is MgATP. The K_m values reported for the adenyl cyclases from E. coli (156)

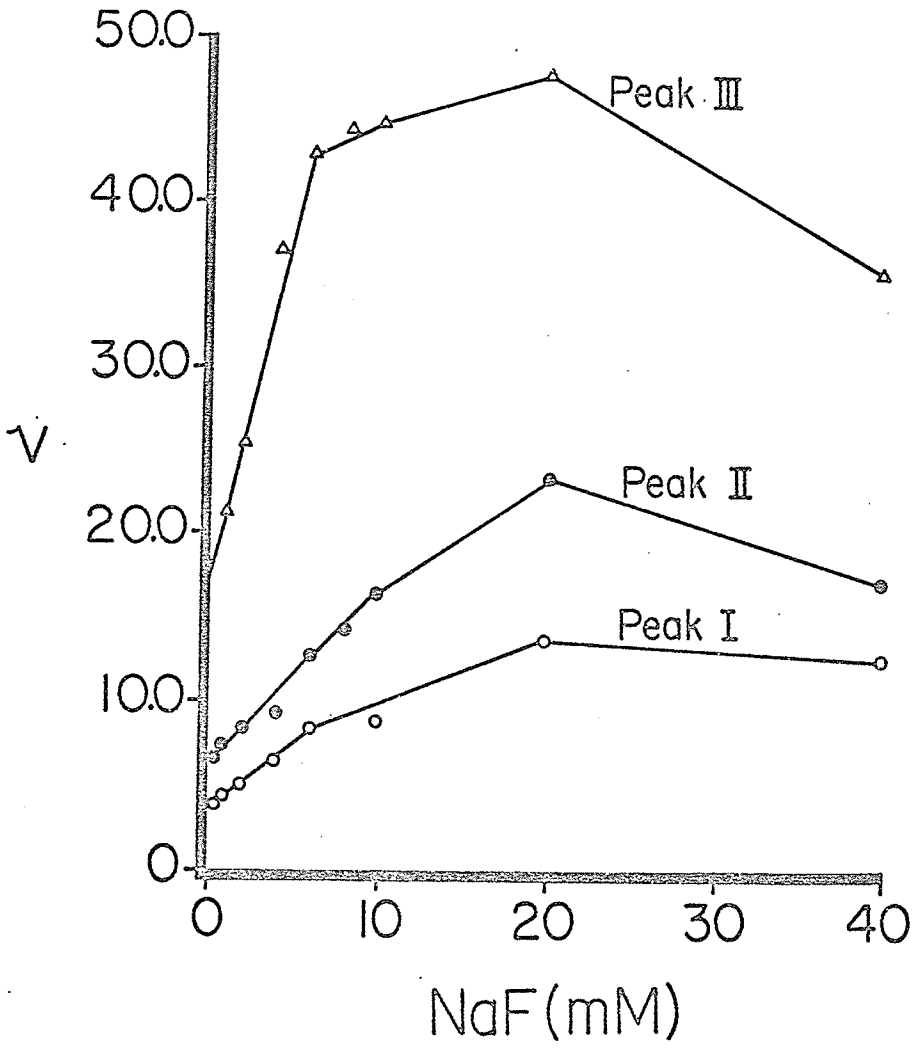


Fig. 4.12. Effect of NaF on the activity of the purified S. salivarius adenyl cyclases. The enzymes were incubated with the required NaF concentration for 10 min with Assay C. Assays were carried out at pH 7.5 for peak I and at pH 8.0 for peaks II and III.

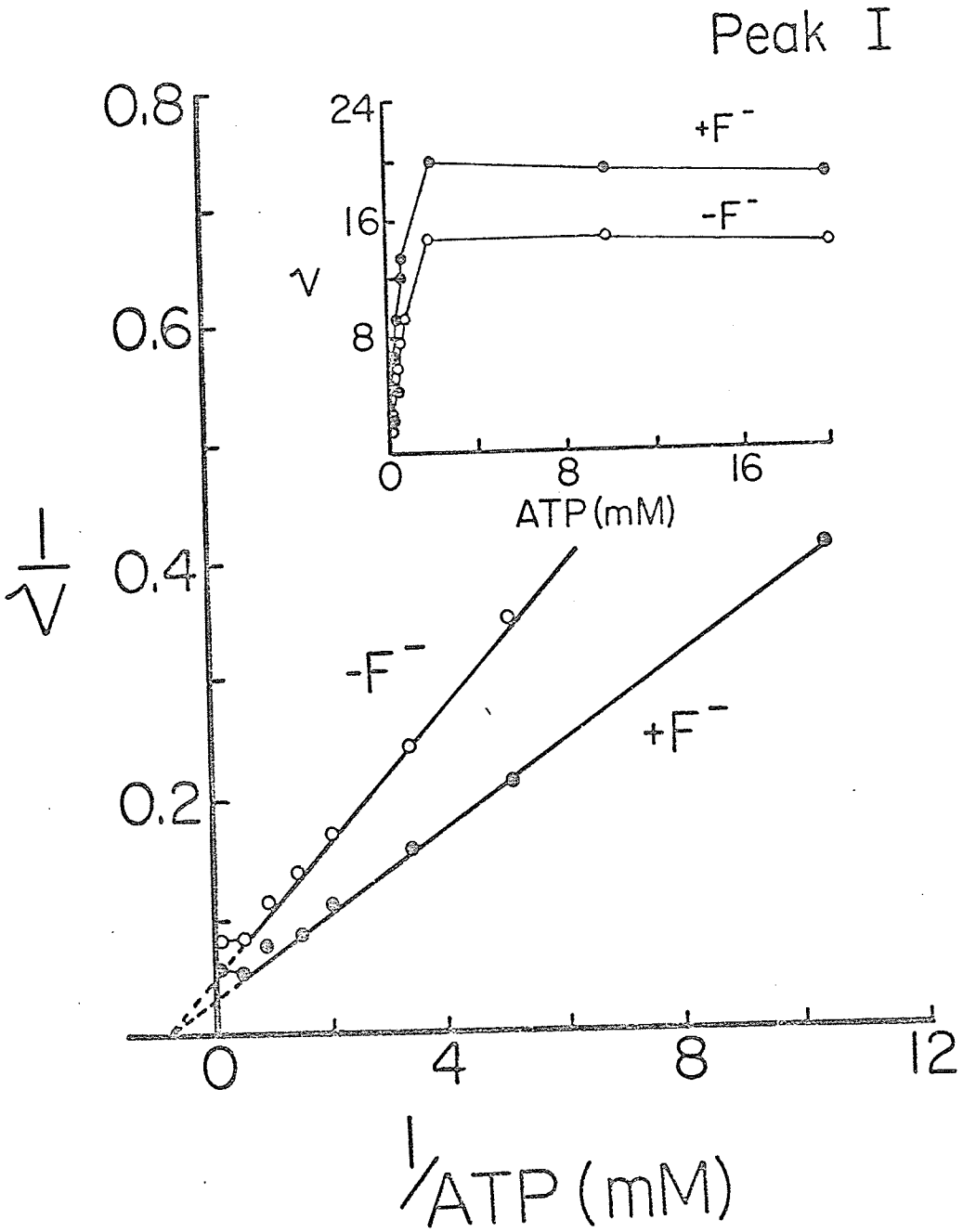


Fig. 4.13. Effect of ATP concentration on the activity of purified S. salivarius adenyl cyclase I, with and without 20 mM NaF. Assay C was employed at pH 7.5.

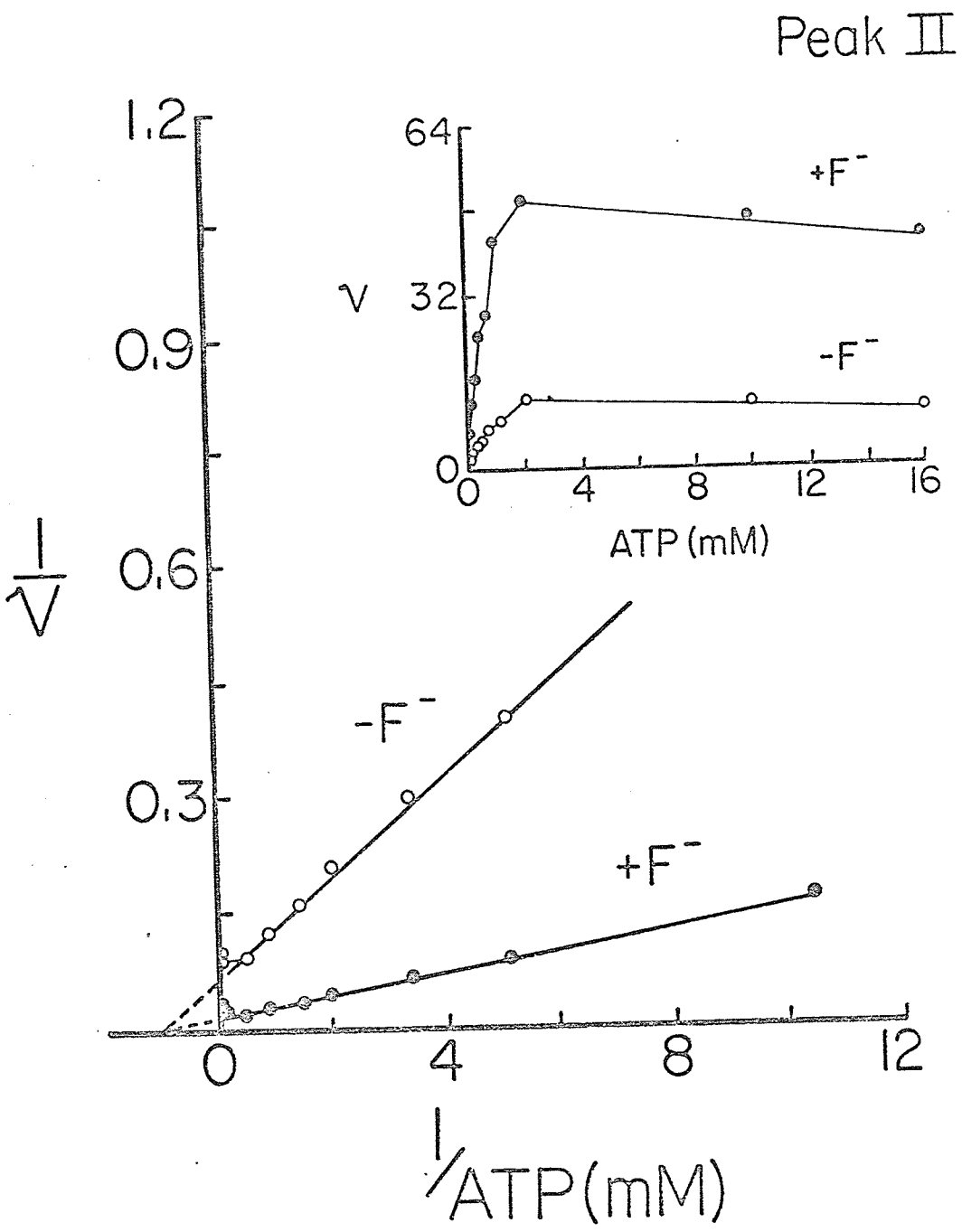


Fig. 4.14. Effect of ATP concentration on the activity of purified S. salivarius adenyl cyclase II, with and without 20 mM NaF. Assay C was employed at pH 8.0.

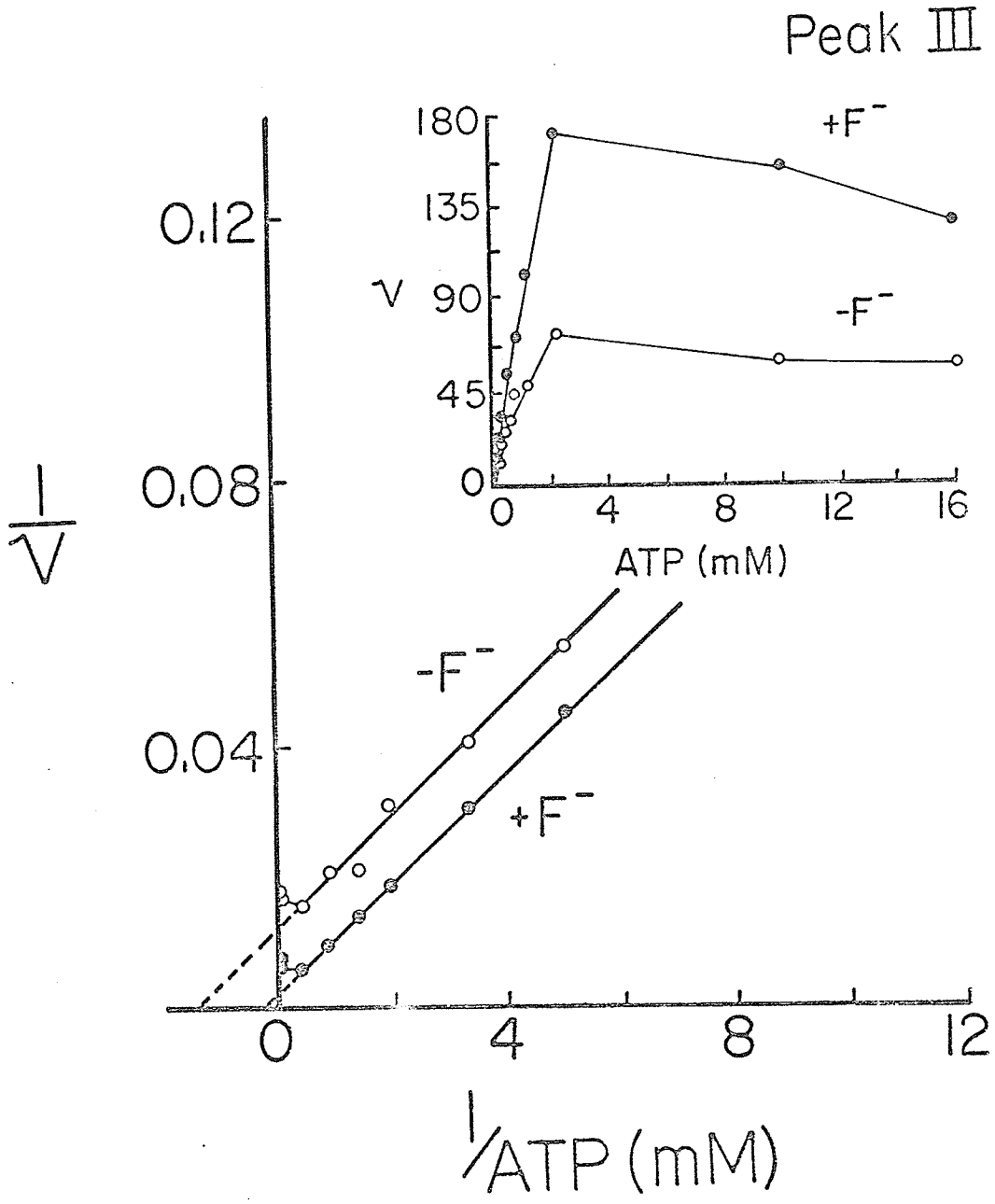


Fig. 4.15. Effect of ATP concentration on the activity of purified S. salivarius adenyl cyclase III, with and without 20 mM NaF. Assay C was employed at pH 8.0.

TABLE 4.8

Summary of apparent K_m and V_{max} values for the three adenyl cyclase preparations (Peaks I, II and III) isolated from S. salivarius[a].

	Peak		
	I	II	III
Apparent K_m (mM) for ATP			
- fluoride	1.0	1.0	0.7
+ fluoride	1.0	1.0	3.3
V_{max}			
- fluoride	17.5[a]	14.7	82.2
+ fluoride	25.9	72.0	407.8
Fold activation by fluoride	1.5	4.9	5.0

[a] Values were determined from the double reciprocal plots in Fig. 4.13 (Peak I), Fig. 4.14 (Peak II) and Fig. 4.15 (Peak III).

and from B. liquefaciens (148) were also found to be 1 mM, while those reported for the animal enzymes ranged from 0.08 to 0.159 mM (175,183,206).

Hill plots (203) of our data for enzymes I, II and III gave slope values (n) of 1 in each case, whether incubated in the presence or absence of 20 mM NaF (Fig. 4.16). This indicated that the enzyme may have only one binding site for the substrate (ATP) and that NaF does not interact with ATP at this site.

F. Involvement of -SH groups in catalysis

The three adenyl cyclase preparations were treated with two -SH inhibitors, N-ethylmaleimide (NEM) and p-hydroxymercuribenzoate (pHMB), to test whether -SH groups are involved in enzyme activity. As shown in Fig. 4.17, both of these inhibitors had a significant effect on enzyme activity at concentrations up to 1 mM, where low or negligible activity was observed. Cysteine limited enzyme inhibition significantly at inhibitor concentrations less than 0.1 mM. These observations, coupled with the stabilizing effect of cysteine on the enzyme, suggests that -SH groups are involved in enzyme function.

