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ADENYL CYCLASE OF *BREVIBACTERIUM LIQUEFACIENS*

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SUMMARY

Adenyl cyclase has been purified about 100-fold from cell-free extracts of *Brevibacterium liquefaciens*. The purified enzyme preparation catalyzed the conversion of ATP (or dATP) to cyclic 3′,5′-AMP (or cyclic 3′,5′-dAMP) and pyrophosphate. In addition to Mg^{2+}, pyruvate or another α-keto-monocarboxylic acid was required for the reaction. None of the other nucleoside triphosphates tested served as substrate.

INTRODUCTION

Cyclic 3′,5′-AMP is well known as a mediator of hormonal actions[1] and a regulator of enzyme reactions[2,3]. Although the capability of forming cyclic 3′,5′-AMP from ATP has been found in many animal tissues, purification of the enzyme (adenyl cyclase) was found to be difficult because of its association with a particulate fraction and its ready inactivation upon solubilization[4]. In recent years, several workers found that cyclic 3′,5′-AMP is also present in microorganisms[5,6]. In order to study the mechanism of cyclic 3′,5′-AMP formation and its physiological role, we have purified the adenyl cyclase activity which was found in the 105 000 × *g* supernatant fraction of cell-free extracts of *Brevibacterium liquefaciens*. Initial attempts at purification of the enzyme by Sephadex column chromatography resulted in almost complete loss of enzyme activity. Subsequently, it was revealed that the low recovery of activity was related to the removal, during purification, of a factor which was later identified as pyruvate[7].

In the present paper, we describe details of the purification procedure and properties of adenyl cyclase of *Brevibacterium liquefaciens*. Although pyruvate was found to be essential for the reaction, it was not metabolized during incubation. The enzyme preparation also catalyzed the conversion of dATP to cyclic 3′ 5′-dAMP, indicating the possibility that the deoxy-analogue of cyclic 3′,5′-AMP may occur in nature.

EXPERIMENTAL PROCEDURE

Chemicals

[8-^{14}C]ATP, [8-^{14}C]dATP, [8-^{14}C]ADP, [8-^{14}C]AMP and lithium pyruvate were purchased from Schwartz BioResearch, Inc. Sodium α-ketobutyrate, sodium α-

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ketovalerate, α-ketoisovalerate, phosphoenolpyruvate, α-ketoglutarate, oxaloacetate, coenzyme A, β-hydroxybutyrate, pyridine nucleotides, phenylpyruvate, adenine derivatives, nucleoside triphosphates, crystalline lactate dehydrogenase and snake venom (*Naja naja*) were purchased from Sigma. The purity of dATP was examined by paper chromatography with solvent system (d) in the text, and was found to be practically free of ATP. Lithium hydroxypyruvate was obtained from Nutritional Biochemicals Co. Acetylphosphate was a product of Worthington Biochemicals Co. α-Hydroxybutyrate was obtained from California Corp. for Biochemical Research. L-Epinephrine was purchased from Merck. The *trans*- and *cis*-isomers of the 2,4-dinitrophenylhydrazone of pyruvate, α-ketobutyrate, α-ketovalerate, α-ketoisovalerate and α-ketocaproate were the gifts of Dr. H. KATSUKI, Department of Chemistry, Faculty of Science, Kyoto University. All other chemicals were of reagent grade.

