JOURNAL OF BACTERIOLOGY, Aug. 1967, p. 317-322

Vol. 94, No. 2
Printed in U.S.A.

Formation of Adenosine Cyclic 3',5'-Phosphate by Nonproliferating Cells and Cell-free Extract of *Brevibacterium liquefaciens*

MISAO IDE, AKIHIRO YOSHIMOTO,[1] AND TADASHI OKABAYASHI
Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku, Osaka, Japan

Received for publication 19 May 1967

The formation of adenosine cyclic 3',5'-phosphate by *Brevibacterium liquefaciens* ATCC 14929 was studied with the use of nonproliferating cells and cell-free extract. With nonproliferating cells provided by deprivation of sulfate, the formation of this nucleotide was accelerated by adding some amino acids and sugars. Among amino acids tested, alanine and asparagine were most effective. Pentoses were more favorable than hexoses and other sugars. Formation of adenosine cyclic 3',5'-phosphate was observed also with chloramphenicol-treated cells. Experiments on cell-free extract showed that addition of alanine or pyruvate stimulated the formation of adenosine cyclic 3',5'-phosphate from adenosine-5'-triphosphate. When alanine was added to the cell-free system, shaking of the reaction mixture further increased the amount of the nucleotide, but pyruvate was far more effective than alanine. No synergistic effect of alanine and pyruvate was observed. Some enzyme activity was observed which decomposed adenosine cyclic 3',5'-phosphate, but it was weak as compared with adenyl cyclase activity in the presence of pyruvate. From the results obtained, it appears that pyruvate may act as an activating factor of adenyl cyclase in *Brevibacterium liquefaciens*.

Previously, we reported the occurrence of adenosine cyclic 3',5'-phosphate (cyclic 3',5'-AMP) in culture fluid of *Brevibacterium liquefaciens* isolated from sewerage (8, 10). This observation has extended the distribution of this nucleotide from the animal kingdom to the microbial field. In consideration of the suggested roles of cyclic 3',5'-AMP and the effect of several hormones on adenyl cyclase, an enzyme which catalyzes the formation of cyclic 3',5'-AMP from adenosine triphosphate (ATP) in animals (14), the occurrence of this nucleotide in the microbial field is rather surprising, and it seems important to investigate the factors affecting cyclic 3',5'-AMP formation in microorganisms.

In a previous paper, we demonstrated that, in the growing cells of this bacterium, cyclic 3',5'-AMP formation is under the control of several factors (9). Thus, a specific amino acid, alanine or asparagine, was required for accumulation of a large amount of cyclic 3',5'-AMP, and addition of a hexose also caused a dramatic increase in cyclic 3',5'-AMP formation.

The present study was carried out to obtain more intimate knowledge of these factors by use of nonproliferating cells and cell-free extracts of *B. liquefaciens*.

[1] Present address: Faculty of Agriculture, Kyushu University, Fukuoka, Japan.

MATERIALS AND METHODS

Bacterial strain. B. liquefaciens ATCC 14929 (7) was used throughout this work.

Media and cultural conditions. B. liquefaciens pregrown overnight in bouillon medium was inoculated into the cultivating media. The standard media contained the following components: DL-alanine, 4 g; glucose, 2 g; K_2HPO_4, 0.7 g; KH_2PO_4, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; and water, 100 ml. When DL-asparagine was used in place of DL-alanine, $MgSO_4 \cdot 7H_2O$ was reduced to 0.01 g. The cells pregrown on alanine medium were designated as alanine-grown cells, and the cells pregrown on asparagine medium were designated as asparagine-grown cells. Both cells behaved differently in response to the various experimental conditions. Cultivation of the bacteria was carried out on a reciprocal shaker for 40 hr at 27 C.