G. Reverse reaction (ATP formation)

The enzyme from all three peaks was capable of synthesizing ATP from cyclic AMP and pyrophosphate in the presence of Mg^{2+} (Table 4.9). However, whereas NaF stimulated the forward reaction, the reverse reaction was inhibited by NaF with inhibition being particularly severe for the enzyme in peak III (73%). Determination of the equilibrium constant (K) and ΔF^{o} for each of the enzyme preparations demonstrated that ATP formation

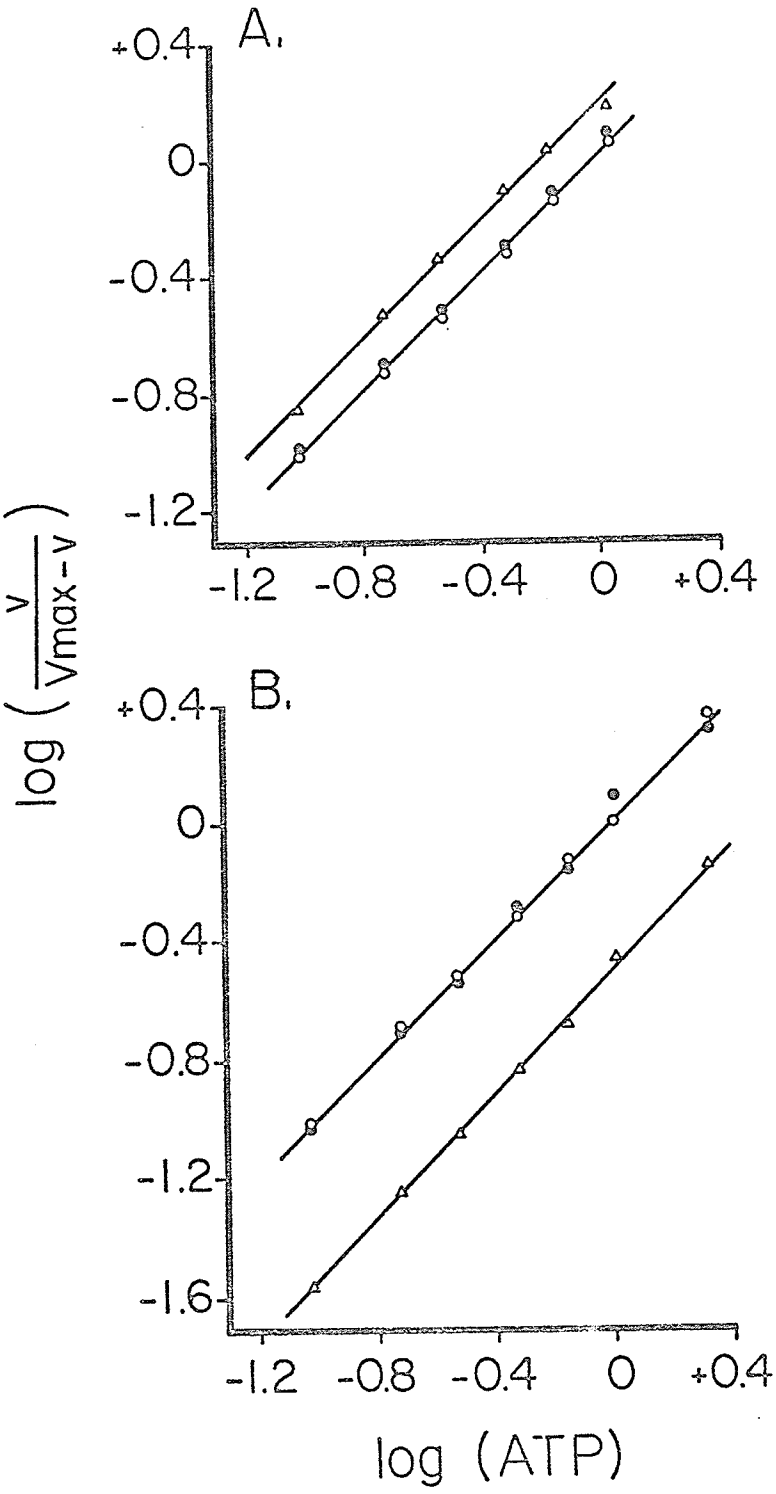


Fig. 4.16. Hill plots for the purified S. salivarius adenyl cyclases without (A) and with (B) fluoride. The data is taken from Figures 4.13, 4.14 and 4.15. Enzyme I, ○; Enzyme II, ●; and Enzyme III, △ .

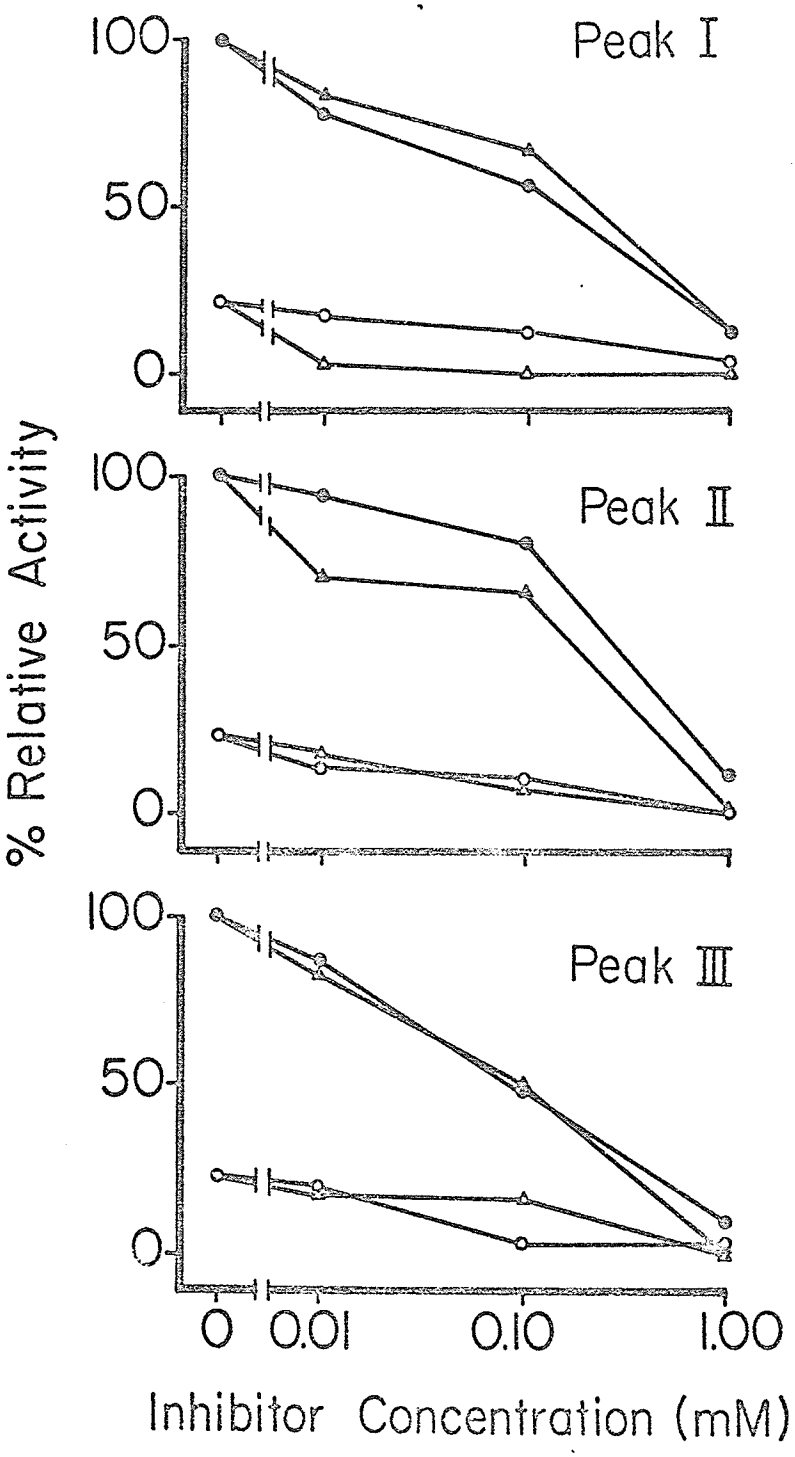


Fig. 4.17. Effect of sulfhydryl inhibitors on the activity of purified S. salivarius adenyl cyclase. Enzyme assays were carried out at pH 7.5 for peak I and pH 8.0 for peaks II and III. NEM, ○; NEM + cysteine (1 mM), ◍; pHMB, △; pHMB + cysteine (1 mM), ▲.

TABLE 4.9

ATP synthesis by purified adenyl cyclase from S. salivarius[a].

	Peak		
	I	II	III
Rate of ATP formation			
- fluoride	1.00[b]	3.70	3.03
+ fluoride	0.86	2.64	0.83
% inhibition by fluoride	14	29	73
K	63[c]	64	43
ΔF^{o}	-2.55[d]	-2.56	-2.32

a Adenyl cyclase activity was determined in the direction of ATP synthesis (reverse reaction) by using cyclic ^{3}H-AMP and pyrophosphate as substrates in absence and presence of NaF (20 mM). To determine the rate of the reverse reaction, the enzyme from peak I (1 µg) was assayed at pH 7.5 whereas peak II (0.62 µg) and peak III (0.38 µg) were assayed in pH 8 buffer.

b nmoles ATP formed/mg protein/min.

c The equilibrium constant (K) was determined both in the forward and reverse directions and employed 0.2 mg, 60 µg and 50 µg of protein from peaks I, II and III at the appropriate pH, respectively. The values represent the mean of 6 determinations, three in each direction.

d K cal/mole.

from cyclic AMP and pyrophosphate was favored energetically. K values were determined in both the forward (cyclic AMP formation) and reverse (ATP synthesis) directions and ranged from 43 to 64. These values were higher than the K value (0.065 in the forward direction) reported for the purified B. liquefaciens adenyl cyclase (151).

V. REGULATION OF PURIFIED ADENYL CYCLASE

In order to understand the regulation of adenyl cyclase activity in S. salivarius, the effect of inorganic phosphate (P_i) and pyrophosphate (PP_i), and various nucleotides and glycolytic intermediates on the activity of enzyme III was examined. This preparation was selected because it was the most homogeneous. Assay C was used to determine adenyl cyclase activity in all cases.

A. Effect of nucleotides

Studies with bacterial extracts (148,158,159) and animal tissues (245,246) have demonstrated the inhibition of adenyl cyclase by a variety of nucleotides. In view of the possible role of nucleotides in the control of this enzyme "in vivo", the effect of all the tri-, di-, and monophosphate nucleotides of adenosine, guanosine, uridine, inosine and cytidine on the activity of purified S. salivarius adenyl cyclase III was examined. In general, all of these nucleotides inhibited enzyme activity. For example, the double reciprocal plot of the effects of the nucleoside triphosphates and ADP (1 mM) on the activity of the purified enzyme, as a function of the ATP concentration, demonstrated that these compounds were competitive

inhibitors (Fig. 4.18 (A)).

By replotting the slopes and intercepts vs inhibitor concentration (Fig. 4.18(B)) according to the method of Cleland (204), it can be seen that the intercepts are independent of inhibitor concentration, whereas the slopes are a linear function of the inhibitor concentration. This linear relationship indicates that the inhibition is fully competitive, i.e., the inhibitors compete directly for the ATP binding site on the enzyme. This conclusion was also confirmed by plotting v_o/v_o-v_i vs $1/I$ as suggested by Webb (205). The inhibitor dissociation constant (K_i) for each inhibitor was determined by Dixon plots (202) of $1/v$ vs $[I]$, as well as by extrapolation of the slope replot to the horizontal intercept (204). The mean K_i value for each inhibitor is listed in Table 4.13 in order of decreasing affinity for the enzyme.

The inhibition of purified S. salivarius adenyl cyclase III by the other nucleoside diphosphates is shown in Table 4.10 for one concentration of ATP (0.6 mM). In all cases, the degree of inhibition increased with increasing concentrations of the inhibitor, although it is evident that GDP had a significant inhibitory effect at the lowest concentration employed (0.1 mM).

Competitive inhibition of the purified enzyme was also noted for all of the nucleoside monophosphates, except AMP, when incubated at a concentration of 5 mM (Fig. 4.19(A)). Although the lines in the double reciprocal plot intersected slightly to the left of the ordinate, analysis of the data by student's t-test following analysis of variance indicated that the intercepts for these nucleoside monophosphates were not significantly different than that for the control ($P>0.1$). Replots of the

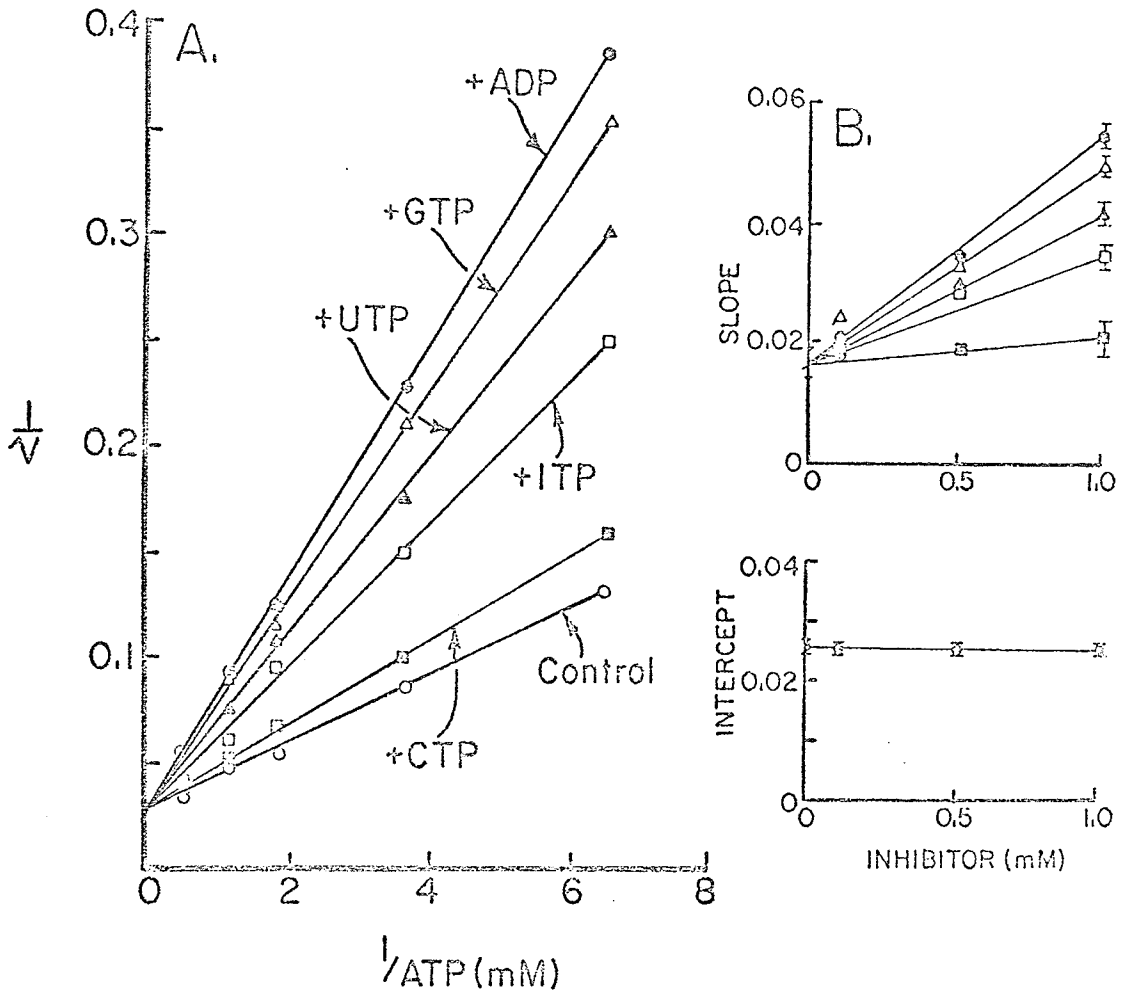


Fig. 4.18. The inhibition of purified S. salivarius adenyl cyclase III by ADP and various nucleoside triphosphates. (A) double reciprocal plots. ADP and the various nucleoside triphosphates were incubated at a concentration of 1 mM with Assay C. Enzyme activity (v) is expressed as nmoles of cyclic AMP formed/mg protein/min. (B) replots of the slopes (K_m/V_{max}) and intercepts (1/v) (obtained from the double reciprocal plot) vs inhibitor concentration. The standard errors were calculated for all points in the slope replot, but were indicated (vertical bars) for the 1 mM inhibitor concentration only to simplify graphical presentation. The vertical bars in the intercept replot represent the mean standard error for all of the inhibitors tested.

TABLE 4.10

Effect of nucleoside diphosphates on the activity of purified S. salivarius adenyl cyclase III.

Additions	Concentration (mM)	Enzyme activity	Percent inhibition
None	-	13.28 ± 0.32[a]	-
GDP	0.1	8.47 ± 0.12	36
	0.5	8.42 ± 0.12	37
	5.0	7.67 ± 0.09	42
IDP	0.5	13.07 ± 0.18	2
	1.0	12.12 ± 0.15	9
	5.0	6.57 ± 0.12	51
CDP	0.1	10.80 ± 0.09	19
	1.0	10.58 ± 0.22	20
	5.0	5.72 ± 0.13	57
UDP	0.1	11.70 ± 0.18	12
	1.0	8.30 ± 0.09	38
	5.0	4.65 ± 0.10	65

[a] nmoles cyclic AMP formed/mg protein/min.

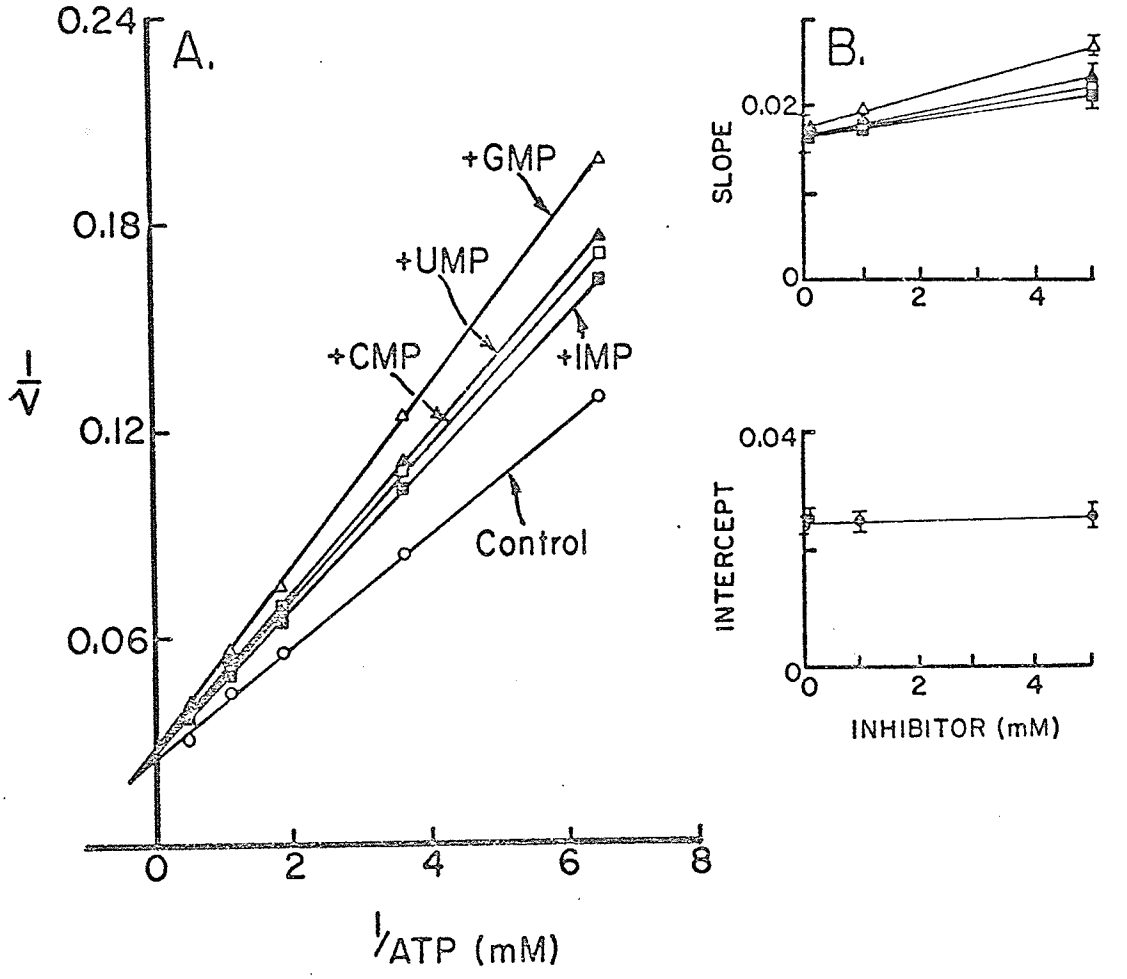


Fig. 4.19. The inhibition of purified S. salivarius adenyl cyclase III by various nucleoside monophosphates at a concentration of 5 mM. (A) Double reciprocal plot. (B) Replots of slopes and intercepts vs inhibitor concentration. Statistical analyses were the same as for Fig. 4.18.

intercepts and slopes (Fig. 4.19(B)) confirmed this and further indicated that the inhibition was completely competitive since only the slope replots varied with inhibitor concentration and did so in a linear fashion (204). The K_i values are listed in Table 4.13 and indicate that the nucleoside monophosphates generally have less affinity for the enzyme than the triphosphate derivatives.