Assay of adenyl cyclase

Adenyl cyclase was assayed by measuring the amount of cyclic 3',5'-[^{14}C]AMP formed from [^{14}C]ATP after separation by paper chromatography. In a routine assay, 0.2 μmole of [8-^{14}C]ATP (specific activity, 150 mμC/μmole), 3 μmoles of $MgSO_4$, 0.5 μmole of lithium pyruvate, 0.3 μmole of NaF, 10 μmoles of Tris–HCl (pH 9.0) and enzyme protein, in a final volume of 0.1 ml were incubated for 30 min at 33°. The reaction was terminated by heating in a boiling-water bath for 2 min, and the precipitate was removed by centrifugation. Then aliquots of the supernatant fluid were spotted on Whatman No. 3 MM filter paper together with carrier cyclic 3',5'-AMP and EDTA. After the chromatogram was developed with 1 M ammonium acetate–ethanol (30:75) for 16–20 h at room temperature, the spot corresponding to cyclic 3',5'-AMP was cut out, immersed in a scintillator solution[8] and the radioactivity was determined with Packard Tri-Carb or Nuclear Chicago liquid scintillation spectrometer. One unit of enzyme was defined as the amount producing 1 mμmole of cyclic 3',5'-AMP per min under standard conditions. Specific activity was expressed as units per mg of protein. Protein was determined by the method of LOWRY *et al.*[9]. Cyclic 3',5'-[^{14}C]AMP recovered from the paper chromatograms during routine assay, was identified by paper chromatography and enzyme digestions as described below.

Assay of other enzymes

Adenosine triphosphatase was estimated by incubating an enzyme sample in a reaction mixture containing 2.5 μmoles of ATP, 15 μmoles of $MgSO_4$, 50 μmoles of Tris–HCl (pH 9.0) in a final volume of 0.5 ml. After 30 min of incubation at 33°, P_i released was determined by the method of FISKE AND SUBBAROW[10]. Pyrophosphatase was assayed in a similar fashion, except that 2.5 μmoles of sodium pyrophosphate was used as the substrate. Cyclic 3',5'-phosphodiesterase activity was determined by measuring the released P_i with the use of excess snake venom phosphomonoesterase. The reaction mixture contained 2.5 μmoles of cyclic 3',5'-AMP, 15 μmoles of $MgSO_4$, 50 μmoles of Tris–HCl (pH 9.0), 50 μg of snake venom (*Naja naja*) and enzyme sample, in a total volume of 0.5 ml, and was incubated for 30 min at 33°. After addition of 0.1 ml of 1 M HCl, the precipitate was removed by centrifugation, and aliquots of the supernatant fluid were analyzed for P_i by the method of FISKE AND SUBBAROW[10].

Paper chromatography

Ascending paper chromatography was carried out for the identification of nucleotides and related compounds with the following solvent systems, (a) *n*-butanol–acetic acid–water (5:2:3, by vol.) (b) iso-propanol–ammonia–water (7:1:2, by vol.), (c) ethanol–1 M ammonium acetate (75:30, v/v), (d) saturated $(NH_4)_2SO_4$–1 M sodium acetate–isopropanol (80:18:2, by vol.). Identity and purity of commercial samples of α-keto acids were checked as their 2,4-dinitrophenylhydrazones by the use of ascending paper chromatography, conducted with *n*-butyl alcohol saturated with 3 % ammonia as solvent.

Determination of pyruvate

Pyruvate was determined spectrophotometrically by measuring the oxidation of NADH at 340 mμ in the presence of lactate dehydrogenase[11] as follows. To the reaction mixture containing 0.3 μmole of NADH and 60 μg of lactate dehydrogenase in 2.9 ml of 0.3 M potassium phosphate (pH 7.5), was added 0.1 ml of the sample solution to be analyzed, and, after 2 min, the amount of pyruvate in the sample solution was estimated from the change in absorbance at 340 mμ.

Determination of pyrophosphate

Pyrophosphate was assayed by measuring the orthophosphate according to the method of FISKE AND SUBBAROW after hydrolysis with yeast pyrophosphatase which was purified by the method of HEPPEL AND HILMOE[12]. The reaction mixture contained, in 3.0 ml, 300 μmoles of Tris–HCl (pH 7.5), 150 μmoles of $MgSO_4$, 10 μg of yeast pyrophosphatase, and the sample solution. After incubation for 30 min at 33°, aliquots were used for the determination of orthophosphate.

Growth of organism

Brevibacterium liquefaciens (ATCC 14929) was kindly provided by Dr. T. OKABAYASHI of the Shionogi Research Laboratory. The organism was grown for 44 h at 25° with vigorous shaking in a medium containing 7 g of K_2HPO_4, 3 g of KH_2PO_4, 20 g of DL-alanine, 20 g of glucose and 0.2 g of $MgSO_4 \cdot 7H_2O$ per l of distilled water. The cells were harvested by centrifugation for 20 min at 6000 × *g*. The yield of wet packed cells was about 15 g per liter of the medium.