Nonproliferating cells. In most experiments, the nonproliferating system was obtained by incubating the washed cells in sulfur-deprived media. The composition of the sulfur-deficient standard medium is as follows: DL-alanine, 200 mg; glucose, 100 mg; K_2HPO_4, 35 mg; KH_2PO_4, 15 mg; $MgCl_2 \cdot 6H_2O$, 0.82 mg; total volume, 5 ml; *p*H, 8.4 (adjusted by KOH). When DL-asparagine was used in place of

DL-alanine, $MgCl_2 \cdot 6H_2O$ was reduced to 0.41 mg. Replacement of amino acid or sugar will be described in each case. Chloramphenicol treatment was undertaken in lieu of sulfate deprivation, where $MgCl_2 \cdot 6H_2O$ was replaced by 1 mg of $MgSO_4 \cdot 7H_2O$ and 100 μg of chloramphenicol per ml was added to the medium. All these media were sterilized. Cells grown for 40 hr were harvested by centrifugation, washed twice with water to remove any cyclic 3',5'-AMP or other substances in the culture fluids, and were suspended in the media mentioned above. The concentration of the cells was adjusted to an optical density of 4.0 at 650 mμ with a Hitachi spectrophotometer. Unless otherwise noted, the media were shaken in test tubes for 40 hr at 27 C. In every experiment, four parallel test tubes were run for each condition to minimize the fluctuation of the results. After incubation, the parallel suspensions were combined and assayed for cyclic 3',5'-AMP.

Cell-free system. Cells grown for 40 hr were harvested by centrifugation and washed with water and 0.1 M tris(hydroxymethyl)aminomethane (Tris)chloride buffer (*p*H 8.0); 15 g (wet weight) of the cells was suspended in 50 ml of the same buffer. The suspension was treated with an Ohtake Sonicator (10 kc) for 20 min, and, after centrifugation at 35,000 $\times$ *g* for 20 min at 3 C, the resulting supernatant fluid was used as the enzyme preparation. Protein was determined by the biuret method (3). The basal reaction mixture consisted of 0.1 M Tris chloride buffer (*p*H 8.0), 0.03 M $MgSO_4$, 3.3 mM ATP (Na salt), and enzyme preparation containing 13.3 mg of protein; the total volume was adjusted to 5 ml. Several substances were added to this mixture to test their effects as described below. The mixture was incubated in test tubes for 120 min at 37 C with or without shaking.

Cyclic 3',5'-AMP degrading enzyme activity. The basal reaction mixture consisted of 0.241 mM cyclic 3',5'-AMP, 0.03 M $MgSO_4$, 0.1 M Tris chloride buffer (*p*H 8.0), and enzyme preparation containing 13.3 mg of protein; total volume was adjusted to 5 ml. Several substances were added to this mixture to test their effects, as described below. The mixture was incubated in test tubes for 120 min at 37 C with or without shaking.

Assay of cyclic 3',5'-AMP. In nonproliferating-cell experiments, cyclic 3',5'-AMP was determined by anion-exchange chromatography, as described previously (9). To 13 ml of the suspension prepared by combining four parallel test tubes, 1.8 ml of 60% perchloric acid was added, and, after standing for 30 min at 0 C, it was centrifuged. The supernatant fluid was treated with charcoal and subjected to chromatography. In cell-free experiments, the reaction mixture was centrifuged after adding 1 ml of 1 N HCl, and the supernatant fluid was desalted by use of Norit A (American Norit Co., Inc., Jacksonville, Fla.) according to Threlfall (15) with the following modification: isooctyl alcohol was used instead of 2-octanol, and 4 ml of ethyl alcohol-concentrated ammonia-water (50:2:48, v/v) instead of 10% aqueous pyridine was used. The desalted solution was evaporated under vacuum, and the residue was assayed for cyclic 3',5'-AMP by thin-layer chromatography according to the method previously reported (17), with a slight modification: the thin-layer plate was developed first with methanol-water (60:40, v/v) instead of water, and then with ammonium carbonate-acetone-water (0.96 g:75 ml:25 ml). By this procedure, cyclic 3',5'-AMP was clearly separated from all the nucleotides, nucleosides, and bases tested so far, including adenine, guanine, cytosine, uracil, and their nucleosides and nucleotides, as well as hypoxanthine, xanthine, inosine, and xanthosine. Corresponding areas were cut out and eluted with 0.1 N HCl. Cyclic 3',5'-AMP was determined by measuring the absorbancy at 260 mμ of the eluted solution with the use of a molecular extinction coefficient of 14,500 (12). The ratio of the absorbancy at 250, 260, 280, and 290 mμ was estimated for confirmation. Cyclic 3',5'-AMP was identified by cellulose thin-layer chromatography after Norit treatment, by use of the following solvent systems: ethyl alcohol-1 M ammonium acetate buffer, *p*H 7.5 (75:30), 2-propanol-concentrated ammonia-water (70:10:20), saturated ammonium sulfate-1 M sodium acetate-2-propanol (80:20:2), and 1-butanol-acetic acid-water (50:20:30). For further identification, hydrolysis with barium hydroxide (5) was carried out, and the resultant 3'-, and 5'-AMP were detected by cellulose thin-layer chromatography with the following solvent system: saturated ammonium sulfate-1 M sodium acetate-2-propanol (80:20:2).