Similar statistical treatment of the kinetic data obtained with the enzyme incubated with AMP indicated that this compound inhibited the enzyme in a non-competitive fashion (Fig. 4.20(A)). Both the slope and intercept replot curves were hyperbolic indicating the inhibition was actually hyperbolic non-competitive. The data were further analyzed by determining the interaction constants α and β as suggested by Webb (205), where α represents the change in both the K_m and K_i, while β represents the rate of breakdown of the enzyme-inhibitor-substrate complex to products relative to the rate for the uninhibited enzyme-substrate complex. Since the value for β was essentially equal to zero (0.018 ± 0.006), while the value for α (3.72 ± 0.25) was less than ∞ but greater than 1, the actual inhibition by AMP was of the mixed type, i.e., partially competitive and completely non-competitive (205). The K_i value for AMP was 2.5 mM indicating that it had the greatest affinity for the enzyme of all the nucleoside monophosphates tested.

Although the inhibition of adenyl cyclase activity by ADP and various nucleoside triphosphates has now been established with extracts from B. liquefaciens (148), E. coli (158), N. erythropolis (159), and with enzyme III from S. salivarius in this study, the effect of the nucleoside monophosphates on other microbial preparations is not clear. While AMP

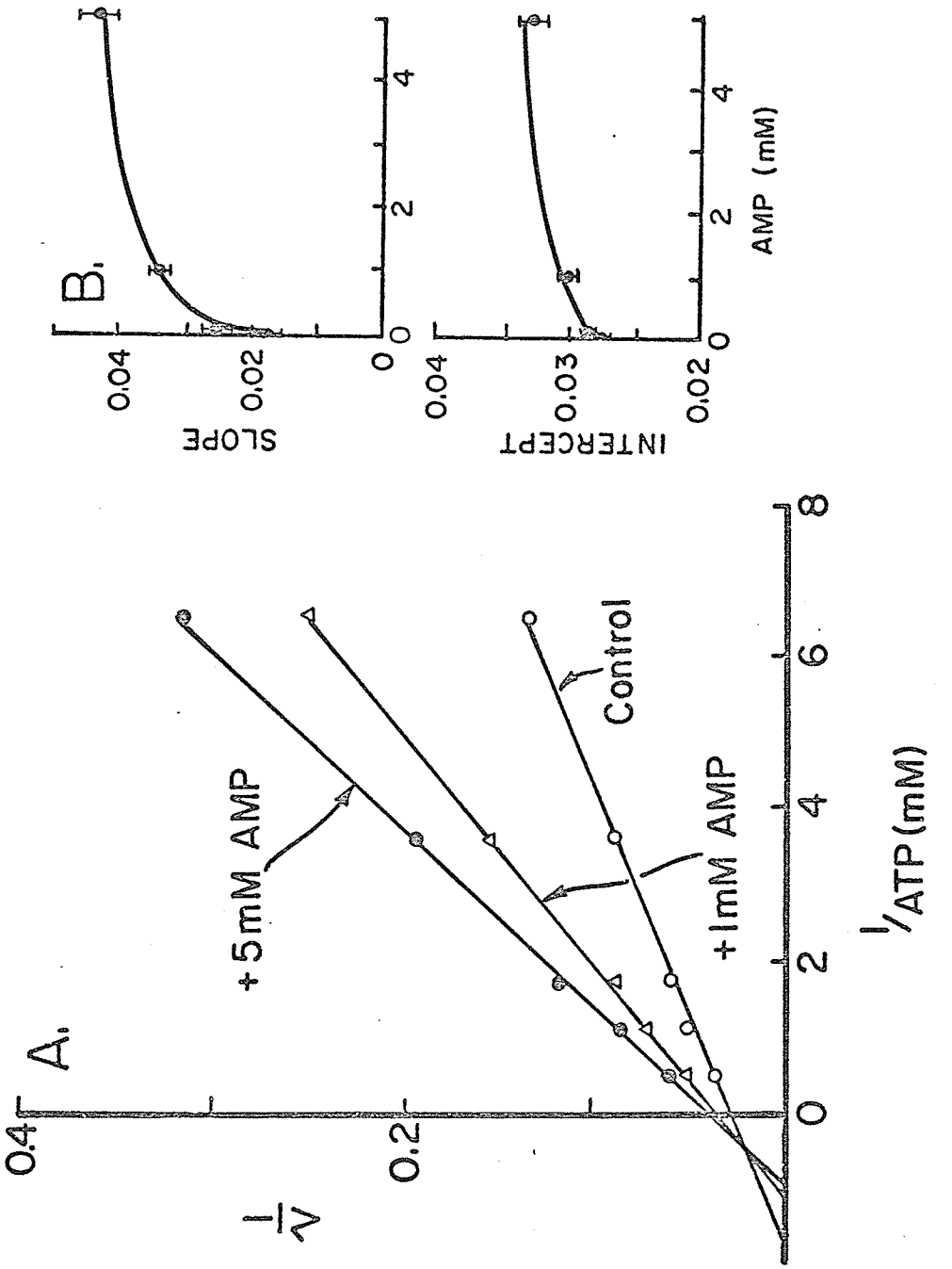


Fig. 4.20 The inhibition of purified S. salivarius adenyl cyclase III by AMP.
(A) Double reciprocal plots.
(B) Replots of slopes and intercepts vs AMP concentration.

inhibited the enzyme in B. liquefaciens (148), the enzyme in E. coli (156) was activated by GMP. It is clear from the results with purified S. salivarius adenyl cyclase III that the adenine and guanine nucleotides were the most inhibitory of all the nucleotides tested.

The involvement of nucleoside diphosphate glucose compounds in mammalian (247) and bacterial (248) glycogen metabolism is well known. Since cyclic AMP is known to regulate glycogen metabolism in mammalian tissue (6), the possibility exists that the nucleoside diphosphate glucose compounds, particularly ADP-glucose (248), might influence adenyl cyclase activity in bacteria. As shown in Table 4.11, all of the nucleoside diphosphate glucose compounds tested inhibited purified S. salivarius adenyl cyclase III activity at concentrations from 0.1 to 5 mM when incubated with 0.6 mM ATP. Inhibition ranged from 11 - 30% at the lowest concentration tested (0.1 mM) to 41 - 72% at the 5 mM level. In all cases UDP-glucose was the most potent inhibitor followed by ADP-glucose at concentrations above 0.1 mM.

Various cyclic 3',5'-nucleoside monophosphates, on the other hand, had relatively little effect on the S. salivarius enzyme (Table 4.12). Of the six cyclic derivatives tested, cyclic 3',5'-UMP was the most inhibitory, although inhibition was not particularly severe even at the 5 mM level (11%). Similar results have been reported for the effect of cyclic AMP and cyclic 3',5'-GMP on E. coli adenyl cyclase (158).

B. Effect of inorganic phosphate and pyrophosphate

Adenyl cyclase, in addition to synthesizing cyclic AMP, also produces equimolar amounts of inorganic pyrophosphate. In S. salivarius,

TABLE 4.11

Effect of nucleoside diphosphate glucose compounds on the activity of purified S. salivarius adenyl cyclase III.

Additions	Concentration (mM)	Enzyme activity	Percent inhibition
None	-	13.28 ± 0.32[a]	-
ADP-glucose	0.1	11.80 ± 0.27	11
	1.0	7.35 ± 0.22	45
	5.0	5.45 ± 0.18	59
GDP-glucose	0.1	11.43 ± 0.40	14
	1.0	9.32 ± 0.18	30
	5.0	7.83 ± 0.07	41
CDP-glucose	0.1	11.22 ± 0.22	16
	1.0	8.37 ± 0.09	37
	5.0	6.77 ± 0.03	49
UDP-glucose	0.1	9.32 ± 0.14	30
	1.0	8.10 ± 0.11	39
	5.0	3.70 ± 0.12	72

[a] nmoles cyclic AMP formed/mg protein/min.

TABLE 4.12

Effect of cyclic 3',5'-nucleoside monophosphates on the activity of purified S. salivarius adenyl cyclase III.

Additions	Concentration (mM)	Enzyme activity	Percent inhibition
None	-	13.28 ± 0.32[a]	-
Cyclic 3',5'-AMP	1	13.05 ± 0.12	2
	5	12.78 ± 0.09	4
Cyclic 3',5'-GMP	1	12.62 ± 0.10	5
	5	12.93 ± 0.09	3
Cyclic 3',5'-IMP	1	13.17 ± 0.03	1
	5	13.17 ± 0.11	1
Cyclic 3',5'-CMP	1	12.60 ± 0.09	5
	5	12.22 ± 0.12	8
Cyclic 3',5'-UMP	1	12.38 ± 0.20	7
	5	11.85 ± 0.09	11
Cyclic 3',5'-TMP	1	12.40 ± 0.18	7
	5	12.20 ± 0.18	8

[a] nmoles cyclic AMP formed/mg protein/min.

this inorganic pyrophosphate is probably degraded readily to inorganic phosphate since inorganic pyrophosphatase is present in abundant quantities in this organism (Table 4.5). As was the case for E. coli adenyl cyclase (157), the purified S. salivarius enzyme III was inhibited by low concentrations of inorganic phosphate (Fig. 4.21(A)) and inorganic pyrophosphate (Fig. 4.21(B)). The double reciprocal plots of the effect of these inhibitors on the S. salivarius enzyme indicate that the inhibition was competitive, however, only the inhibition by P_i was fully competitive as seen by the slope and intercept replots. The K_i for P_i was 1.40 mM (Table 4.13). The inhibition by inorganic pyrophosphate, on the other hand, was hyperbolic with respect to the slope replot indicating that the inhibition was partially competitive, i.e., PP_i binds at a site different than that for ATP (204). The inhibition constant (K_i) for PP_i was only 0.23 mM indicating that of all the inhibitors tested, this compound had the greatest affinity for the enzyme.

C. Effect of glycolytic intermediates

In 1965 Makman and Sutherland (93) reported that the presence of exogenous glucose caused a decrease in the cellular concentration of cyclic AMP in E. coli cells. Since then several reports have appeared which have indicated that glucose, or possibly metabolites of glucose, had a significant effect in regulating the synthesis of various inducible enzymes by causing the extrusion of cyclic AMP from the cell and by preventing its formation (97,134). In view of these reports and the fact that members of the genus, Streptococcus, are homofermentative organisms, we have examined the effects of various glycolytic intermediates on the formation

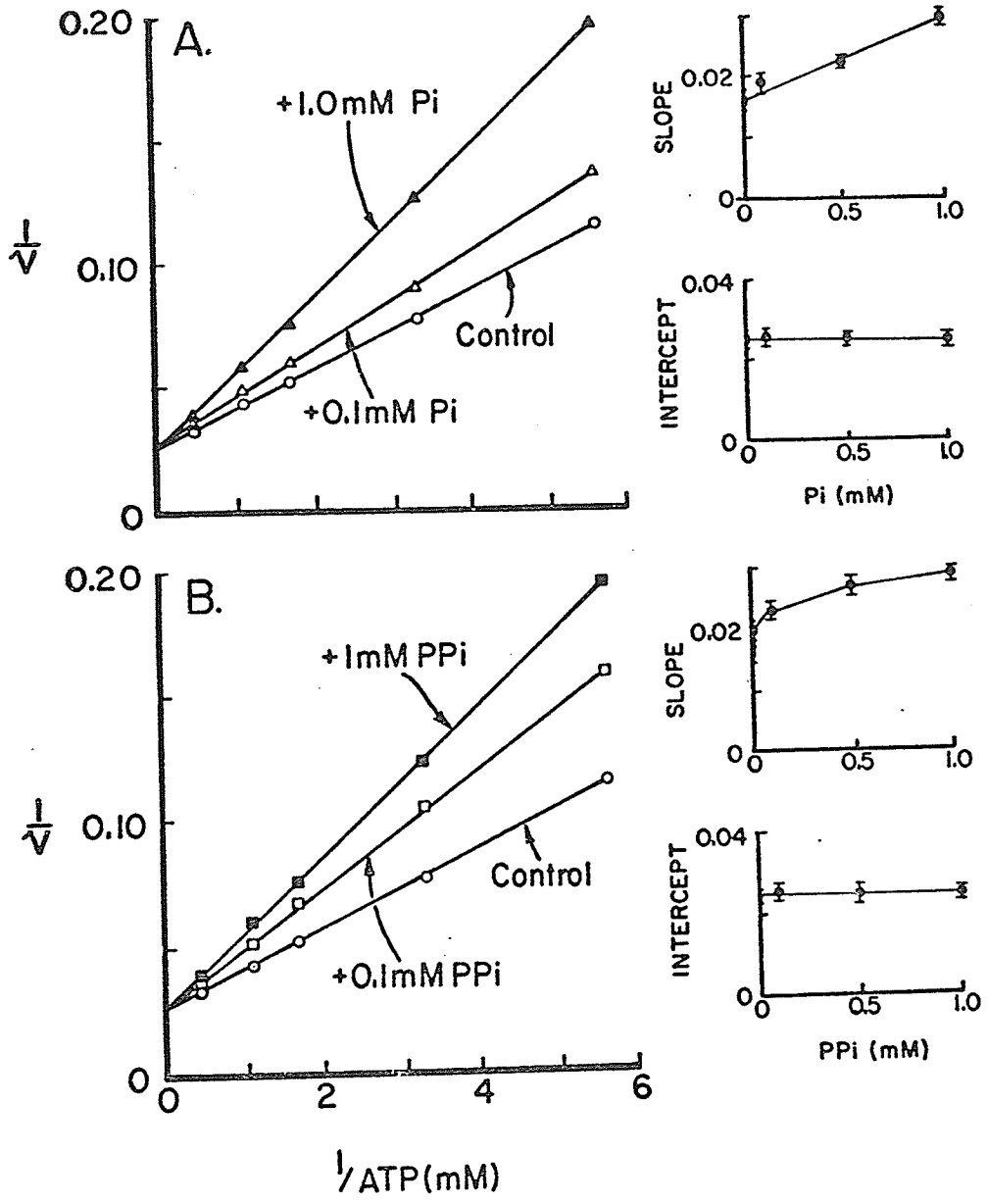


Fig. 4.21. The inhibition of purified S. salivarius adenyl cyclase III by (A) inorganic phosphate (P_i) and (B) inorganic pyrophosphate (PP_i). The small inset plots represent the replots of the slopes and intercepts determined from the corresponding double reciprocal plot.

of cyclic AMP by purified S. salivarius adenyl cyclase III.

As shown in Fig. 4.22(A), glucose-6-P, glucose-1-P, 2-P-glycerate and pyruvate were weak activators of the enzyme (i.e., up to 40%) in the concentration range from 0.1 to 10 mM, although these compounds were essentially ineffective below the 0.01 mM level. Pyruvate was the weakest activator, which is in contrast to its requirement by other bacterial adenyl cyclase preparations (148,159). Activation of the enzyme by glucose-6-P, glucose-1-P and 2-P-glycerate was significant between 0.1 and 10 mM (at 0.1 mM, P=0.02; at 1 and 10 mM, P=0.01), while the activation by pyruvate was significantly different than the control only at levels of 1 and 10 mM (P=0.05). The above compounds, when tested at various ATP concentrations, were shown to increase the maximum velocity (V_{max}) of the enzyme without affecting the apparent K_m for ATP (Fig. 4.22 B,C,D and E). The activation constants (K_a) for these compounds are listed in Table 4.13 and indicate that, of these four compounds, 2-P-glycerate was the most potent activator. Weak activation was also observed with acetate, while citrate and lactate, at concentrations between 0.1 and 1 mM, inhibited the enzyme up to 50%. Dihydroxyacetone-P, 2,3-diphosphoglycerate and 3-P-glycerate had no effect on enzyme activity. The enzyme in E. coli has been found to be inhibited by pyruvate and acetate, but unaffected by glucose-6-P and glucose-1-P (158).