RESULTS

Enzyme purification

All procedures were carried out at 0–4°.

Cell-free extracts

Washed cells, 100 g, were suspended in 500 ml of 0.1 M Tris–HCl (pH 8.3) and were disrupted in a 10-kcycles Raytheon sonic disintegrator for 20 min. The supernatant fluid was separated from the residue by centrifugation for 20 min at 8000 × *g*. Unbroken cells were resuspended in 500 ml of 0.1 M Tris–HCl (pH 8.3) and

were subjected to sonic oscillation, then centrifuged in the same way. The supernatant solutions thus obtained were combined and were centrifuged for 60 min at 78 000 $\times g$. Subsequent manipulations were carried out in the presence of 1 mM β-mercaptoethanol.

Streptomycin treatment

To 1 l of the yellow supernatant were added 40 ml of 1 M HCl in order to adjust the pH to 6.5 and 65 ml of 5 % streptomycin sulfate with stirring. After 60 min the precipitate was removed by centrifugation for 30 min at 12 000 $\times$g. The pH value of the solution was adjusted to 8.3 with about 40 ml of 1 M KOH.

$(NH_4)_2SO_4$ fractionation

Solid $(NH_4)_2SO_4$ (225) g was added to 1150 ml of the above supernatant with stirring, and the resulting precipitate was removed by centrifugation for 30 min at 14 000 $\times g$. Then 130 g of solid $(NH_4)_2SO_4$ was addded to the supernatant solution with stirring, and, after 30 min, the precipitate was collected by centrifugation and was dissolved in 40 ml of 0.1 M Tris–HCl (pH 7.0). The solution was passed through a Sephadex G-25 column (2 cm $\times$ 15 cm) which had been equilibrated with 0.1 M Tris–HCl (pH 7.0) to remove $(NH_4)_2SO_4$.

Aluminum hydroxide C_γ gel treatment

To the effluent solution, 40 ml of aluminum hydroxide C_γ gel (11 mg/ml) were added with stirring. After 10 min, the gel was collected by centrifugation and washed with 200 ml of 2 mM sodium pyrophosphate. The enzyme was then eluted from the gel with 150 ml of 20 mM sodium pyrophosphate, and the solution was dialyzed for 4 h against 0.1 M Tris–HCl (pH 8.3).

DEAE-Sephadex column chromatography

The dialyzed fraction was applied to a column (1.5 cm $\times$ 5.0 cm) of DEAE-Sephadex (A-50) which had been equilibrated with 0.1 M NaCl in 0.1 M Tris–HCl (pH 8.3). The column was washed with 350 ml of 0.1 M NaCl in Tris–HCl (pH 8.3), and elution was carried out with 0.16 M NaCl in 0.1 M Tris–HCl (pH 8.3). The fractions of highest specific activity were combined and the enzyme was precipitated by the addition of $(NH_4)_2SO_4$ (50 % satn.). This fraction was used for all kinetic studies unless otherwise specified. Typical results of enzyme purification are shown in Table I. The overall purification was usually 100 fold with a yield of about 20 %.

TABLE I

ENZYME PURIFICATION

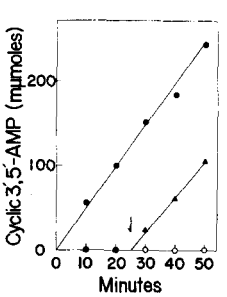
	Total protein (mg)	*Specific activity (units/mg protein)*			
		Cyclase	*3',5'-Diesterase**	*ATPase*	*Pyrophosphatase*
Cell-free extracts	4200	2.3	1.3	89	57
Streptomycin supernatant	4020	2.1	0.8	51	83
$(NH_4)_2SO_4$	445	9.5	0.0	19	11
C_γ gel eluate	78	27.4	0.0	16	5
DEAE-Sephadex	10	222.0	0.0	3	1

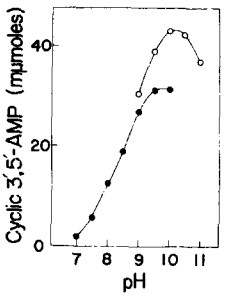
* 3',5'-diesterase, cyclic 3',5'-phosphodiesterase.