RESULTS

Validity of the modified assay method. The experiment indicated in Table 1 was performed to confirm the validity of the modified thin-layer technique as the assay method to be used. Authentic cyclic 3',5'-AMP was mixed with 6.5 μmoles of 5'-AMP, 6.3 μmoles of ATP, 900 μmoles of DL-alanine, 60 μmoles of $MgSO_4$, 0.1 M Tris chloride buffer, *p*H 8.0 (final concentration), and enzyme preparation (13.3 mg of protein) pretreated with 0.1 ml of 1 N HCl. Total volume was adjusted to 2 ml. The solution was treated with Norit, eluted with ethanolic ammonia, and assayed for cyclic 3',5'-AMP by the thin-layer method. As shown in Table 1, almost quantitative recovery of cyclic 3',5'-AMP was obtained from the mixture containing ATP, $MgSO_4$, Tris chloride buffer, alanine, and deproteinized cell extract. In the routine assay of cyclic 3',5'-AMP in the reaction mixtures, the absorbancy at different wavelengths was estimated to confirm the absence of interfering substances. Further identification of cyclic 3',5'-AMP was carried out by thin-layer chromatography and by hydrolysis as described in Materials and Methods, and it was shown that all the samples tested contained no ultraviolet-absorbing materials other than cyclic 3',5'-AMP. It was concluded, therefore, that no interfering substance exists in the spot for cyclic 3',5'-AMP in the thin-layer chromatogram.

TABLE 1. *Assay of cyclic 3',5'-AMP in standard samples by thin-layer chromatography*

Cyclic 3',5'-AMP added	Cyclic 3',5'-AMP determined	Recovery
mμmoles	*mμmoles*	%
0	0	
51.8	47.0	91.0
98.8	90.8	91.9
250	254	101.6
528	504	95.5
980	964	98.9

Some conditions for cyclic 3',5'-AMP formation by nonproliferating cells. Cells cultivated for 24 to 62 hr exhibited an almost constant capacity to make cyclic 3',5'-AMP. When these cells were incubated in the sulfur-deprived medium, cyclic 3',5'-AMP increased as the incubation time increased, and reached a plateau at about 70 hr. During this period, no growth was observed, as determined by optical-density measurement at 650 mμ. Magnesium ions had no effect on cyclic 3',5'-AMP formation, and practically the same amount of cyclic 3',5'-AMP formed with or without this ion. The effect of phosphate ions was different, according to the cultural condition of the cells used. When alanine-grown cells were used, phosphate could be replaced by 0.1 M Tris chloride buffer (*p*H 8.4) without affecting the amount of cyclic 3',5'-AMP. When asparagine-grown cells were used, a change of phosphate to Tris chloride buffer reduced the nucleotide formation by one-half. These observations with magnesium and phosphate are somewhat different from the results obtained with growing cells (9).