A more complicated effect was observed with the S. salivarius enzyme in the presence of fructose-6-P, fructose-1,6-P_2,glyceraldehyde-3-P and P-enolpyruvate. These compounds were both activators and inhibitors of the enzyme depending on the concentration of the compound employed in the assay mixture. The lower concentrations (<1 mM) of fructose-6-P and

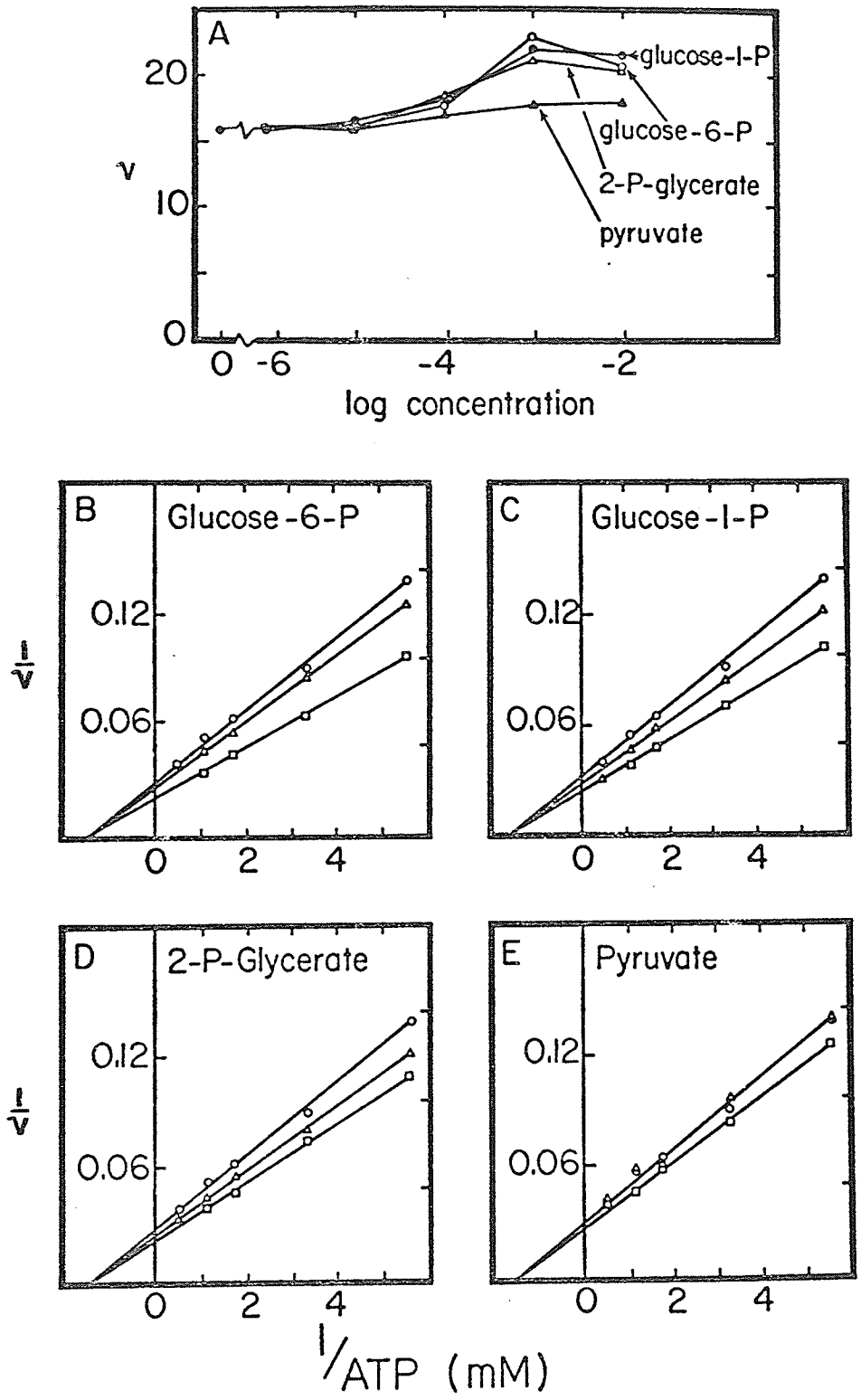


Fig. 4.22. Effect of increasing concentrations of glucose-6-P, glucose-1-P, 2-P-glycerate and pyruvate on the activity of purified S. salivarius adenyl cyclase III. Symbols in B-E: control (O), 0.1 mM (Δ), 1 mM (□). Adenyl cyclase activity was determined with Assay C.

TABLE 4.13

Values of the various inhibition constants (K_i) and activation constants (K_a) for various effectors of purified adenyl cyclase III of S. salivarius.

Effectors	K_i (mM)	K_a (mM)
PP_i	0.23 ± 0.03	-
ADP	0.43 ± 0.03	-
GTP	0.52 ± 0.03	-
UTP	0.60 ± 0.02	-
ITP	0.93 ± 0.04	-
P_i	1.40 ± 0.23	-
AMP	2.50 ± 0.16	-
CTP	3.25 ± 0.31	-
GMP	7.20 ± 0.50	-
UMP	10.50 ± 0.39	-
CMP	13.00 ± 1.19	-
IMP	14.40 ± 0.32	-
2,P-glycerate	-	0.13 ± 0.02
Glucose-1-P	-	0.24 ± 0.03
Glucose-6-P	-	0.55 ± 0.07
Pyruvate	-	1.12 ± 0.09

fructose-1,6-P_2 activated the enzyme whereas the enzyme was inhibited at concentrations above 1 mM (Fig. 4.23, 4.24, respectively). However, the activation by fructose-1,6-P_2 was significant only at the 0.05 mM level (P=0.1), while the activation by fructose-6-P was significant between 0.01 mM and 0.3 mM (0.01 mM, P=0.05; 0.05 mM, P=0.02; 0.1 mM, P=0.02; and 0.3 mM, P=0.1). On the other hand, the inhibition by fructose-1,6-P_2 was significant between 1 and 10 mM (1 mM, P=0.05; 5 and 10 mM, P=0.01), while the inhibition by fructose-6-P was significant only at the 5 and 10 mM levels (P=0.01). These compounds altered both the maximum velocity of the enzyme and the apparent K_m for ATP.

Glyceraldehyde-3-P activated the enzyme at concentrations below 0.05 mM and greater than 1 mM, while inhibiting the enzyme slightly at a concentration of 0.1 mM (Fig. 4.25). Statistical analysis, indicated that the activations at 0.01 mM (P=0.02), 0.05 mM (P=0.1), 5 mM (P=0.1) and 10 mM (P=0.05) were significant. The inhibition at the 0.1 mM concentration was only significant at the 10% level (P=0.1). In contrast, P-enolpyruvate was ineffective at 0.01 mM, activated the enzyme at 0.1 mM (P=0.1) and 0.3 mM (P=0.02) but was inhibitory at 5 mM (P=0.05) and 10 mM (P=0.02) (Fig. 4.26). Although the effects of P-enolpyruvate and glyceraldehyde-3-P were opposite at the same concentrations, kinetically they were similar. The activating concentrations of these two compounds increased the V_{max} of the enzyme without affecting the apparent K_m for ATP. However, at inhibiting concentrations, i.e., at 0.1 mM for glyceraldehyde-3-P and above 1 mM for P-enolpyruvate, the maximum velocity was decreased and the apparent K_m value increased, although the change in V_{max} for glyceraldehyde-3-P was small (7.2%).

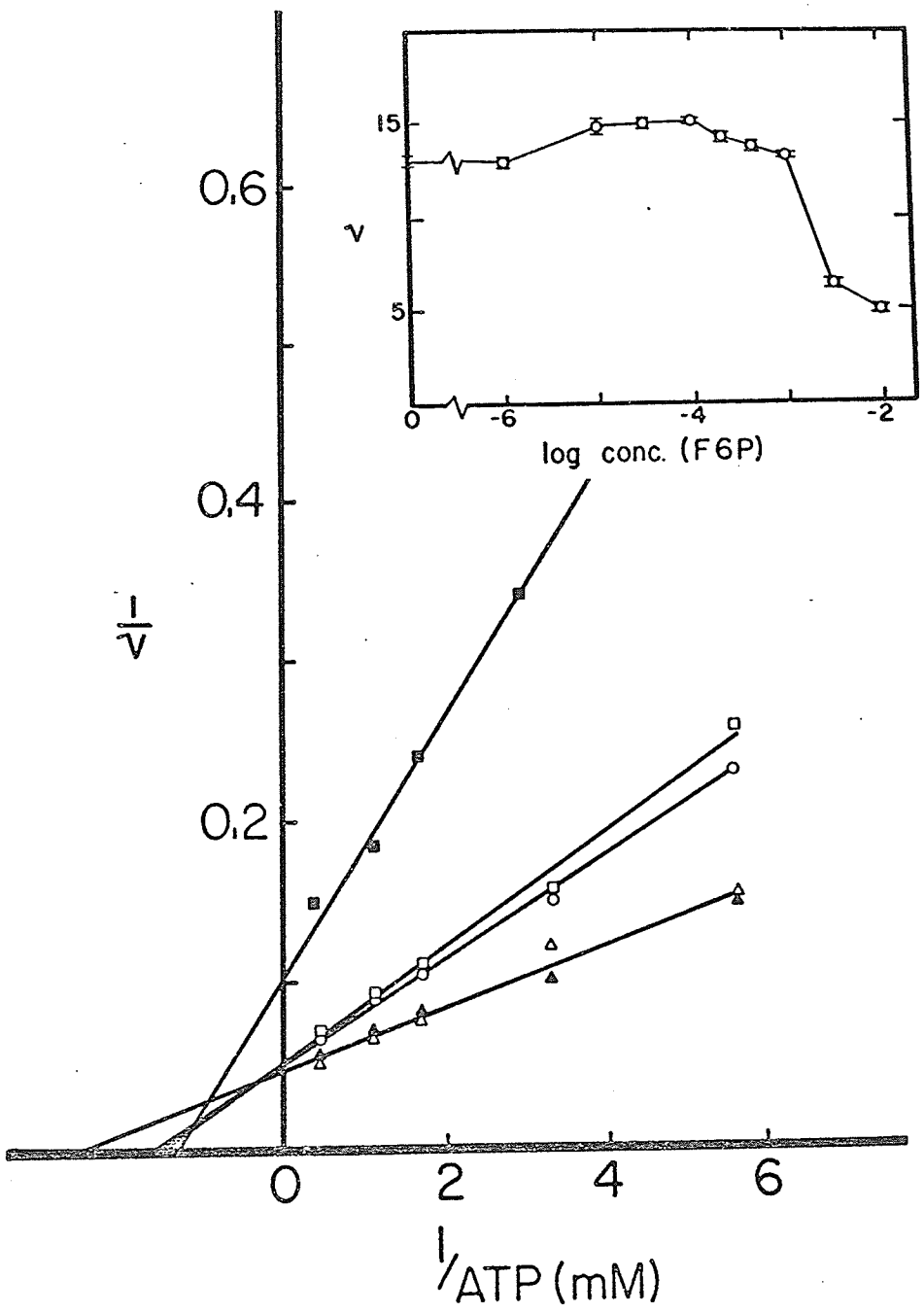


Fig. 4.23. Effect of fructose-6-P (F6P) on the activity of purified S. salivarius adenyl cyclase III. Control (○), 0.01 mM (△), 0.1 mM (▲), 1 mM (□) and 5 mM (■). The inset shows the effect of increasing fructose-6-P concentration on enzyme activity at one ATP concentration (0.6 mM).

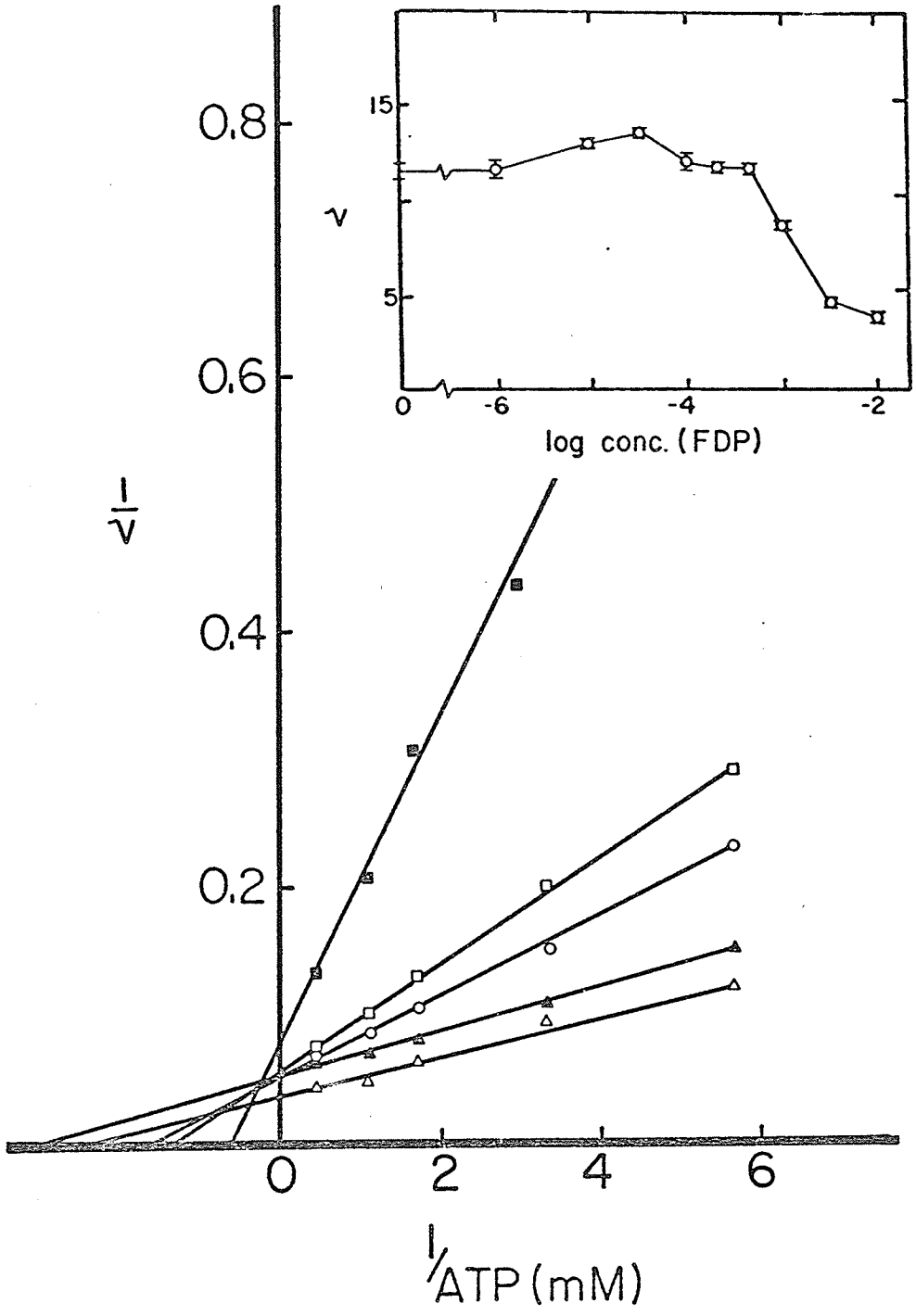


Fig. 4.24. Effect of fructose-1,6-P_2 (FDP) on the activity of purified S. salivarius adenyl cyclase III. Control (O), 0.01 mM (Δ), 0.1 mM (▲), 1 mM (□) and 5 mM (■). The inset shows the effect of increasing fructose-1,6-P_2 concentration on enzyme activity at one ATP concentration (0.6 mM).

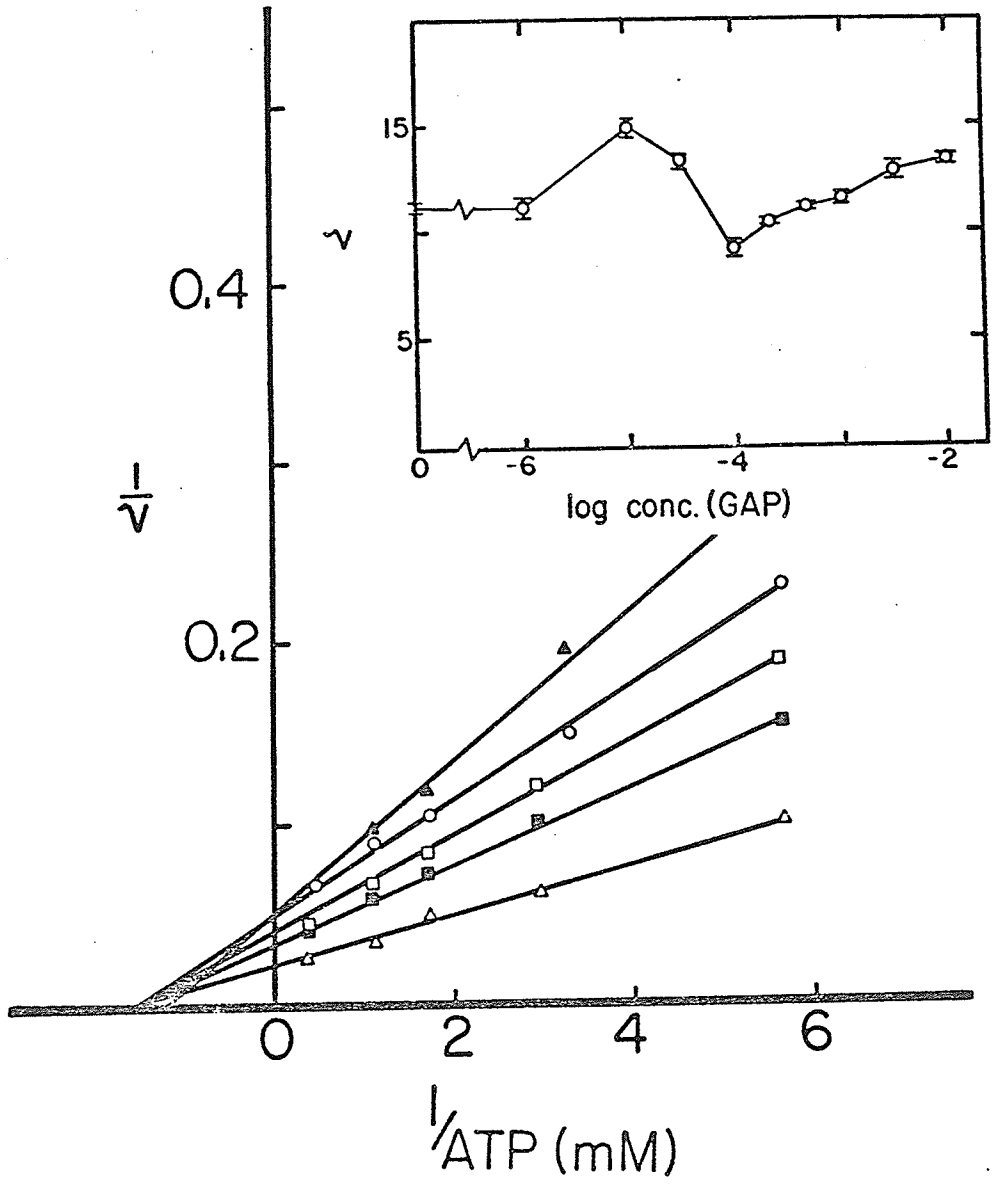


Fig. 4.25. Effect of glyceraldehyde-3-P (GAP) on the activity of purified S. salivarius adenyl cyclase III. Control (O), 0.01 mM (△), 0.1 mM (▲), 1 mM (□) and 5 mM (■). The inset shows the effect of increasing glyceraldehyde-3-P concentration on enzyme activity at one ATP concentration (0.6 mM).