Cyclic 3′,5′-phosphodiesterase, ATPase and inorganic pyrophosphatase activities, which were present in the crude extract, were almost negligible in the final preparation.

Requirement for α-keto acids

As has been previously reported[7], pyruvate was required for full enzyme activity. In the absence of pyruvate, as shown in Fig. 1, the reaction did not take place to a significant extent. When pyruvate was added either at zero time or after a certain period of time, the reaction took off immediately. The rate of the reaction was maximal at about pH 10.0 with glycine–KOH buffer (Fig. 2). In the absence of pyruvate, little activity was observed over the entire pH range tested. α-Ketobutyrate





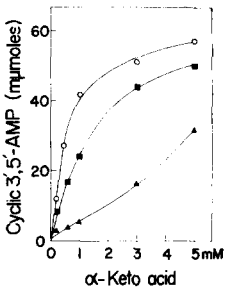


Fig. 1. Time course of the reaction in the presence or absence of pyruvate. The reaction mixture containing 0.9 μmole of [8-^{14}C]ATP, 9 μmoles of $MgSO_4$, 30 μmoles of Tris-HCl (pH 9.0) and 60 μg of enzyme protein with or without 2.1 μmoles of lithium pyruvate, in 0.3 ml, was incubated at 33°. Aliquots were taken at 10-min intervals to determine the amount of cyclic 3′,5′-[^{14}C]AMP formed. Incubation was performed in the presence (●–●) or absence (○–○) of pyruvate. Pyruvate was added as indicated by an arrow after 25 min incubation (▲–▲).

Fig. 2. Effect of pH on the rate of reaction. Enzyme activities were determined under standard conditions except that 0.1 M Tris–HCl buffer (●–●) or 0.1 M glycine-KOH buffer (○–○) was employed. Each tube contained 7 μg of enzyme protein.

Fig. 3. Dependence of the rate on the varying concentration of α-keto acids. Enzyme activity was determined by the standard method, except that pyruvate (○–○) was replaced by α-ketobutyrate (■–■) or α-ketovalerate (▲–▲). The enzyme protein used was 10 μg.

was about 80 % as active as pyruvate at 5 mM as shown in Fig. 3, though the apparent K_m value for α-ketobutyrate was twice as much as that of pyruvate. α-Ketovalerate was approx. 45 % as active. Other α-keto-monocarboxylic acids such as α-ketoisovalerate and α-ketocaproate were partially effective. Phosphoenolpyruvate, acetylphosphate, lactate, DL-alanine, α-ketoglutarate, *n*-butyrate, acetate, acetoacetate, hydroxypyruvate, coenzyme A, cocarboxylase, β-hydroxybutyrate, phenylpyruvate, NAD, NADH, NADP, NADPH, carbonate, malate, cysteine, β-mercaptoethanol were ineffective at 5.0 mM. Although oxaloacetate was about 5 % as active as pyruvate at 5 mM, it may be due to the spontaneous formation of pyruvate during incubation. L-Epinephrine was not effective at 0.1 mM.

Substrate specificity

ADP was about 20 % as active as ATP as substrate, with the enzyme preparation of $(NH_4)_2SO_4$ fraction. With the enzyme purified through the DEAE-Sephadex step, however, the rate of cyclic 3'-5'-[^{14}C]AMP formation from [^{14}C]ADP was found to be approx. 4 % of that from [^{14}C]ATP (Table II). ADP must be converted to

TABLE II

SUBSTRATE SPECIFICITY

The reaction mixture contained 0.2 μmole of ^{14}C-labeled compound (specific activity 300 mμC/μmole), 0.5 μmole of lithium pyruvate, 3 μmoles of $MgSO_4$ and 10 μg of enzyme protein in 0.1 ml of 0.1 M Tris–HCl (pH 9.0) and was incubated for 30 min at 33°.