Effect of amino acids. As in the case of the growth experiment (9), alanine and asparagine showed prominent stimulatory effects on cyclic 3',5'-AMP formation (Table 2). The effect of each amino acid is, however, somewhat different, depending upon the condition of pregrowth of *B. liquefaciens*. Thus, when the cells pregrown on asparagine medium were incubated in the medium fortified with various amino acids, only asparagine stimulated the formation of cyclic 3',5'-AMP. Alanine had no effect. It was noticed further that some other amino acids inhibited the cyclic 3',5'-AMP formation. With alanine-grown cells, the stimulatory effect of alanine was most pronounced. In this case, however, asparagine and some other amino acids were also stimulatory. Some differences were observed with optical isomers; the interpretation of this effect will be made later in the case of alanine isomers.

Effect of sugars. In a previous paper (9), we reported that the addition of sugars stimulated the formation of cyclic 3',5'-AMP by a growing culture of *B. liquefaciens*, and that the effect of glucose and mannose was most prominent among the sugars tested. The stimulatory effect of sugars was also evident in the nonproliferating cell experiment. In this case, the effects of pentoses, such as arabinose, xylose, and ribose, were larger than those due to hexoses (Table 3).

TABLE 2. *Effect of amino acids on cyclic 3',5'-AMP formation by sulfate-starved cells of Brevibacterium liquefaciens*

Amino acid added to medium without sulfate[a]	Cyclic 3',5'-AMP	
	Alanine-grown cells[b]	Asparagine-grown cells[b]
	mμmoles/ml	*mμmoles/ml*
Experiment 1		
DL-Alanine	493	110
DL-Asparagine	258	316
L-Arginine		93
DL-Aspartic acid	50	35
DL-Glutamic acid	35	140
DL-Glutamine		93
Glycine		0
DL-Histidine	168	137
L-Lysine		37
L-Proline		109
DL-Serine	187	142
DL-Threonine	143	105
DL-Valine		98
NH_4Cl		23
None	0	115
Experiment 2		
DL-Alanine	493	
D-Alanine	600	
L-Alanine	360	
DL-Asparagine		206
D-Asparagine		62
L-Asparagine		220
None	0	57

[a] DL-Alanine in the standard medium without sulfate was replaced by the same amount (200 mg in 5 ml medium) of the indicated amino acid.
[b] See Materials and Methods.

Cyclic 3',5'-AMP formation in the presence of chloramphenicol. Growth of *B. liquefaciens* was inhibited by 10 μg/ml or lower concentrations of chloramphenicol. As shown in Table 4, the cells were also able to accumulate cyclic 3',5'-AMP in the presence of higher concentrations of chloramphenicol, exhibiting essentially the same features as sulfate-deprived cells.

Cell-free system. When the enzyme preparation of *B. liquefaciens* was incubated in the reaction mixture containing ATP, Mg^{++}, and Tris chloride buffer, trace amounts of cyclic 3',5'-AMP were formed. The formation was accelerated consid-

TABLE 3. *Effect of sugars on cyclic 3',5'-AMP formation by sulfate-starved cells of Brevibacterium liquefaciens*

Sugar added to sulfateless medium[a]	Cyclic 3',5'-AMP	
	Alanine-grown cells	Asparagine-grown cells
	mμmoles/ml	*mμmoles/ml*
Glucose	208	258
Mannose	75	266
Galactose	87	0
Fructose	158	227
Arabinose	378	307
Xylose	410	465
Ribose	285	635
Maltose	171	279
Lactose	38	14
Sucrose	225	275
None	52	6

[a] Glucose in the standard sulfateless medium was replaced by the same amount (100 mg in 5 ml medium) of the indicated sugar.

TABLE 4. *Formation of cyclic 3',5'-AMP by Brevibacterium liquefaciens in the presence of chloramphenicol*

Addition[a]	Cyclic 3',5'-AMP	
	Chloramphenicol treatment[b]	Sulfate deprivation
	mμmoles/ml	*mμmoles/ml*
DL-Alanine + glucose	221	370
DL-Alanine	80	47
DL-Asparagine + glucose	253	230
DL-Asparagine	13	9

[a] DL-Alanine (200 mg) and glucose (100 mg) in 5 ml of the standard sulfateless medium were partially omitted or replaced as indicated.