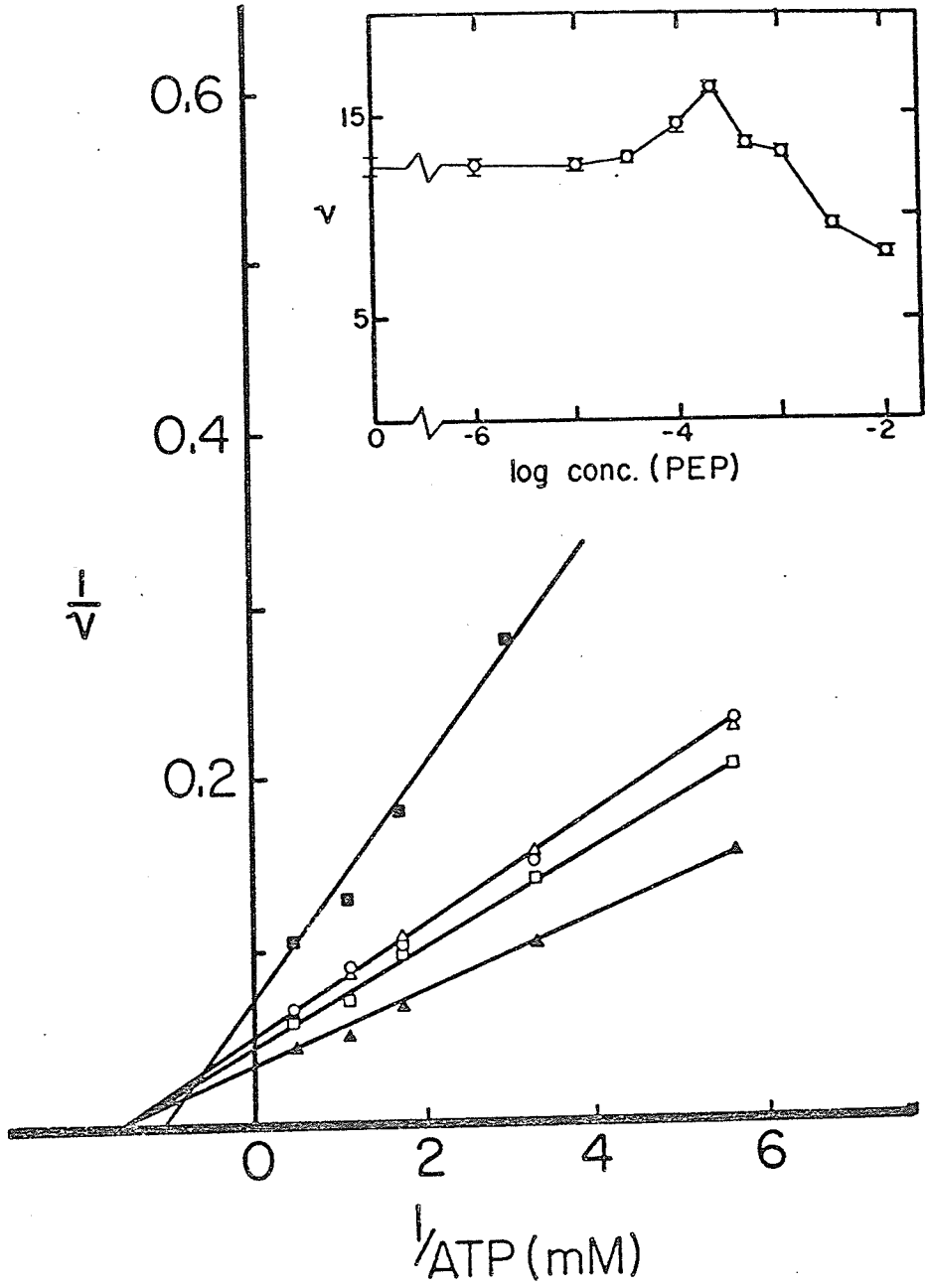


Fig. 4.26. Effect of P-enolpyruvate (PEP) on the activity of purified S. salivarius adenyl cyclase III. Control (O), 0.01 mM (Δ), 0.1 mM (▲), 1 mM (□) and 5 mM (■). The inset shows the effect of increasing P-enolpyruvate concentration on enzyme activity at one ATP concentration (0.6 mM).

VI. DISCUSSION

The repeated recovery of adenyl cyclase from S. salivarius in three distinct peaks upon Sephadex G-200 chromatography has raised the obvious question as to whether the activity observed in each of the peaks was that of the same enzyme or whether three isozymes exist. Clearly, enzyme III is distinctly different from enzymes I and II with respect to its kinetic properties, both in the presence and absence of fluoride (Table 4.8). Differences between the enzyme in peaks I and II can also be seen. While the K_m values were the same with and without fluoride, fluoride increased the V_{max} of enzyme II three times more than it did that of enzyme I. Furthermore, differences in the rates of the reverse reaction (i.e., ATP synthesis) and the degree of fluoride inhibition of this process, coupled with different K values strongly suggests that these three enzymes are different. One cannot, however, completely exclude the possibility that the contaminating proteins in peaks I and II may be altering some of the kinetic parameters. It should be noted, however, that two different adenyl cyclase systems have been observed in animal systems (207,208).

The physiological significance of different isozymes possessing adenyl cyclase activity is unknown, but may be a reflection of the association of the enzyme with different subcellular fractions. For example, the loss of enzyme activity in crude extracts during RNase treatment suggests that the enzyme may be bound to, or associated with, RNA. As one would assume that the rather harsh treatment of the cell during breakage by ultrasound (and beads) would disrupt the protein synthesizing machinery, this association may be distinct from its normal

relationship to mRNA and ribosomes during protein synthesis. In addition, a portion of the total activity may have also been associated with the cell membrane before sonic breakage as has been observed with the enzyme from E. coli (155,156).

The adenyl cyclases from S. salivarius differ in a variety of respects from the other bacterial enzymes studied. While all of the microbial enzymes including those from S. salivarius required a divalent ion such as Mg^{2+} for activity, the enzyme from the gram-negative bacteria had optimum pH values at least 1 or 2 units higher (i.e., 9-10) than that observed in the present study. The enzyme from B. liquefaciens, in addition, required pyruvate, or a related α-keto acid for activity (148). Of the three reports concerning the isolation of adenyl cyclase from E. coli (155-157), two of these found the enzyme particulate (155,156), while the third isolated the enzyme from the soluble fraction of the cell (157). In this latter study, adenyl cyclase was inhibited by NaF in direct contrast to the results obtained with S. salivarius.

The fluoride effect on enzyme III was unusual in that it not only caused an increase in the V_{max} but also an increase in the K_m (ATP) for the enzyme (Table 4.8). Several studies with adenyl cyclases from animal tissues (175,206) have demonstrated increased V_{max} values in the presence of fluoride without affecting the K_m of the enzyme. These observations are similar to those obtained with the S. salivarius enzymes in peaks I and II. Clearly, the effect is a complex one, since fluoride not only increased the velocity of the reaction in the direction of cyclic AMP synthesis, but it also inhibited the reverse reaction (Table 4.9). In fact, with enzyme III the fluoride concentration which increased the V_{max} for cyclic AMP synthesis 5-fold also inhibited the reverse reaction

by 73%. Further work will be necessary to elucidate the precise mechanism of fluoride action.

The inhibition of S. salivarius adenyl cyclase III by nucleotides was in general agreement with results obtained with the enzyme isolated from other sources. Adenyl cyclase from thyroid glands was inhibited by deoxyATP, TTP and UTP, but was unaffected by CTP, GTP or ITP (246). In fat cell membrane, the enzyme activity was inhibited by GTP, GDP, GMP, CTP and UTP but cyclic GMP and guanosine had no effect (245). In contrast, the enzyme from rat adrenal or kidney was not affected by GTP. In E. coli, the enzyme was inhibited by ADP, UTP, CTP and GTP (158), but was activated by GMP (156). The inhibition of adenyl cyclase in N. erythropolis (159) by GTP, UTP, CTP, ITP, 3'AMP along with the inhibition of B. liquefaciens enzyme (148) by AMP, dAMP, ADP, dATP and GTP is also in general agreement with S. salivarius enzyme. The reason for difference in the type of inhibition observed with AMP and other nucleoside monophosphates is not clear, but it is possible that AMP may have a complex inhibiting effect on this enzyme.

Although pyruvate-activated (or dependent) adenyl cyclase activity has been observed in B. liquefaciens and several other organisms (159), this compound was a very weak activator of S. salivarius adenyl cyclase III. Further differences can be noted, e.g., the S. salivarius enzyme was affected by a number of glycolytic intermediates, some of which had no effect on the E. coli enzyme (158). However, despite these differences, inhibition by PP_i has been observed with both the E. coli (157) and S. salivarius enzymes. This functional similarity suggests a possible regulatory role for inorganic pyrophosphate on the microbial

adenyl cyclase reaction. The low K_i (0.23 mM) for this compound indicates that this product of the adenyl cyclase reaction must be removed rapidly "in vivo" to avoid extensive inhibition of cyclic AMP formation. The presence of a highly active inorganic pyrophosphatase with a low apparent K_m for PP_i (0.1 - 0.2 mM depending on Mg^{2+} concentration (249)) indicates that pyrophosphate probably does not accumulate to any great extent but is converted to P_i, which is less inhibitory (K_i = 1.40 mM). Thus, the control of inorganic pyrophosphatase activity "in vivo" may be an important factor in the synthesis of cyclic AMP.

Although the exact role of cyclic AMP in S. salivarius has not been determined, the fact that the equilibrium for the adenyl cyclase reaction favors ATP synthesis suggests that this enzyme may play an important function in energy metabolism. As Greengard, Hayaishi and Colowick (151) have shown, the 3'-OH phosphate ester bond of cyclic AMP is a high energy bond, giving a free energy of 11-12 Kcal/mole on hydrolysis, compared to 10 Kcal/mole for the α-β bond (250), or 8 Kcal/mole for the β-γ bond (251) of ATP. Thus, regardless of whether cyclic AMP is converted to ATP and thereby conserving energy, or whether it is hydrolyzed to AMP by phosphodiesterase with the release of 11-12 Kcal/mole, it can be regarded as an energy storage compound. From the evidence, presently available, it would seem that cells of S. salivarius probably do not acquire energy from the latter reaction since cyclic nucleotide phosphodiesterase is apparently absent from these cells (Chapter 5).

The fact that a large number of nucleotides (Fig. 4.18, 4.19, 4.20 and Table 4.10, 4.11, 4.12) and glycolytic intermediates (Fig. 4.22 - 4.26), as well as inorganic phosphate and pyrophosphate (Fig. 4.21),

affect the activity of purified adenyl cyclase III suggests that the mechanism regulating cyclic AMP synthesis in S. salivarius may be a complex one.

CHAPTER 5

FATE AND EFFECTS OF CYCLIC AMP

I. INTRODUCTION

II. THE SEARCH FOR CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

- A. Enzyme activity during growth
- B. Crude extract studies
  - a) Effect of pH
  - b) Effect of divalent ions and reducing agents
  - c) Effect of various metabolites
  - d) Extract fractionation
  - e) Other possible end-products
- C. Effect of snake venoms, phosphodiesterase activator and protein kinase inhibitor
- D. *In vivo* experiments

III. THE FATE OF CYCLIC AMP

IV. THE EFFECTS OF CYCLIC AMP

- A. Phosphofructokinase
- B. Glycogen phosphorylase
- C. P-enolpyruvate phosphotransferase system

V. DISCUSSION

I. INTRODUCTION

The content of cyclic AMP in cells is regulated by two processes: (a) by the rate of its synthesis from ATP by adenyl cyclase and (b) by the rate of its removal within or from the cell. Two mechanisms have been demonstrated for the removal of cyclic AMP from microbial cells. The first of these involves the degradation of cyclic AMP to AMP by cyclic nucleotide phosphodiesterase (1,2,5,252), while the second results in the extrusion of this nucleotide into the medium (93).

With the exception of red cells, the presence of cyclic nucleotide phosphodiesterase has been reported in all mammalian tissues tested with brain containing the highest enzymatic activity (18,253-255). Subcellular fractionation of various tissues has indicated that the enzyme is partially soluble and partially particulate (18,209,256), the relative amount of enzyme activity in the fractions depending on the source of the enzyme. Maximum activation of enzyme activity in these tissues was observed with Mg^{2+} or Mn^{2+}, while other ions were less effective (179,256-262). The optimum Mg^{2+} concentration varied from 0.08 - 10 mM, again depending on the source of the enzyme (179,253,258,259,262). Kakiuchi, Yamazaki and Teshima (263) recently isolated two phosphodiesterases from rat brain by column chromatography with Sepharose 6B. Enzyme I was Mg^{2+}-dependent whereas enzyme II activity was dependent on Ca^{2+} and Mg^{2+}. An earlier study by Kakiuchi and Yamazaki (264) demonstrated that the presence of a heat-stable, non-dialyzable factor was essential for the activity of enzyme II.

The presence of cyclic nucleotide phosphodiesterase has also been observed in lower forms of life, such as in yeast (165,265), slime

mold (166-168), several marine organisms (260) and several bacteria (7,159-164). While the enzyme is present in some strains of E. coli, other strains of this organism possess very low or negligible quantities of the enzyme (7,161). However, the degradation of cyclic AMP to AMP is not the only mechanism for its removal from bacterial cells since Makman and Sutherland (93) have demonstrated its extrusion into the medium with resting cells of E. coli. These authors observed that in the presence of glucose, cyclic AMP rapidly disappeared from the cells and instantly appeared in the medium. Such an effect was also observed in the presence of acetate and succinate.

As a companion study to the synthesis of cyclic AMP by adenyl cyclase in S. salivarius, a study was undertaken to determine the mechanism of cyclic AMP removal from cells of this organism. As a first step, the cellular content of cyclic AMP was measured during carbohydrate fermentation by non-proliferating cells grown with glucose, fructose, mannose or raffinose as the sole carbon source. In these experiments, late exponential-phase cells of S. salivarius were harvested, washed and finally suspended at a concentration of 10 mg dry wt/ml in 50 mM (pH 7.0) phosphate buffer (171). The cells were then preincubated anaerobically for 20 min at 37^{o}C in buffer alone followed by the addition of the appropriate carbon source. One ml samples were withdrawn throughout the incubation period for cyclic AMP analysis (Fig. 5.1). Following the depletion of the first carbon source, glucose was added to the cell suspension and the sampling continued. In these experiments, the incubation mixture was maintained at a constant pH (7.2 ± 0.1) with 1 N NaOH, added by means of a magnetic valve coupled to a Radiometer Model

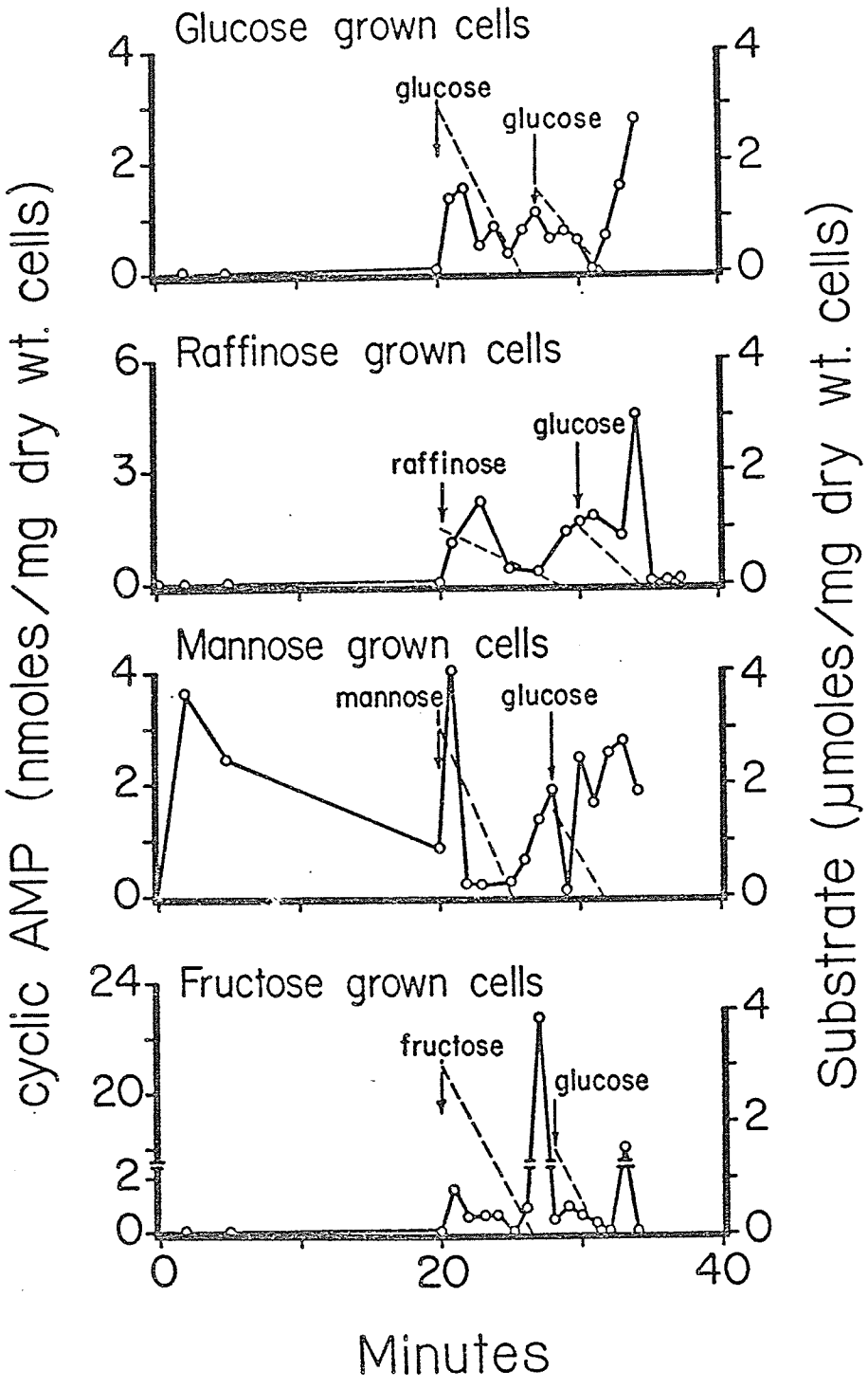


Fig. 5.1. Cyclic AMP levels during carbohydrate metabolism in non-proliferating cells of S. salivarius grown with different sugars. All sugars added to the cells at the time indicated by the arrows.

26 pH-meter-titrator assembly (Radiometer, Copenhagen). The rate of cellular carbohydrate metabolism was monitored by the amount of NaOH required to keep the pH constant. In some experiments, the amount of glucose utilized was confirmed by determining the glucose content in the samples by the glucose-oxidase method (see Methods).

The samples withdrawn for cyclic AMP assay throughout the incubation period were stored at $0^{o}C$ until the end of the experiment and then centrifuged at 3,000 g for 10 min. The supernatant was decanted and the sedimented cells extracted with 1 N perchloric acid for 30 min followed by neutralization. Cyclic AMP was isolated from the extracts by passing them through small columns (0.5 x 6 cm) of Dowex 50-X8 (100-200 mesh) and treating the cyclic AMP-containing fractions with $Ba(OH)_2$-$ZnSO_4$. The samples were adjusted to pH 8 before being analyzed for cyclic AMP by the method of Brooker, Thomas and Appleman (195) (Method (A)).