	Cyclic 3',5'-[^{14}C]AMP (mμmoles)	*Relative (%)*
[^{14}C]ATP	54.9	100
[^{14}C]ADP	2.1	4
[^{14}C]ADP+ATP*	3.3	6
[^{14}C]AMP	0.0	0
[^{14}C]AMP+ATP*	1.2	2

* 0.2 μmole of ATP was included

ATP before participating in the cyclase reaction, since the enzyme preparation still contained a trace of adenylate kinase activity. AMP was completely inert as substrate both in the presence and absence of ATP. The apparent K_m values for ATP, as calculated from the LINEWEAVER–BURK plot[14], were approx. 1.0 mM in the presence of 30 mM of $MgSO_4$, and were not altered significantly by the change in the amount of pyruvate included (Fig. 4). The apparent K_m values for dATP* were

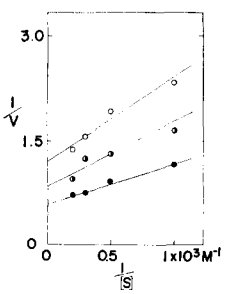


Fig. 4. LINEWEAVER–BURK plots for ATP in the presence of 5 mM (●–●), 2 mM (◑–◑) or 1 mM pyruvate (○–○). Velocity is expressed in mμmoles of cyclic 3',5'-AMP formed per min per 0.1 ml of reaction mixture, which contained 10 μg of enzyme protein.

* Cyclase activity was assayed with [8-^{14}C]dATP as substrate with the standard assay system.

found to be almost identical to those for ATP. dATP inhibited the conversion of [^{14}C]ATP to cyclic 3',5'-[^{14}C]AMP competitively. Other nucleoside triphosphates, including GTP, ITP, UTP, CTP, TTP, dGTP, dUTP and dCTP, could not serve as substrate as examined by paper chromatography[13] either with purified or cruder enzyme preparations.

Stoichiometry of reaction

When ATP was incubated with the enzyme preparation in the presence of 2 mM (Expt. 1 in Table III) or 0.67 mM (Expt. 2) of pyruvate, ATP consumed coin-

TABLE III

STOICHIOMETRY OF THE REACTION

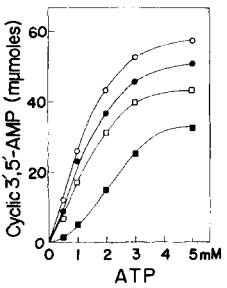
The reaction mixture containing, in a final volume of 0.6 ml, 4.8 μmoles of ATP, 36 μmoles of $MgSO_4$, 60 μmoles of Tris–HCl (pH 9.0), 300 μg of enzyme protein and 1.2 μmole (Expt. 1) or 0.4 μmole (Expt. 2) of lithium pyruvate, was incubated for 60 min at 33°. After the reaction was terminated with 0.2 ml of 1 M HCl, the precipitate was removed by centrifugation, washed with water and the total volume was made up to 1.2 ml. Separate 0.4-ml aliquots were used for the determination of nucleotides, pyrophosphate and pyruvate as described in the text.

Expt. No.	*Incubation time* (*min*)	*ATP* (μ*moles*)	*Cyclic 3',5'-AMP* (μ*moles*)	*PP_i* (μ*moles*)	*Pyruvate* (μ*moles*)
1	0	4.8	—	0.0	1.20
	60	2.8	2.0	2.1	1.18
2	0	4.8	—	0.0	0.40
	60	3.7	1.1	1.0	0.38

cided with the amount of cyclic 3',5'-AMP and pyrophosphate formed. ATP and cyclic 3',5'-AMP were separated on a Dowex 1-X8 column (0.9 cm × 4.0 cm) eluted with a gradient between 0.05 M LiCl and 0.25 M LiCl in 0.003 M HCl and were determined by absorbance at 260 mμ. The amounts of pyruvate were determined by the enzymic procedure[11] as described in the text. Approx. 0.02 μmole of pyruvate disappeared in each case.