[b] Chloramphenicol concentration: 100 μg/ml.

erably by the addition of either isomer or alanine (Table 5). The reaction rate remained constant during the assay period. Further increase occurred when the reaction mixture was shaken in the open test tube. These observations led us to the assumption that an oxidation product of alanine might be a cofactor of the bacterial adenyl cyclase. The experiment presented in Fig. 1 was undertaken to confirm this assumption. A glance at the figure makes it quite evident that pyruvate has a more prominent stimulatory effect than alanine. Further experiments showed that there was no synergistic effect of pyruvate and alanine. This may imply that the stimulatory effect of alanine may be attributed to that of pyruvate.

TABLE 5. *Cyclic 3',5'-AMP formation from ATP by cell-free extract of Brevibacterium liquefaciens*[a]

Addition and treatment	Cyclic 3',5'-AMP formed[b]
Experiment 1	
DL-Alanine, without shaking	1.8
L-Alanine, without shaking	1.5
D-Alanine, without shaking	1.0
None, without shaking	±
DL-Alanine, with shaking	13.3
L-Alanine, with shaking	11.4
D-Alanine, with shaking	10.4
None, with shaking	±
Experiment 2	
Pyruvate + DL-alanine, with shaking	22.3
Pyruvate + DL-alanine, without shaking	20.2
Pyruvate, with shaking	24.1
Pyruvate, without shaking	24.1
DL-Alanine, with shaking	11.9
DL-Alanine, without shaking	1.7

[a] Concentration of the added components: DL-, D-, or L-alanine, 0.45 M; sodium pyruvate, 0.1 M.

[b] Expressed as millimicromoles per 2 hr per milligram of protein; ± denotes that only trace amount of cyclic 3',5'-AMP formation occurred.

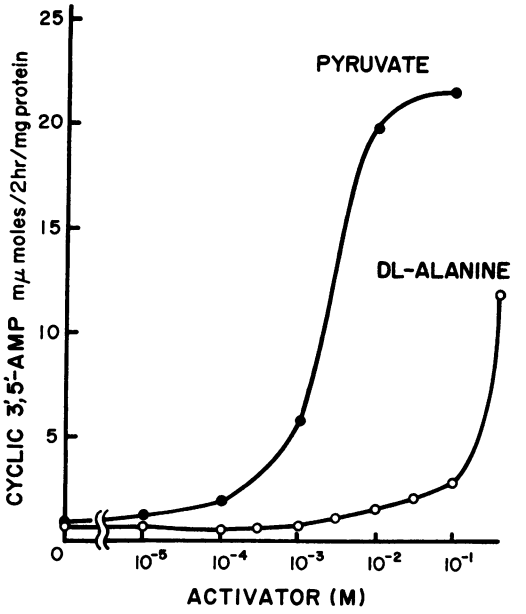

FIG. 1. *Effect of DL-alanine and pyruvate on the formation of cyclic 3',5'-AMP in cell-free extract of Brevibacterium liquefaciens. Symbols: ○ = DL-alanine with shaking; ● = pyruvate, without shaking.*

In a separate experiment, we observed that the cell-free extract of *B. liquefaciens* had phosphodiesterase activity, depending upon its culture condition (*unpublished data*). In this experiment, also, we observed weak but appreciable cyclic 3',5'-AMP degrading enzyme activity in the cell-free extract (Table 6). Pyruvate appeared to be inhibitory to this degradation, but it is also possible that pyruvate stimulated cyclic 3',5'-AMP synthesis from the substrate remaining in the crude extract. This apparent inhibitory action of pyruvate was much weaker than the stimulatory activity in cyclic 3',5'-AMP formation. Alanine showed no inhibitory action on the degradation of cyclic 3',5'-AMP. These results suggest that the effect of alanine is displayed after conversion to pyruvate, and that pyruvate acts primarily as a stimulating factor of cyclic 3',5'-AMP synthesis rather than as an inhibitor of the degrading enzyme.