As shown in Fig. 5.1, little cyclic AMP was formed during the preincubation period in buffer except for the cells grown in mannose. In these latter cells, the cyclic AMP level increased rapidly initially and then slowly decreased during the remainder of the preincubation period. With all of the sugars tested, the cellular cyclic AMP level increased just after the addition of the carbon source and immediately following its depletion. By far the greatest amount of cyclic AMP (22.8 nmoles/mg dry wt cells) was formed by fructose-grown cells following the complete exhaustion of fructose by the resting cells. Although, no consistent cyclic AMP profiles were observed with the various sugars, some differences were observed. For example, after the addition of

glucose to mannose-grown cells the cyclic AMP levels decreased instantly and then reached another steady-state level. With the other cell types, the cyclic AMP content decreased slowly during the entire period of glucose utilization and then increased after the depletion of glucose.

These preliminary experiments demonstrated that the cyclic AMP content "in vivo" fluctuates rapidly during the metabolism of sugars. In fact, cyclic AMP could be synthesized and removed at rates as high as 22 nmoles/mg cells/min (fructose-grown cells). Although, it is clear that the formation of cyclic AMP in these cells was due to the action of adenyl cyclase, the mechanism for the decrease in the cellular cyclic AMP content was not clear. As mentioned previously, this may have been due to (i) its conversion to AMP by phosphodiesterase and/or (ii) the extrusion of this nucleotide from the cells into medium. In order to determine whether the observed rapid loss of cellular cyclic AMP was due to a phosphodiesterase, a study was undertaken to isolate and characterize this enzyme from S. salivarius. In the following section, we will discuss all of the variables examined in an effort to detect the activity of phosphodiesterase in extracts of this organism. Despite an extensive search, cyclic nucleotide phosphodiesterase activity was not detected in S. salivarius.

II. THE SEARCH FOR CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

In all of the experiments outlined below, unless otherwise specified, extracts were prepared from the late exponential phase cells of S. salivarius by sonication (Branson Sonifier) for 3 min at $0^{o}C$ with glass beads (cells:beads = 3:1) followed by centrifugation at 30,000 g

for 20 min. Three assay procedures, (A(P), B(P) and C(P)), were employed to assay for phosphodiesterase activity throughout the work.

A. Enzyme activity during growth

As shown in Chapter 4, the activity of adenyl cyclase in S. salivarius was relatively dependent on the phase of the growth (Fig. 4.1). Thus, it was conceivable that phosphodiesterase might also be active at different phases of the growth. To test this possibility, cells were harvested by centrifugation at various points during growth in 0.3% glucose-TYE broth and cell-free extracts prepared. Phosphodiesterase activity was sought in the whole homogenate, the 30,000 g supernatant and the 30,000 g pellet suspended in Tris-HCl buffer (50 mM, pH 7.5). No detectable phosphodiesterase activity was observed in any of these samples. Similar results were also observed with extracts prepared from late exponential-phase cells grown in TYE broth with different concentrations of glucose (0.05-0.5%).

To test for the possibility that cyclic AMP phosphodiesterase might be repressed by glucose under the growth conditions used in the above studies, cells of S. salivarius were grown with fructose, galactose, lactose, maltose or sucrose at concentrations of 0.1 and 0.3%. The extracts prepared from these various cells were also devoid of phosphodiesterase activity.

Since it was conceivable that cyclic AMP might induce the synthesis of cyclic phosphodiesterase, dibutyryl cyclic AMP, at concentrations of 1 and 5 mM, was added to cells of S. salivarius growing in glucose-TYE broth. The dibutyryl derivative was used because previous experiments had shown that S. salivarius cells are impermeable to extracellular cyclic AMP.

However, despite the use of dibutyryl cyclic AMP, phosphodiesterase activity was not observed in cells receiving this compound nor was the rate and total growth of the cells altered compared to control cells grown without the cyclic nucleotide.

The method of cell breakage was also varied in order to determine whether it influenced the activity of phosphodiesterase in extracts of S. salivarius. For this purpose, late exponential phase cells were disrupted by ultrasound in the Branson and Biosonik Sonifiers for periods ranging from 0.5 min to 30 min, with and without 5 μm and 25 μm glass beads in various cell to bead ratios. The variously prepared extracts again showed no activity of phosphodiesterase.

B. Crude extract studies

a) Effect of pH. The pH optimum for phosphodiesterase in various mammalian systems ranges from pH 7-9 (179,256-261). The optimum pH for the enzyme in various microbial extracts is in the same range, e.g., 8.5 for the Saccharomyces carlsbergensis (165), 8.4 for E. coli (160) and 7.5 - 8.5 for Serratia marcescens (164).

In an attempt to detect phosphodiesterase activity in extracts of S. salivarius, assays were carried out at pH values between 5.0 and 10.5 with the following buffers: sodium acetate-acetic acid (pH 5-6), β-glycerophosphate buffer (pH 6-7), Tris-β-glycerophosphate (pH 6.5-8.5), glycylglycine buffer (pH 7-8.5), Tris-HCl (pH 7-9.5) and glycine-NaOH (pH 9-10.5). No detectable enzyme activity was observed in any of the assay mixtures employing these buffers.

b) Effect of divalent ions and reducing agents. The activity of mammalian phosphodiesterase is stimulated by divalent ions such as Mg^{2+} or Mn^{2+} (179,252,256-264). In extracts of Saccharomyces carlsbergensis (165) phosphodiesterase was activated by Mn^{2+} ions, while the Serratia marcescens enzyme (164) was stimulated by Fe^{2+}, Ca^{2+} and Ba^{2+}, but not by Mg^{2+} and Mn^{2+}. In some strains of E. coli (7), enzyme activity was dependent on Fe^{2+} and in the presence of reducing agents such as mercaptoethanol.

In view of these reports, the effect of Mn^{2+}, Ca^{2+}, Fe^{2+}, Sn^{2+}, Ba^{2+}, Zn^{2+} and Sr^{2+} ions at concentrations of 1 mM and 5 mM was examined with extracts prepared from the late exponential-phase cells of S. salivarius. In all cases phosphodiesterase activity was not observed. Furthermore, the addition to the extract preparations of EDTA or EGTA, with and without several divalent ions, Mg^{2+} alone at concentrations from 0.1 - 100 mM or reducing agents (e.g., mercaptoethanol, mercaptoacetic acid, cysteine, glutathione and dithiothreitol) added separately or in combination with various divalent ions, had no effect.

c) Effect of various metabolites. Since various cellular metabolites are important in the regulation of adenyl cyclase activity in S. salivarius (Chapter 4), it was postulated that a phosphodiesterase in this organism might also be regulated in a similar manner. This suggestion was supported by the observations of Monard, Janecek and Rickenberg (162), who demonstrated the activation of E. coli phosphodiesterase with several phosphorylated hexoses and pentoses. With this in mind, phosphodiesterase activity was sought by assaying the 30,000 g supernatant of sonically disrupted cells in presence of glucose, glucose-1-P, glucose-6-P, fructose-6-P,

fructose-1,6-P_2, glyceraldehyde-3-P, P-enolpyruvate, pyruvate, NAD, NADP, NADH, NADPH and several other nucleotides. None of these compounds elicited phosphodiesterase activity in the extract preparation.

The hydrolysis of cyclic AMP by cyclic nucleotide phosphodiesterase in rat tissues (266) and in rat thymic lymphocytes (267) has been shown to be stimulated by low amounts (to 20 μM) of cyclic GMP when added with cyclic AMP to the assay mixture. However, whether added separately, or in combination at concentrations between 1 μM to 10 mM, these two cyclic nucleotides did not elicit phosphodiesterase activity in crude preparations of S. salivarius.

d) Extract fractionation. Since the purification of adenyl cyclase from S. salivarius demonstrated that enzyme activity could be suppressed by the presence of inhibitor(s) in the crude extract preparations (Table 4.4), it was conceivable that phosphodiesterase activity might have been similarly affected. To test for this, a sample of the whole homogenate and the 30,000 g supernatant, obtained from the whole homogenate, were fractionated by ammonium sulfate fractionation, by gel filteration on Sephadex G-200 and by ion exchange chromatography on DEAE-cellulose. Ammonium sulfate precipitation was carried out in increments of 10% from 20-80% saturation. The fractions obtained were dialyzed overnight against Tris-HCl buffer (50 mM, pH 7.5), before analysis. The samples were run in the descending direction on a Sephadex G-200 (2.5 x 42 cm) with Tris-HCl buffer, while the proteins adsorbed onto DEAE-cellulose were eluted in a stepwise fashion with increasing concentrations of NaCl in 50 mM Tris-HCl buffer (pH 7.5). All of the fractions collected during these three procedures were devoid of phosphodiesterase activity.

e) Other possible end-products. In the above experiments, phosphodiesterase activity was primarily measured by one of two assays (B(P) and C(P)) involving cyclic AMP depletion. Assay B(P) involved measuring the cyclic AMP content following the precipitation of other nucleotides by $Ba(OH)_2$-$ZnSO_4$ treatment, while assay C(P) involved the separation of the assay components by paper chromatography. The latter method was particularly useful since cyclic AMP depletion and AMP formation could be measured at the same time, although AMP (and adenosine) were never formed with preparations of S. salivarius. However, since Gulyassy and Oken (268) and Therriault and Winters (269) have shown that the degradation of cyclic AMP in a crude phosphodiesterase system may result in the formation of other products such as inosine and hypoxanthine, we decided to pursue this possibility further. To this end, S. salivarius extracts were assayed with cyclic ^{3}H-AMP as the substrate. After the termination of the reaction, the assay components were isolated by 2-dimensional thin layer chromatography (TLC) using glass fiber paper impregnated with Silica gel (ITLC-type SG - Gelman Instrument Co.). The paper was developed with the solvents described by Therriault and Winters (269) (solvent I, n-butanol:iso-propanol: NH_4OH = 70:20:10, and solvent II, n-butanol:iso-propanol:H_2O = 50:25:25). The results obtained from this experiment confirmed the earlier observationns, i.e., that the substrate cyclic AMP was not hydrolyzed to adenosine, inosine, hypoxanthine, xanthine, 5'-IMP and 5'-AMP.

C. Effect of snake venoms, phosphodiesterase activator and protein kinase inhibitor

Cheung (270) partially purified an inactive phosphodiesterase from bovine brain which could be activated by preincubating the enzyme with

different snake venoms. This activation was not due to the protection of the enzyme by proteins contained in the venom or to the relief of product inhibition by the action of venom 5'-nucleotidase activity on AMP. This possibility was tested by preincubating S. salivarius crude extracts with two snake venoms (Crotalus atrox and Naja naja) at 37°C for 10 min and then assaying for phosphodiesterase activity. No enzyme activity was observed as a result of this treatment.

Cheung (271,272) further reported the presence of a protein activator for the brain phosphodiesterase which was removed during the purification procedures. The purified enzyme was fully active only in the presence of this activator. A protein activator of bovine heart phosphodiesterase was kindly made available to us by Dr. J. H. Wang, Department of Biochemistry, Faculty of Medicine, to test with crude extracts of S. salivarius. The addition of the activator, however, did not elicit enzyme activity in the S. salivarius preparations. Similar negative results were also obtained when protein kinase inhibitor, isolated from bovine skeletal muscle, was added to our assay system. This inhibitor was prepared for the assay of cyclic AMP by the method of Gilman (196).

D. In vivo experiments

Since phosphodiesterase was not detected in cell-free extracts of sonically-disrupted cells of S. salivarius, it was concluded that, if phosphodiesterase was present, it may have been destroyed during sonification. To test this possibility, cyclic AMP degradation was measured under "in vivo" conditions with cells treated with 0.5% toluene for 30 min at 37°C to make them permeable to cyclic AMP. This procedure was considered

gentle enough to preserve enzyme activity, if it existed, and yet make cyclic AMP available to the cellular contents. Enzyme activity was sought by using assays A(P) and C(P) as described in Methods, except that the toluene-treated cells replaced the extract preparations in the reaction mixture. Phosphodiesterase activity was not detected.

Further to the previously mentioned experiments, many of the tests performed with the S. salivarius extracts were also carried out with extracts of strains AHT, BHT, HHT and 6715 of the cariogenic oral microbe, S. mutans, in an effort to detect phosphodiesterase activity. In all cases, phosphodiesterase activity was not observed.

Thus, the aforementioned experiments clearly indicate that S. salivarius, and at least some other members of the genus Streptococcus, are devoid of cyclic nucleotide phosphodiesterase activity.

III. THE FATE OF CYCLIC AMP

Since phosphodiesterase was not present in S. salivarius, the obvious question to be asked is what mechanism is responsible for the rapid loss of cyclic AMP from non-proliferating cells previously observed in Fig. 5.1. Early work by Makman and Sutherland (93) with E. coli has indicated that the loss of cyclic AMP from the cells was due to its extrusion into the medium. In order to determine whether such a mechanism might exist in S. salivarius, the intracellular and extracellular content of cyclic AMP was measured in systems employing growing and non-proliferating cells.

The first series of experiments involved assaying the cyclic AMP

content of cells growing in a glucose-TYE broth. For this, two flasks containing 0.2% glucose-TYE broth were inoculated with an overnight culture and samples (5 ml) withdrawn from both flasks periodically by sterile syringe and the contents immediately filtered to separate the cells from the supernatant (medium) by the celite-filter procedure outlined in Methods. One flask served as the control while the other flask received 12 mM NaF when the cells were in mid-exponential phase. The cells in the celite matrix on the filter were extracted with perchloric acid, neutralized and treated with $Ba(OH)_2$-$ZnSO_4$ followed by adjustment of the pH to 4.0. The medium in the filtrate was concentrated to dryness by flash evaporation after $Ba(OH)_2$-$ZnSO_4$ treatment and suspended in 50 mM sodium acetate-acetic acid buffer (pH 4.0). Both fractions were then analyzed for cyclic AMP by the method of Gilman (196) (Methods B).

Unlike the resting cell experiments shown previously in Fig. 5.1, the cyclic AMP levels in the cells were relatively constant throughout the experiment falling off as the cells reached the stationary phase (Fig. 5.2(D)). The cyclic AMP levels in the medium were essentially constant throughout the growth period (Fig. 5.2(C)). The addition of NaF to the cells in the exponential phase resulted in complete inhibition of growth (Fig. 5.2(A)) and glucose uptake (Fig. 5.2(B)), while the cyclic AMP content in the medium increased rapidly (Fig. 5.2(C)). This increase in the extracellular cyclic AMP occurred without a significant change in the cyclic nucleotide content of the cells (Fig. 5.2(D)). When the intracellular levels of cyclic AMP were expressed in terms of a constant unit of cell weight (i.e., nmoles/mg dry wt cells), the cellular cyclic AMP content was observed to decrease throughout the growth period until the onset of

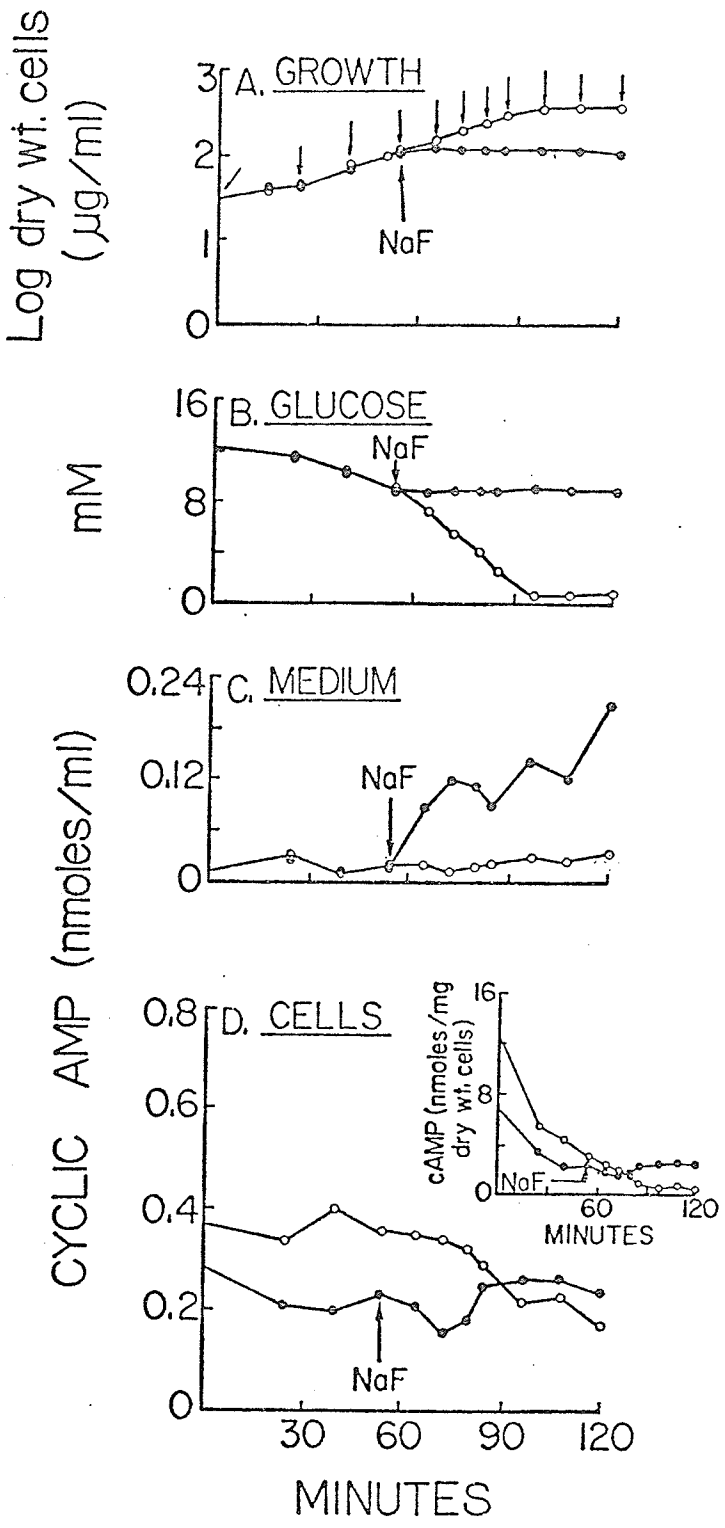


Fig. 5.2. The distribution of cyclic AMP in the cells and in the medium during growth of S. salivarius in 0.2% glucose-TYE broth, with and without the addition of 12 mM NaF. The addition of NaF has been indicated by the arrow. Control: -O-O- , NaF: -◎-◎-. Cellular cyclic AMP content represents the amount containing in the cell obtained from 1 ml of suspension.

stationary phase (Fig. 5.2(D))-inset) suggesting that little, if any, cyclic AMP was synthesized during growth.

When the experiment was repeated with the addition of a lower concentration (0.1%) of glucose to growth medium 35 min after the addition of the inoculum, somewhat similar results were obtained. However, while the addition of NaF to the cells in the exponential phase showed the same effect on cell growth (Fig. 5.3(A)) and on glucose uptake (Fig. 5.3(B)) as in the previous experiment, a lag period of 55 min was observed after the NaF addition before the increase in the cyclic AMP content of the medium was observed (Fig. 5.3(C)). The reason for this phenomenon is unknown.