Cation requirement

Mg^{2+} was essential for the reaction[7]. Mn^{2+} and Co^{2+} could replace Mg^{2+}, and were shown to be about 80 and 25 % as active at 3 mM, respectively. Ca^{2+} was not effective at any concentration tested. When equivalent amounts of Mg^{2+} and ATP were used, and the concentrations of both ATP and Mg^{2+} were varied, the rate *versus* substrate concentration curve was sigmoidal. However, a hyperbolic curve was obtained when Mg^{2+} and ATP were varied at a constant Mg : ATP ratio of 2, 3 or 4 as shown in Fig. 5, indicating that the true substrate of adenyl cyclase is not MgATP but rather a Mg_2ATP complex. It is interesting to note that decreasing the pyruvate concentration caused a sigmoidal response even when Mg : ATP was 4 (Fig. 6).



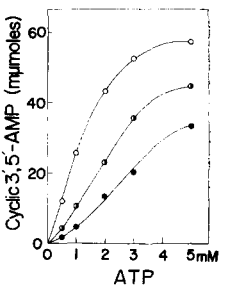


Fig. 5. Effects of Mg:ATP ratio on the rate of reaction. The ratio was varied as follows; $n = 1$ (■–■); $n = 2$ (□–□); $n = 3$ (●–●); $n = 4$ (○–○).

Fig. 6. Effect of pyruvate concentration on enzyme activity. 5 mM (○–○), 2 mM (◑–◑), or 1 mM (●–●) pyruvate was included in the reaction mixture, and the ratio $MgSO_4$:ATP was kept constant at 4.

Inhibitors

The enzyme was inhibited by *p*-chloromercuribenzoate or mersaryl and the inhibition was reversed by the addition of β-mercaptoethanol. Several nucleotides including AMP, dAMP, ADP or GTP were inhibitory, though the mode of inhibition of these compounds has not been extensively examined.

Identification of cyclic 3',5'-AMP

In order to isolate the reaction product, 250 μmoles of ATP, 250 μmoles of lithium pyruvate, 2 mmoles of $MgSO_4$, 150 μmoles of creatine phosphate, 500 μg of creatine kinase and 100 mg of enzyme protein ($(NH_4)_2SO_4$ fraction) in 50 ml of 0.1 M Tris–HCl (pH 8.3) were incubated for 4 h at 33°. The reaction was terminated by the addition of 2 % perchloric acid and the precipitate was removed by centrifugation. After the total volume of the solution was adjusted with water to 200 ml, the mixture was passed through a charcoal column (2 cm × 5 cm). The reaction products were then eluted from the column with 100 ml of ethanol–ammonia–water (50 : 2 : 48, by vol.) and the solvent was evaporated under reduced pressure at 30° (approx. 70 % of the nucleotides were recovered from the charcoal column). The residue was dissolved in 100 ml of water, neutralized with 1 M KOH, and was applied to Dowex 2- X8 column (3.5 cm × 20 cm). With 1 M formate, approx. 35 μmoles of cyclic 3',5'-AMP were eluted from the column, and further purification was achieved by passage through a Dowex 50-X4 (2 cm × 20 cm) column. Crystallization was then carried

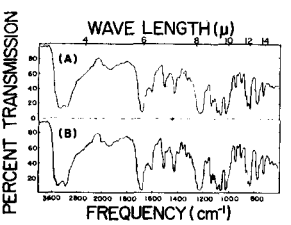


Fig. 7. Infrared spectra of cyclic 3',5'-AMP chemically synthesized (A) and enzymatically formed (B) in KBr disks.

out with aqueous ethanol at pH 2.0. The infrared spectrum of cyclic 3',5'-AMP recrystallized several times, was identical with that of an authentic sample in KBr (Fig. 7). The identity was confirmed upon paper chromatography by the use of different solvent systems (Solvent a, b, c, d) and by ultraviolet absorption spectra at pH 7.0 and 2.0. Further evidence of the identity of the product was provided by enzymic digestion to AMP upon treatment with a rabbit-brain phosphodiesterase[15].