DISCUSSION

Cyclic 3',5'-AMP was first discovered in animals, and its synthesis from ATP (13), degradation to AMP (2), and its physiological roles (14) have been investigated by use of animals. Considerable evidence has accumulated which suggests that cyclic 3',5'-AMP plays some role in the metabolic regulation systems of animals as a mediator of several hormones.

Our previous observation of cyclic 3',5'-AMP formation by *B. liquefaciens* (8, 10) has extended the distribution of this nucleotide to the microbial field, where no such hormones are known to play any physiological role as in animals. Makman and Sutherland (6) also reported the presence of cyclic 3',5'-AMP in *Escherichia coli*. Therefore, it may be expected that this nucleotide occurs rather widely in microorganisms.

So far, little information is available as to the physiological role of cyclic 3',5'-AMP in microorganisms. A preliminary report of Viñuela et al. (16) suggested the activating effect of cyclic 3',5'-AMP on yeast phosphofructokinase, but Atkinson's group (1, 11) failed to observe the effect of this nucleotide on yeast and *Escherichia coli* phosphofructokinase. We also tested the effect of cyclic 3',5'-AMP on *B. liquefaciens* phosphofructokinase, but data obtained up to the present time are not encouraging. (The experiment on *B. liquefaciens* phosphofructokinase was undertaken by M. I., at the Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto. We wish to thank O. Hayaishi for his advice and discussions.) Apart from these reports, it may be assumed that cyclic 3',5'-AMP plays some important roles also in microorganisms.

TABLE 6. *Cyclic 3',5'-AMP degrading enzyme activity in cell-free extract of Brevibacterium liquefaciens*

Addition and treatment[a]	Cyclic 3',5'-AMP decomposed[b]
DL-Alanine, with shaking	5.0
DL-Alanine, without shaking	4.7
Pyruvate, with shaking	3.2
Pyruvate, without shaking	2.5
None, with shaking	5.0
None, without shaking	4.6

[a] Concentration of added components: DL-alanine, 0.45 M; sodium pyruvate, 0.1 M.

[b] Expressed as millimicromoles per 2 hr per milligram of protein.

Our previous study with growing cells (9), and the present investigation on nonproliferating cells, both suggest that cyclic 3',5'-AMP formation is under the specific control of two major biological materials, amino acids and sugars, and, further, the present paper suggests that microbial adenyl cyclase is activated by pyruvate, one of the key intermediates which is situated in the branching point of metabolic pathways, including carbohydrate and amino acid metabolism. In consideration of recent knowledge of the patterns of regulatory systems of organisms, these effects might be regarded as reflections of the role of cyclic 3',5'-AMP in microorganisms, suggesting the presence of some regulatory mechanisms in amino acids and carbohydrate metabolism, where cyclic 3',5'-AMP acts as an intracellular mediator of regulatory signals. Investigations are now being made to verify this assumption.

The effect of pyruvate in vitro may explain the role of alanine observed in experiments in vivo, and the effectiveness of both isomers of alanine may be due to the presence of both D- and L-amino acid oxidases or of alanine racemase.

So far we have failed to demonstrate the stimulatory effects of asparagine and sugars in cell-free systems, but, as these substances do affect the cyclic 3',5'-AMP formation in intact cells, and it seems very difficult to ascribe these effects solely to the effect of pyruvate, it may be possible that there are some unknown mechanisms through which they display their effects on cyclic 3',5'-AMP formation. Makman and Sutherland (6), using nongrowing cells of *E. coli*, suggested that glucose prevented cyclic 3',5'-AMP formation and stimulated its extrusion from the cells. Although there exist some apparent discrepancies between their observation and ours, where glucose and other sugars increased the total

amount of cyclic 3′,5′-AMP, it seems very likely that there is some role of sugars or their metabolites, other than pyruvate, in controlling cyclic 3′,5′-AMP level in microbial cells.

During preparation of this manuscript, Hirata and Hayaishi (4) also observed the effect of pyruvate on adenyl cyclase of our bacterium. Their work is consistent with our suggestion, and provides further evidence that cyclic 3′,5′-AMP formation is under the specific control of this intracellular metabolite.