Since the results obtained with growing cells (Fig. 5.2 and 5.3) showed little, if any, fluctuation in the cellular content of cyclic AMP compared to the earlier experiments with non-proliferating cells (Fig. 5.1), experiments were again undertaken to measure cyclic AMP in a non-proliferating cell system. However, on this occasion, the cells were separated from the medium by the rapid filter-celite method and cyclic AMP was assayed with the more sensitive method of Gilman (196). In these experiments, cyclic AMP was assayed in glucose-grown cells and in the extracellular medium during and after the metabolism of fructose or glucose. As can be seen in Fig. 5.4(A) and 5.4(B), the cyclic AMP level, both within the cells and in the medium, was almost constant during the 10 min preincubation period. The addition of fructose resulted in a linear increase in intracellular level of cyclic AMP until the fructose was depleted (Fig.5.4(A)); no significant change was observed in the cyclic AMP concentration in the extracellular medium during this period. However, immediately following the complete utilization of this sugar, the cyclic AMP was excreted from the cells into the medium. With glucose, on the

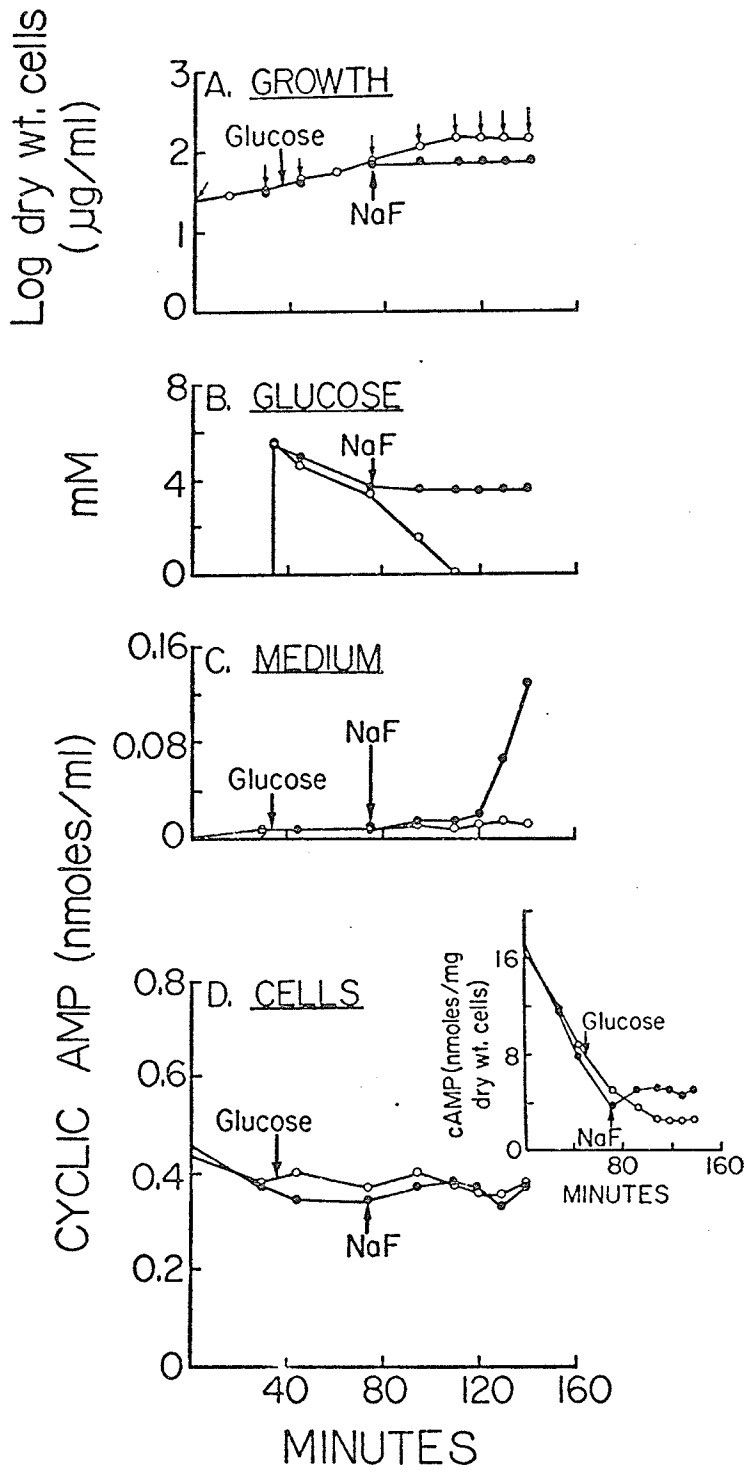


Fig. 5.3. The distribution of cyclic AMP in the cells and in the medium during the growth of S. salivarius in 0.1% glucose-TYE broth, with and without the addition of 12 mM NaF. The addition of glucose and NaF has been indicated by the arrows. Control:-O-O-, NaF:-●-●-.

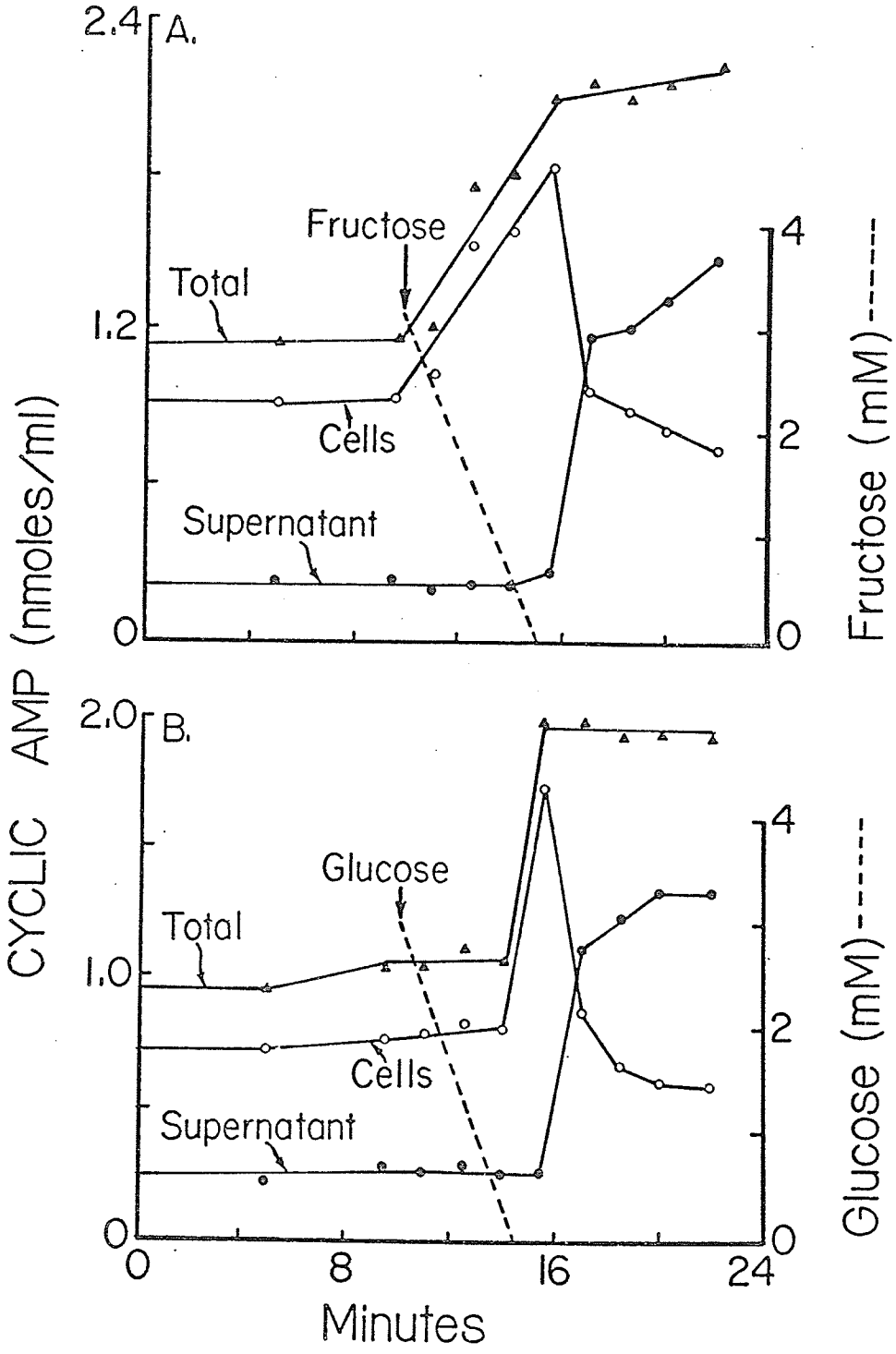


Fig. 5.4. The distribution of cyclic AMP in the cells and in the medium during the metabolism of fructose (A) and glucose (B) by the non-proliferating cells (2 mg/ml) of S. salivarius at a constant pH of 7.2 ± 0.1.

other hand, the cyclic AMP content, both within the cells and in the medium, did not change until glucose was completely depleted (Fig.5.4(B)). At this point, the cellular cyclic AMP increased rapidly for a short period and then was excreted into the medium at an equally rapid rate.

The more recent results obtained with resting cells in Fig. 5.4, differed from those observed in the earlier experiments (Fig. 5.1). Since the later experiments employed improved procedures for the separation of the cells from the medium, as well as the isolation and assay of cyclic AMP, they probably reflect more accurately the distribution of cyclic AMP than the earlier results. With these improved techniques it was possible to obtain consistent results between experiments.

IV. THE EFFECTS OF CYCLIC AMP

Although the work of Pastan, Perlman and their associates have established the role of cyclic AMP in the regulation of inducible enzyme synthesis in unicellular organisms, relatively little is known about its function on other cellular processes. Since we have, to date, been unable to demonstrate inducible enzyme synthesis in *S. salivarius*, probably because energy metabolism by this organism is restricted to the fermentation of relatively few carbohydrates, we were unable to pursue the function of cyclic AMP in this direction. As an alternative project, the effect of cyclic AMP on the activity of a number of regulatory enzymes was examined. For this study, phosphofructokinase and glycogen phosphorylase were selected because of their known regulation by cyclic AMP in other systems (Chapter 2), as well as the P-enolpyruvate

phosphotransferase system (PEP-PTS) for glucose transport in S. salivarius (273). This latter system was selected because of the current interest in our laboratory on the factors regulating glucose transport in this organism. No attempt was made to study these effects in detail since the purpose of these experiments was to indicate the direction of future research.

A. Phosphofructokinase

Mansour and several other workers (65-85) have demonstrated the activation by cyclic AMP of phosphofructokinase from various mammalian tissues, as well as the reversal of the ATP inhibition of this enzyme by this nucleotide. Since the substrate (fructose-6-P) and product (fructose-1,6-P_2) of the phosphofructokinase reaction had a significant regulatory effect on the activity of purified adenyl cyclase in S. salivarius (Fig. 4.23 and 4.24), it was postulated that the activity of phosphofructokinase might be regulated by cyclic AMP in a reciprocal fashion. This possibility was examined by measuring the activity of phosphofructokinase in crude extracts of S. salivarius in the presence of increasing concentrations of cyclic AMP. As indicated in Table 5.1, the enzyme was activated by cyclic AMP and the activation increased with increasing concentrations of this nucleotide, at least to a level of 1 mM.

B. Glycogen phosphorylase

Unlike mammalian glycogen phosphorylases (6), microbial phosphorylases, with the exception of Neurospora crassa (274), appear

TABLE 5.1

Effect of cyclic AMP on phosphofructokinase activity in crude extracts of S. salivarius.

Cyclic AMP concentration (mM)	Enzyme activity	Relative activity
0	5.3[a]	1.0
0.001	10.3	1.9
0.01	18.6	3.5
0.1	25.1	4.7
1.0	32.8	6.2

[a] nmoles of fructose-1,6-P_2 formed/mg protein/min.

to exist only in one active form (184,275-277). The glycogen phosphorylase of N. crassa exists in two interconvertible forms: one active (AMP-dependent) form and the other inactive in the absence of AMP. Like the majority of microbial enzymes, glycogen phosphorylase in S. salivarius appears to exist only in one active form since preincubation with ATP and Mg^{2+} (and an ATP-regenerating system) did not increase enzyme activity (Table 5.2). In fact, such treatment resulted in a loss of activity which was particularly severe for the extract preparation obtained from stationary phase cells. The observed inhibition, increased with the time of preincubation and was considerably augmented by 1 mM cyclic AMP. Similar inhibiting effects were also observed with cyclic AMP at concentrations as low as 10 µM when incubated with ATP-Mg^{2+}. In addition, preincubation of the crude extracts for 30 min with no addition, followed by a further 10 min incubation period in the presence of ATP-Mg^{2+} and cyclic AMP produced the same results, further differentiating this enzyme from the one in N. crassa (274).

Although nucleotides were not required for enzyme activity, charcoal treatment of the crude extracts with 5% Norit A following preincubation with and without ATP-Mg^{2+} resulted in the loss of some activity (Table 5.3). With this treatment, the inhibition of the enzyme by ATP-Mg^{2+} was abolished when compared to the charcoal-treated enzyme incubated in the absence of ATP. However, the addition of 1 mM and 10 µM cyclic AMP to the preincubation medium in combination with ATP-Mg^{2+} resulted in the usual inhibition of glycogen phosphorylase activity in both the control and charcoal-treated extracts. Surprisingly, the addition to the preincubation medium of cyclic AMP alone was not

TABLE 5.2

Effect of preincubation with ATP and cyclic AMP on the activity of glycogen phosphorylase in crude extracts of S. salivarius obtained from late exponential phase (exp. 1) and stationary phase cells (exp. 2).

Preincubation additions	Time of preincubation (min)			
	0	2	5	10
Exp. 1				
None	0.80[a]	0.71	0.71	0.72
+ ATP[b]	-	0.77	0.64	0.28
+ ATP + cyclic AMP (1 mM)	-	0.23	0.21	0.19
Exp. 2				
None	0.50	0.47	0.46	0.47
+ ATP	-	0.13	0.10	0.11
+ ATP + cyclic AMP (1 mM)	-	0.10	0.09	0.08

[a] After the preincubation period, the samples were dialyzed for 4 hours with two changes of Tris-HCl buffer (50 mM, pH 7.0) and assayed for glycogen phosphorylase activity. Units are nmoles of glucose-^{14}C incorporated into glycogen/mg protein/30 min.

[b] In all cases when ATP was employed in the preincubation medium, ATP regenerating system was included in the experiment. The reaction mixture contained: 2.5 mM ATP, 10 mM $MgCl_2$, 5 mM P-enolpyruvate, 20 mM KCl, 10 µg commercial pyruvate kinase and crude extract.

TABLE 5.3

Effect of preincubation with ATP and cyclic AMP on the activity of glycogen phosphorylase in crude extracts of S. salivarius subsequently treated with charcoal.

Preincubation additions	Phosphorylase activity	
	Control	Charcoal treatment[a]
None	1.00[b]	0.71
ATP[c]	0.86	0.73
ATP + cyclic AMP (10 μM)	0.44	-
ATP + cyclic AMP (1 mM)	0.39	0.34
Cyclic AMP (10 μM)	0.96	-
Cyclic AMP (1 mM)	1.21	1.17

a The samples were treated with 5% charcoal (Norit A) after preincubation for 30 min as indicated.

b nmoles of glucose-^{14}C incorporation into glycogen/mg protein/30 min.

c Conditions as in Table 5.2.

inhibitory at both concentrations, in fact, this nucleotide actually activated the enzyme at the 1 mM level.

The effect of cyclic AMP in relation to AMP and NaF was further examined with a 200-fold purified glycogen phosphorylase (278) prepared from S. salivarius. The enzyme was activated 2-fold by 1 mM cyclic AMP in the absence of AMP and NaF, but cyclic AMP was completely ineffective in the presence of AMP and NaF. The combined effect of AMP and cyclic AMP on enzyme activity is illustrated more completely in Fig. 5.5. It can be seen that, as a function of the AMP concentration (Fig. 5.5(A)), 1 mM cyclic AMP had no additional effect on the enzyme compared to the activity with AMP alone. As shown above, 1 mM cyclic AMP activated the enzyme only in the absence of AMP; a higher concentration (5 mM) of this nucleotide was slightly inhibitory. The inhibition is more clearly observed when the values are plotted as a function of the cyclic AMP concentration (Fig. 5.5(B)). In the presence of 1 and 5 mM AMP, increasing levels of cyclic AMP resulted in a slight but steady decline in activity, which, under these conditions, was still greater than the activity with cyclic AMP alone. The influence of NaF on varying levels of both AMP and cyclic AMP is also shown in this figure. The combination of AMP + NaF produced twice the activation of the S. salivarius phosphorylase, and at a lower AMP level (Fig. 5.5(A)), than did the combination of cyclic AMP + NaF (Fig. 5.5(B)).

