PYRUVATE DEPENDENT ADENYL CYCLASE ACTIVITY OF *BREVIBACTERIUM LIQUEFACIENS*[1/]

Masaharu Hirata[2/] and Osamu Hayaishi

Department of Medical Chemistry
Kyoto University Faculty of Medicine
Kyoto, Japan

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Although much has been published concerning the metabolism and function of cyclic 3',5'-AMP in animal tissues, little is understood as to the significance of this compound in microorganisms. Recently Makman and Sutherland (1965) and Okabayashi, Yoshimoto and Ide (1963) reported the presence of cyclic 3',5'-AMP in the culture medium of *E. coli* and *Brevibacterium liquefaciens* respectively. In the latter, the addition of DL-alanine to the growth medium led to the accumulation of a large amount of cyclic 3',5'-AMP. In the present paper the partial purification of adenyl cyclase from *B. liquefaciens* is described. In addition to Mg^{++}, an α-keto acid, such as pyruvate, α-ketobutyrate or oxalacetate, was shown to be almost absolutely required for this activity.

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2/ On leave from the Shionogi Research Laboratory, Shionogi and Company, Ltd., Osaka, Japan.

B. liquefaciens (ATCC 14929) was grown for 62 hours at 25° with vigorous shaking in a medium containing 7 g of K_2HPO_4, 3 g of KH_2PO_4, 40 g of DL-alanine, 20 g of glucose, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter of distilled water. Cells were disrupted by sonic oscillation and particles were removed by centrifugation at 10,000 X g for 20 minutes and then at 105,000 X g for 60 minutes. The yellow supernatant, thus obtained, was passed through a Sephadex G-25 column in order to remove low molecular weight substances and was then subjected to ammonium sulfate fractionation after the streptomycin treatment. Ammonium sulfate fraction (35 - 55% saturation) was used as the enzyme source throughout the experiments. This fraction contained considerable amounts of nucleotidase and adenylate kinase activity, while the phosphodiesterase activity was almost negligible at pH 9.0 at which the cyclase assay was performed. The assay of enzyme activity was carried out as follows. The incubation mixture contained, in 0.15 ml, 100 mM of Tris-HCl buffer, pH 9.0, 100 mM of $MgSO_4$, 5 mM of lithium pyruvate, 2 mM of ATP-8-^{14}C (specific activity 66 mμc/μmole, Schwarz BioResearch, Inc.), and enzyme protein. Following incubation at 33° for 30 minutes, the reaction was stopped by heating at 100° for 2 minutes and the precipitate was removed by centrifugation. Aliquots of the supernatant fluid were spotted on Whatman No. 3 MM filter paper together with a carrier cyclic 3',5'-AMP and EDTA. The chromatogram was developed with 1 M ammonium acetate-ethanol (30:75) (Paladini and Leloir, 1952) at 25° for 16 - 20 hours. Then the spot corresponding to cyclic 3',5'-AMP was cut out, immersed in a scintillator solution (Wang and Jones, 1959) and the radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer.

As shown previously by Rall and Sutherland (1958) in the case of mammalian adenyl cyclase, Mg^{++} was essential for full enzyme activity (Table I). In addition to Mg^{++}, however, pyruvate marked-

ly stimulated the rate of reaction with the microbial enzyme system.

Table I.

System	Cyclic 3',5'-AMP (mμmole/mg protein/minute)
Complete	7.13
- Magnesium ion	0.00
- Pyruvate	0.01
Heated enzyme	0.00

The assay was carried out as described in the text. Each tube contained 195 μg of enzyme protein.

^{14}C-labeled cyclic 3',5'-AMP prepared by a large scale incubation mixture was identical with the authentic cyclic nucleotide as judged by its behavior upon paper electrophoresis (at pH 9.5 and 3.5) and paper chromatography with four solvent systems (n-butanol-acetate-water 5:2:3, saturated ammonium sulfate-1 M sodium acetate-isopropanol 80:18:12, ethanol-1 M ammonium acetate 75:30, isopropanol-concentrated ammonium-water 7:1:2), and its conversion to 5'-AMP upon treatment with the rabbit brain phosphodiesterase (Drummond and Perrott-Yee, 1961). Further proof for the identity of the enzymatic product was provided by the ultraviolet absorption spectra of the crystalline material at pH 7.0 and 2.0, and by infrared absorption spectra in KBr.

The rate of the reaction was maximum in the presence of about 5 mM pyruvate as shown in Fig. 1. α-Ketobutyrate was almost as active as pyruvate, while oxalacetate was about 30% active as pyruvate at this concentration. Phosphoenolpyruvate, coenzyme A, cocarboxylase, acetyl-phosphate, pyridine nucleotides, acetate, acetoacetate, α-ketoglutarate, lactate, cysteine, β-mercaptoethanol or carbonate could not replace pyruvate.

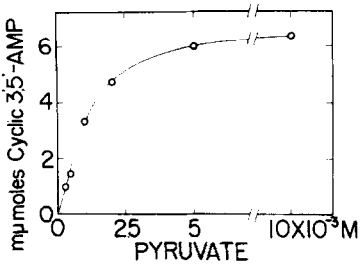

Fig. 1. Effects of varying concentrations of pyruvate on the rate of reaction. The conditions were identical with those described in Table I.

When 15 mμmoles of cyclic 3',5'-AMP were produced from 300 mμmoles of ATP in the presence of 30 mμmoles of pyruvate, the amount of pyruvate, as determined by the enzymatic procedure (Kornberg, 1955), did not change significantly during the course of the reaction indicating that pyruvate was not metabolized by this enzyme system but functioned catalytically or as a metabolic regulator. In contrast to the adenyl cyclase of animal tissues, the adenyl cyclase of B. liquefaciens is not stimulated by the addition of catecholamines either with crude or purified enzyme preparations. The role of pyruvate in this reaction is currently under investigation in our laboratory.

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REFERENCES

Drummond, G. I., and Perrott-Yee, S., J. Biol. Chem., 236, 1126 (1961).

Kornberg, A., in S. P. Colowick and N. O. Kaplan eds., Methods in Enzymology, Vol. I, Academic Press, New York, 1955, p. 441.

Makman, R. S., and Sutherland, E. W., J. Biol. Chem., 240, 1309 (1965).

Okabayashi, T., Yoshimoto, A., and Ide, M., J. Bacteriol., 86, 930 (1963).

Paladini, A. C., and Leloir, L. F., Biochem. J., 51, 426 (1952).

Rall, T. W., and Sutherland, E. W., J. Biol. Chem., 232, 1065 (1958).

Wang, C. H., and Jones, D. E., Biochem. Biophys. Res. Communs., 1, 203 (1959).