LITERATURE CITED

1. ATKINSON, D. E., AND G. M. WALTON. 1965. Kinetics of regulatory enzymes. J. Biol. Chem. **240**:757–763.
2. BUTCHER, R. W., AND E. W. SUTHERLAND. 1962. Adenosine 3′,5′-phosphate in biological materials. I. Purification and properties of cyclic 3′,5′-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3′,5′-phosphate in human urine. J. Biol. Chem. **237**:1244–1250.
3. GORNALL, A. G., C. J. BALDAWILL, AND M. M. DAVID. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751–766.
4. HIRATA, M., AND O. HAYAISHI. 1965. Pyruvate dependent adenyl cyclase activity of *Brevibacterium liquefaciens*. Biochem. Biophys. Res. Commun. **21**:361–365.
5. LIPKIN, D., W. H. COOK, AND R. MARKHAM. 1959. Adenosine-3′,5′-phosphoric acid: a proof of structure. J. Am. Chem. Soc. **81**:6198–6203.
6. MAKMAN, R. S., AND E. W. SUTHERLAND. 1965. Adenosine 3′,5′-phosphate in *Escherichia coli*. J. Biol. Chem. **240**:1309–1314.
7. OKABAYASHI, T., M. IDE, T. HATTORI, AND A. YOSHIMOTO. 1962. Occurrence of nucleotides in the culture fluids of microorganisms. IV. On the characters of *Brevibacterium liquefaciens* sp. n. and its cultural conditions for the excretion of nucleotides. Ann. Rept. Shionogi Res. Lab. **12**:191–197.
8. OKABAYASHI, T., M. IDE, AND A. YOSHIMOTO. 1963. Excretion of adenosine 3′,5′-phosphate in the culture broth of *Brevibacterium liquefaciens*. Arch. Biochem. Biophys. **100**:158–159.
9. OKABAYASHI, T., M. IDE, AND A. YOSHIMOTO. 1964. Occurrence of nucleotides in culture fluids of microorganisms. VI. Culture condition of *Brevibacterium liquefaciens* sp.n. for excretion of adenosine cyclic 3′,5′-phosphate. Amino Acid Nucleic Acid (Tokyo) **10**:117–123.
10. OKABAYASHI, T., A. YOSHIMOTO, AND M. IDE. 1963. Occurrence of nucleotides in culture fluids of microorganisms. V. Excretion of adenosine cyclic 3′,5′-phosphate by *Brevibacterium liquefaciens* sp. n. J. Bacteriol. **86**:930–936.
11. RAMAIAH, A., J. A. HATHAWAY, AND D. E. ATKINSON. 1964. Adenylate as a metabolic regulator. J. Biol. Chem. **239**:3619–3622.
12. SMITH, M., G. I. DRUMMOND, AND H. G. KHORANA. 1961. Cyclic phosphates. IV. Ribonucleoside-3′,5′ cyclic phosphates. A general method of synthesis and some properties. J. Am. Chem. Soc. **83**:698–706.
13. SUTHERLAND, E. W., T. W. RALL, AND T. MENON. 1962. Adenyl cyclase. I. Distribution, preparation, and properties. J. Biol. Chem. **237**:1220–1227.
14. SUTHERLAND, E. W., AND G. A. ROBISON. 1966. The role of cyclic-3′,5′-AMP in response to catecholamines and other hormones. Pharmacol. Rev. **18**:145–161.
15. THRELFALL, C. J. 1957. An analytical procedure for the acid-soluble phosphorus compounds in rat-skeletal muscle. Biochem. J. **65**:694–699.
16. VIÑUELA, E., M. L. SALAS, M. SALAS, AND A. SOLS. 1964. Two interconvertible forms of yeast phosphofructokinase with different sensitivity to endproduct inhibition. Biochem. Biophys. Res. Commun. **15**:243–249.
17. YOSHIMOTO, A., M. IDE, AND T. OKABAYASHI. 1964. Detection and assay of adenosine cyclic 3′,5′-phosphate by the thin-layer chromatographic method. Amino Acid Nucleic Acid (Tokyo) **10**:124–128.