Identification of cyclic 3',5'-dAMP

The incubation mixture (2.0 ml) containing 6 μmoles of dATP, 20 μmoles of lithium pyruvate, 200 μmoles of Tris–HCl (pH 9.0) and 240 μg of enzyme protein, was incubated for 60 min at 33°. After the reaction was terminated by the addition of 0.5 ml of 1 M HCl, cyclic 3',5'-dAMP was isolated and identified as has been previously reported[13].

DISCUSSION

The adenyl cyclase system of animal tissues was first characterized by SUTHERLAND, RALL AND MENON[4]. When particulate preparations were incubated with ATP and Mg^{2+}, cyclic 3',5'-AMP was formed with a concomitant release of pyrophosphate[16], although exact stoichiometry of the reaction was difficult to establish. We have succeeded in purifying adenyl cyclase from the cell-free extracts of *Brevibacterium liquefaciens*, and were able to establish the stoichiometry of the cyclase reaction. ATP was found to be converted stoichiometrically to cyclic 3',5'-AMP and pyrophosphate by the purified enzyme preparation. These results suggest that the reaction sequence probably involves intramolecular phosphorylation of the 3'-OH of the ribose moiety, as has been proposed by LIPKIN, MARKHAM AND COOK[17].

The properties of the Brevibacterium adenyl cyclase and the animal enzyme appear different, particularly with regard to reaction requirements and substrate specificity. The enzyme system of mammalian tissues was found to utilize ATP as substrate and was stimulated by catecholamines[18]. In contrast, the microbial enzyme requires pyruvate for activity, and both ATP and dATP serve as substrates. L-Epinephrine could not replace pyruvate to any extent nor did it stimulate the rate of reaction in the presence of pyruvate. However, this distinction may only be arbitrary, since the mammalian enzyme has not been purified and characterized extensively. In view of the recent finding by LEVINE* that cyclic 3',5'-dAMP was found in rat-liver bile, the occurrence of a deoxy analogue of cyclic AMP in nature deserves further investigation.

Pyruvate is essential for the reaction but is not metabolized during incubation. Although the rate of reaction is dependent upon the concentration of pyruvate, K_m values for ATP are independent of the pyruvate concentrations. The role of pyruvate in the reaction appears, therefore, to be similar to that of *N*-acetylglutamate in the carbamyl phosphate synthetase reaction studied by MARSHALL *et al.*[19]. Since many allosteric effectors have been shown to affect the sedimentation constant of the enzyme, we have examined the effect of pyruvate on the sedimentation pattern.

* Personal communication from Dr. R. A. LEVINE, State University of New York.

The sedimentation coefficient of the enzyme was estimated to be about 4.5 by sucrose-density gradient centrifugation[20] either in the presence or absence of 5 mM pyruvate (unpublished observations). Preliminary experiments have shown that pyruvate does not protect the enzyme from inactivation by heat.

The physiological role of cyclic 3′,5′-AMP in animal tissues has been extensively explored by SUTHERLAND and other investigators. Cyclic 3′,5′-AMP enhances the degradation of glycogen by regulating phosphorylase b kinase activity, on the other hand cyclic 3′,5′-AMP also controls glycogen synthesis through acceleration of transferase I kinase[21]. In *Escherichia coli*, a large quantity of glycogen-like polysaccharide has been found within the cell[22], but the role of the cyclic nucleotide in this phenomenon has not been examined. Several reports have described the physiological significance of cyclic 3′,5′-AMP in microorganisms[23,24]. In the culture media of *Brevibacterium liquefaciens*, a large quantity of cyclic 3′,5′-AMP was shown to accumulate, when grown with DL-alanine as the nitrogen source[25]. This observation may be related to the pyruvate activation of the adenyl cyclase reaction. If alanine is oxidized efficiently by this organism, an elevated concentration of pyruvate might accumulate and facilitate the formation of cyclic 3′,5′-AMP in the cell. Since pyruvate is a key intermediate in the carbohydrate metabolism, cyclic 3′,5′-AMP may play a major role in the metabolism in this microorganism. Further studies are necessary to elucidate the physiological significance of cyclic 3′,5′-AMP in microorganisms.

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