C. P-enolpyruvate phosphotransferase system

Previous research in our laboratory has demonstrated that glucose is transported into cells of S. salivarius by the PEP-PTS (273). This

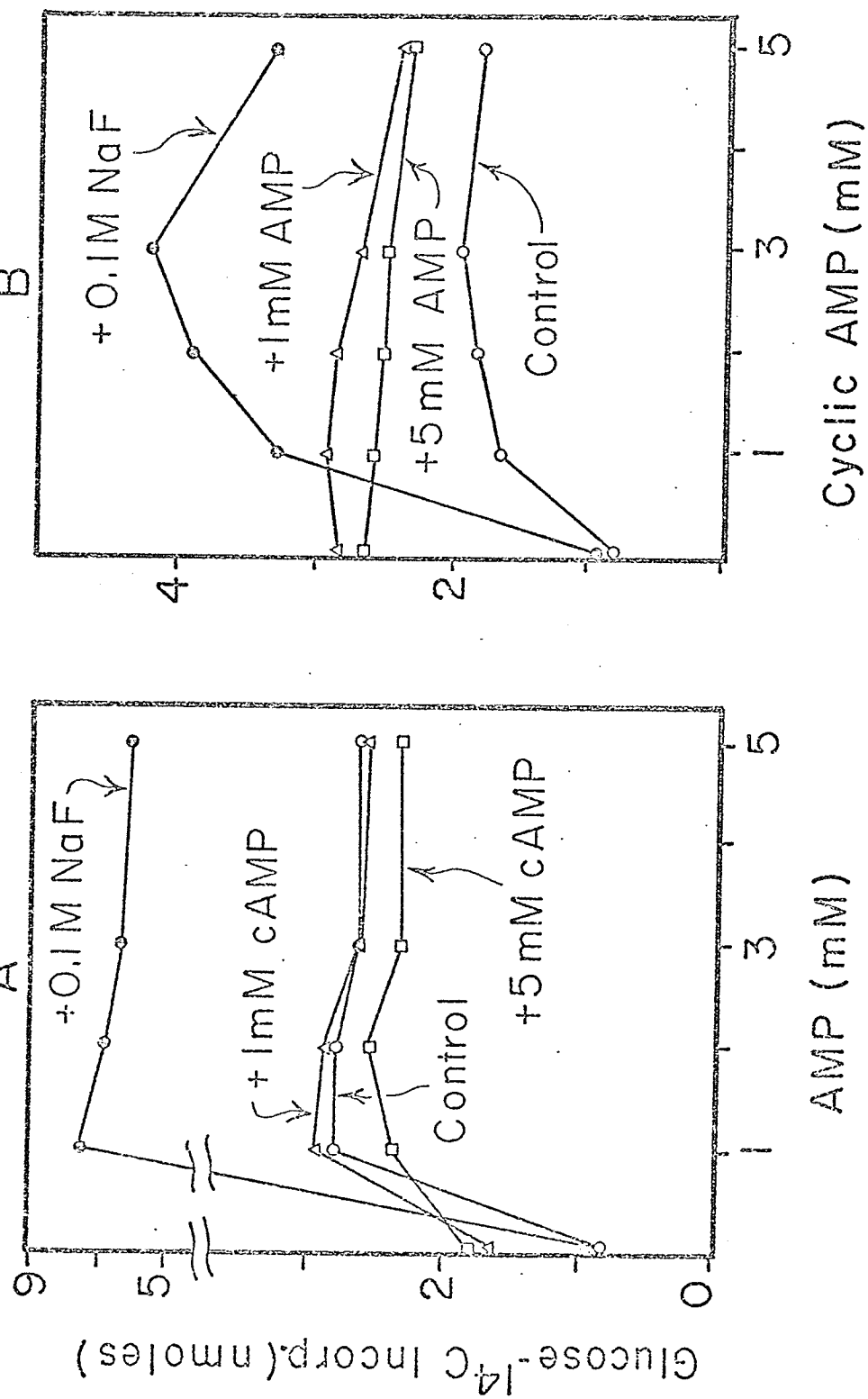


Fig. 5.5. Effect of varying concentrations of AMP (A) and cyclic AMP (B) on the activity of purified S. salivarius glycogen phosphorylase incubated with the other component and NaF. Assay conditions were same as described in Methods except that pH 6.0 β-glycerophosphate buffer was used in place of this buffer at pH 6.8.

system was first observed in E. coli by Kundig, Ghosh and Roseman (122) and later identified as the predominant system for the transport of glucose in a variety of bacteria (123,189,279,280). The activity of the PEP-PTS in toluene-treated cells of S. salivarius was measured by the phosphorylation of 2-deoxyglucose-U-^{14}C in the presence of P-enolpyruvate (273). The effect of cyclic AMP, dibutyryl cyclic AMP and theophylline on the activity of this transport system was determined. As shown in Table 5.4, the inhibition by dibutyryl cyclic AMP and theophylline was greater than the inhibition observed with cyclic AMP. The percent inhibition with the 10 μM dibutyryl cyclic AMP was greater than that observed with 1 mM. It can also be seen in this table that the inhibition by theophylline was partially overcome by cyclic AMP. These results indicated that cyclic AMP may play some role in the regulation of this transport system.

V. DISCUSSION

Since the discovery of cyclic AMP in microbial systems, much of the research in this field has been concentrated on the function of cyclic AMP in the regulation of inducible enzyme synthesis. Less interest has been shown in the regulation of cyclic AMP synthesis by adenyl cyclase and even less interest in the factors controlling the loss of cellular cyclic AMP. Earlier, Makman and Sutherland (93) observed the presence of cyclic AMP both in cells and in the medium of E. coli cultures. However, conflicting observations have been recently reported by Peterkofsky and Gazdar (281). While working on the effect of glucose on cyclic AMP

TABLE 5.4

Effect of cyclic AMP, dibutyryl cyclic AMP and theophylline on transport of 2-deoxyglucose by P-enolpyruvate phosphotransferase system in toluene-treated cells of S. salivarius.

Additions	Rate of phosphorylation	Percent inhibition
None	565.8[a]	-
Cyclic AMP (10 μM)	549.3	2.9
Cyclic AMP (1 mM)	534.9	5.5
Cyclic AMP (5 mM)	481.6	14.9
Dibutyryl cyclic AMP (10 μM)	443.0	21.7
Dibutyryl cyclic AMP (1 mM)	498.4	11.9
Theophylline (1 mM)	439.3	22.4
Cyclic AMP (10 μM) + theophylline (1 mM)	450.7	20.3
Cyclic AMP (1 mM) + theophylline (1 mM)	500.1	11.6

[a] nmoles of 2-deoxyglucose-6-P-^{14}C formed from 2-deoxyglucose-U-^{14}C/mg dry wt cells in 30 min.

metabolism in E. coli, these authors could not detect any cellular cyclic AMP. In this latter study, the cyclic AMP content of the total system (i.e., cells + medium) was determined by boiling the reaction mixture for 3 min to extract any cellular cyclic AMP. The total cyclic AMP content was assayed in the clear supernatant obtained after centrifugation. The extracellular cyclic AMP was determined by the immediate separation of the cells from the exogenous medium by centrifugation followed by boiling of supernatant for 3 min. Cellular cyclic AMP content was taken as the difference between these two fractions. In our experiments with S. salivarius, cellular cyclic AMP could not be extracted by boiling the cell suspension for 3 min in a fashion similar to Peterkofsky and Gazdar (281). It was necessary to treat cells of S. salivarius with 1 N perchloric acid for 30 min in order to extract the cellular cyclic AMP with this organism.

The observation that the cyclic AMP content of both the cells and the exogenous medium were constant during growth of S. salivarius in complex medium consisting of tryptone-yeast extract was surprising (Fig. 5.2 and 5.3). These findings suggest that cyclic AMP probably is not required for the growth of this organism in a nutritionally complete medium. Clearly, much more work is required on the growth of this organism in a defined medium, as well as on its genetics, before definitive answers will be obtained.

The effect on cyclic AMP synthesis by the addition of NaF to growing cultures (Fig. 5.2 and 5.3) was not completely surprising since NaF was known to stimulate adenyl cyclase activity in S. salivarius (Fig. 4.4 and 4.12). However, the results demonstrated that this

additional cyclic AMP was formed after the complete inhibition of growth and glucose uptake, and was excreted into medium as soon as it was formed. Previous experiments with resting cells of S. salivarius metabolizing glucose have demonstrated that the addition of NaF to these cells results in the immediate decline in the ATP content of these cells (273). Experiments are in progress to determine whether this loss of ATP is in fact related to the extrusion of cyclic AMP into the medium under identical conditions.

The patterns observed for the cellular and exogenous cyclic AMP levels in experiments with fermenting resting cells of S. salivarius (Fig. 5.1 and 5.4) were different than those observed with growing cells (Fig. 5.2 and 5.3). Of significance in the resting cell experiments was the intracellular increase of cyclic AMP after the addition of the sugar and just after the depletion of the carbon source, as well as its excretion into the medium. Unlike these results, cyclic AMP in cells of E. coli increased on incubation in phosphate buffer but was excreted into the medium after the addition of glucose (93). However, an abrupt increase in the cellular content of cyclic AMP in this organism, just after the depletion of glucose, was in agreement with our present findings with S. salivarius. A similar increase in cyclic AMP was also observed by Peterkofsky and Gazdar (281) with an E. coli system, however, the increase occurred in the extracellular medium and not in the cells. Since cyclic phosphodiesterase is absent in S. salivarius, the cyclic AMP extrusion mechanism is probably the only existing process for the loss of cyclic AMP from within cells of this organism. The results have also indicated that once cyclic AMP is excreted into the extracellular fluid it is no

longer available to the cell because a mechanism does not exist for its transport into the cell. Whether a precise control mechanism exists for the extrusion of cyclic AMP is not, at present, known.

The studies concerning the effects of cyclic AMP on some of the enzymic processes were preliminary in nature but served to indicate a possible role of cyclic AMP in the regulation of phosphofructokinase and P-enolpyruvate phosphotransferase activity. Cyclic AMP activated the partially purified S. salivarius glycogen phosphorylase in the absence of AMP, but had no effect on the enzyme in the presence of AMP (Fig. 5.5). Since the cellular level of AMP fluctuates during metabolism (282,283), cyclic AMP might activate the enzyme at low AMP concentrations. In fact, if the AMP concentration in cells of S. salivarius resembles that for ATP (5 nmoles/mg dry wt cells) (273), cyclic AMP may indeed have a controlling effect. Adenyl cyclase activity in S. salivarius is readily inhibited by AMP and other nucleotides, as well as by certain glycolytic intermediates (Chapter 4), indicating that a complex control system regulates metabolite levels in these cells. These levels, particularly those of AMP, would then directly affect the activity of glycogen phosphorylase.

CHAPTER 6

GENERAL DISCUSSION

I. DISCUSSION

Although considerable evidence is now available demonstrating the role of cyclic AMP in the regulation of inducible enzyme synthesis in bacteria (7,8) relatively little is known about its effect on other cellular processes in micro-organisms. This nucleotide may have an important role in the control of glycolysis in unicellular organisms. This is a reasonable assumption in view of the increasing amount of information available concerning energy metabolism and cyclic AMP. For example, Perlman and Pastan (121) isolated a mutant from E. coli which did not grow on a variety of carbohydrates and which was low in adenyl cyclase activity. However, in the presence of exogenous cyclic AMP, the mutant was able to degrade carbohydrates in a manner similar to that of the wild-type strain. Furthermore, Moses and Sharp (129) have shown that cyclic AMP greatly stimulated glycolysis and profoundly affected the intracellular concentration of several glycolytic intermediates, particularly fructose-1,6-P_2, suggesting the interaction of cyclic AMP and certain enzyme in the glycolytic pathway, such as phosphofructokinase. This is likely a possibility since it has been known for some time that cyclic AMP can reverse the ATP-sensitivity of yeast phosphofructokinase (81) and inhibit yeast glyceraldehyde-3-P dehydrogenase (62). Of course, the role of cyclic AMP in the regulation of glycolysis, glycogenolysis and gluconeogenesis in animal systems is well-known (1,2,284).

The interrelationship between the phosphofructokinase reaction and cyclic AMP is also evident in the results reported for purified S. salivarius adenyl cyclase since fructose-6-P and fructose-1,6-P_2 activated or inhibited the enzyme depending on the concentration of the

intermediate (Fig. 4.23 and 4.24). Clearly, the concentrations of these compounds "in vivo" are probably important to the activity of the enzyme within the cell. Under physiological conditions in S. salivarius, however, the fructose-6-P concentration is probably of lesser importance since the level of this intermediate remains low during active glycolysis (0.6-3.2 nmoles/mg dry wt cells). On the other hand, the intracellular levels of fructose-1,6-P_2 are usually high (29-32 nmoles/mg dry wt cells) during energy metabolism and changes in the concentration of this compound probably play a significant role in the regulation of adenyl cyclase activity.

These and other results (81) suggest that a reciprocal control system might exist in microbial cells regulating both adenyl cyclase and phosphofructokinase activity. In addition to demonstrating the influence of the phosphorylated fructose derivatives on adenyl cyclase activity, we have also demonstrated the stimulation of the phosphofructokinase activity in crude extracts of S. salivarius by cyclic AMP (Table 5.1). In this connection, one cannot exclude the possible effect of fructose-1,6-diphosphatase activity on the level of fructose-1,6-P_2, as well as the effect of cyclic AMP on this latter enzyme. The enzyme in kidney extracts has been shown to be inhibited by this nucleotide (86).

The influence of glyceraldehyde-3-P and P-enolpyruvate on adenyl cyclase activity is of particular interest since both the glyceraldehyde-3-P dehydrogenase and pyruvate kinase reactions constitute major glycolytic control points (62-64). Furthermore, both compounds are at branch points for divergent metabolic pathways: glyceraldehyde-3-P at the point between glycerol synthesis and glycolysis, while P-enolpyruvate is involved

not only in glycolysis and gluconeogenesis, but also in the phosphorylation of carbohydrates in the P-enolpyruvate phosphotransferase transport system (273). Here again, since glyceraldehyde-3-P dehydrogenase is inhibited by cyclic AMP in other microbial systems (62) a reciprocal control system might exist between this enzyme and adenyl cyclase in S. salivarius. The influence of cyclic AMP on pyruvate kinase has not been examined in microbial systems. The transport of glucose in S. salivarius, which does possess P-enolpyruvate phosphotransferase transport system was slightly inhibited by cyclic AMP (Table 5.4). The possible metabolic control of glycolysis "in vivo" by the oscillating levels of fructose-1,6-P_2, ATP and cyclic AMP in relation to phosphofructokinase, glyceraldehyde-3-P dehydrogenase and pyruvate kinase, has been discussed by Yang and Deal (62).

Clearly, the control of adenyl cyclase activity in S. salivarius is not only related to the cellular levels of the critical glycolytic intermediates but also to the whole range of nucleotides which must exist at any one time in the cell. The kinetic data in Table 4.13 indicates that of all the nucleotides tested, ADP probably has the greatest effect "in vivo" since the inhibitor dissociation constant (K_i = 0.43 mM) is significantly lower than the apparent K_m (0.70 mM) for ATP. One might imagine that in addition to the "adenylate charge" control system proposed by Atkinson (282) there might exist another regulatory system controlled by the interaction of both nucleotides and glycolytic intermediates and involving cyclic AMP as a "messenger". Although the reaction in S. salivarius is reversible, one can conceive of the possibility that adenyl cyclase might not only be involved directly in energy metabolism

by regulating the cellular concentration of ATP, but secondarily in response to the cellular levels of nucleotides and metabolites. For example, the inhibition of the enzyme by ADP suggests that when the adenylate charge was low (i.e., AMP + ADP is high) cyclic AMP synthesis would be inhibited thereby reducing the amount of cyclic AMP available to interact with the various glycolytic enzymes. At such low cyclic AMP concentrations, for instance, the activation of phosphofructokinase would be reduced, thereby decreasing the amount of fructose-1,6-P_2 formed which, in turn, would reverse the inhibition of adenyl cyclase (Fig. 4.24). This control system is probably further complicated by the activity of adenylate kinase, which would convert 2 moles of ADP to 1 mole of ATP, the substrate for adenyl cyclase, and 1 mole of AMP, a much less effective inhibitor of the reaction (K_i = 2.50 mM, Table 4.13).

In addition to the effects of glycolytic intermediates and nucleotides, one cannot ignore the possible role of inorganic pyrophosphate on the activity of adenyl cyclase. The low K_i (0.23 mM) for this compound suggests that "in vivo", this product of the adenyl cyclase reaction must be removed rapidly to avoid extensive inhibition of cyclic AMP formation. The presence of a highly active inorganic pyrophosphatase in S. salivarius (Table 4.5) indicates that PP_i probably does not accumulate to any great extent but is converted to P_i, which is less inhibitory (K_i = 1.40 mM).

The results presented in this thesis further suggest that the concentration of cyclic AMP in cells of S. salivarius may be regulated solely through the regulation of adenyl cyclase activity. This postulation is supported by data indicating that (i) adenyl cyclase activity is regulated by a number of nucleotides, glycolytic intermediates, as well as by inorganic

phosphate and pyrophosphate, (ii) cyclic nucleotide phosphodiesterase is absent in S. salivarius and (iii) the presence of an extrusion mechanism for the removal of cyclic AMP. the question remains as to whether the latter extrusion process is controlled or is simply available to keep the cellular cyclic AMP below a certain steady state level. The regulation of the extrusion process would, of course, play a significant role in determining the cellular concentration of this nucleotide.

II. POSSIBLE FUTURE ENDEAVOURS

The present study has answered some questions on the regulation of cyclic AMP synthesis in S. salivarius and on some aspects of its loss from cells. However, like most research projects, it has raised a variety of other questions which can be answered by future research.

Since the purification of adenyl cyclase has indicated the possible presence of three isozymes for the enzyme, further work should establish whether in fact the enzymes I and II are different or similar to the enzyme III, which has already been studied in some detail.

The effect of glycolytic intermediates on enzyme III has suggested that these compounds probably regulate adenyl cyclase activity in S. salivarius. However, the combined effect of these intermediates, when added together with purified adenyl cyclase, is not known. This could readily be undertaken by determining the physiological concentrations of the various glycolytic intermediates under different conditions of growth and examining the effect of these concentrations on cyclic AMP production by adenyl cyclase.

Adenyl cyclase activity was stimulated by NaF in the forward direction (i.e., cyclic AMP synthesis) and inhibited by the same compound in the reverse direction (i.e., ATP synthesis). It would be of interest to pursue the precise mode of NaF action by examining its effect kinetically in both directions, particularly under conditions of varying Mg^{2+} concentrations. Furthermore, as we have also demonstrated the NaF activation of cyclic AMP formation under "in vivo" conditions, it will be of interest to know whether other metabolic inhibitors can mimic the effect of this compound.

Adenyl cyclase isolated from S. salivarius catalyzes the reverse reaction i.e., ATP formation from cyclic AMP and PP_i. The activity of the enzyme in this direction "in vivo" will mainly depend on the presence of cyclic AMP and PP_i in the system. The concentrations of PP_i, a potent inhibitor of adenyl cyclase, will depend mainly on the activity and regulation of inorganic pyrophosphatase, an enzyme involved in the hydrolysis of this compound to P_i. Therefore, a study on the regulation of purified inorganic pyrophosphatase would be of interest. Determination of the "in vivo" concentrations of PP_i under various conditions would also provide useful information.

Furthermore, as there is no apparent activity of cyclic nucleotide phosphodiesterase in S. salivarius, the cyclic AMP levels both in cells and supernatant under a variety of experimental conditions should be pursued. A few such experiments have already been reported in this thesis but the value of this information would be greatly augmented by data on the levels of various glycolytic intermediates and nucleotides existing under the same conditions. The results obtained would undoubtedly provide

additional insight as to the mechanism for the extrusion of cellular cyclic AMP in the medium.

Preliminary data has indicated the possible effect of cyclic AMP on phosphofructokinase, glycogen phosphorylase and on the P-enolpyruvate phosphotransferase system. The effect of cyclic AMP on phosphofructokinase would be most profitably pursued with a purified preparation. In this context, the regulatory properties of the enzyme can be subjected to analysis. A similar study might also be undertaken on the regulatory properties of the two important glycolytic enzymes, glyceraldehyde-3-P dehydrogenase and pyruvate kinase. Studies on the effect of cyclic AMP on glycogen phosphorylase and on the P-enolpyruvate phosphotransferase system in S. salivarius also deserve further attention.

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