*ISOLATION OF ADENYL CYCLASE FROM ESCHERICHIA COLI**

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Abstract.—We have found a soluble cyclase, using for assay radioactively marked ATP as precursor. The reaction product was isolated by thin-layer chromatography and identified by specific degradation. After homogenization, part of the activity remained in the particulate fraction but could be easily extracted. The cyclase was concentrated 100-fold by conventional methods. The enzyme has a Mg^{++} requirement and is inhibited by fluoride and inorganic pyrophosphate.

The production of adenosine 3′,5′-cyclic monophosphate (cyAMP) in *Escherichia coli* was first observed by Makman and Sutherland,[1] who found it abundant in organisms grown in the absence of a carbon source. Addition of glucose caused its secretion into the surrounding medium. Since then, a number of reports have appeared on the effects of cyAMP in microorganisms. With *E. coli*, Perlman and Pastan[2–4] and Ullman and Monod[5] showed a reversion by cyAMP of catabolite repression of inducible enzyme production, particularly β-galactosidase. On the other hand, Okabayashi and his co-workers[6] have studied the production of cyAMP in *Brevibacterium liquefaciens*, and Hirata and Hayaishi[7] have isolated a soluble adenyl cyclase from this organism.

In view of the indications for the production of cyAMP in *E. coli*, it seemed desirable to us to look for the enzyme responsible for its formation. We report here on the isolation of a soluble cyclase from *E. coli* that has properties somewhat different from those of the enzyme isolated from *Brevibacterium liquefaciens*.[7]

Methods.—Standard assay for adenyl cyclase: Adenyl cyclase activity was determined by measuring the amount of cyAMP-P^{32} or cyAMP-H^3 formed from ATP-α-P^{32} or ATP-H^3 using thin-layer chromatography for separation. This was carried out at room temperature on cellulose and polyethylenimine-impregnated cellulose sheets. The development time for the solvents was 3–4 hr, by which time the solvent front had migrated about 18 cm. For the cellulose layer, the following solvents were used: (*A*) 1 *M* NH_4OAc:95% ethanol (30:75, v/v); (*B*) 1 *M* NH_4OAc, pH adjusted to 5.0 with acetic acid:95% ethanol (30:70, v/v); (*C*) isopropanol:conc. NH_4OH:0.1 *M* H_3BO_3 (60:10:30, by volume). For polyethylenimine-cellulose, the solvent used was (*D*) 1 N acetic acid:3 *M* LiCl (9:1, v/v).[8] Solvent system *A* was routinely used in the assay for adenyl cyclase, and *B*, *C*, and *D* were used for identification of the enzymatic reaction product.

The incubation mixture for assay contained, in a final volume of 50 μl: 2.5 μmoles of Tris-HCl, pH 8.5, 1 μmole of $MgSO_4$, 15 mμmoles of ATP-α-P^{32} or ATP-H^3, and enzyme protein. The mixture was incubated at 34° for 30 min and the reaction was terminated by heating for 3 min in a boiling water bath. After centrifugation, 25 μl of the supernatant containing carrier cyAMP was applied to a cellulose thin-layer sheet and developed with solvent *A*. The spot corresponding to cyAMP was cut out and immersed in 1 ml of water; 10 ml of Bray's[9] solution was added, and the radioactivity determined in a liquid scintillation spectrometer. One unit of enzyme is defined as that amount of enzyme that causes the production of 1 μμmole of cyAMP in 30 min at 34°. Specific activity is expressed in units of enzyme per mg of protein. Protein was determined by the method of Lowry *et al.*, [10] with bovine plasma albumin used as standard.

Assay for interfering enzymes: CyAMP phosphodiesterase activity was assayed as described by Butcher and Sutherland[11] by measuring colorimetrically the release of P_i in the presence of 5'-nucleotidase in *Crotalus atrox* venom. The incubation mixture consisted of 10 μmoles of Tris-HCl, pH 8.5, 4 μmoles of $MgSO_4$, 1 μmole of cyAMP, 0.04 mg of *Crotalus atrox* venom, and enzyme fraction in a final volume of 0.2 ml.

ATPase and inorganic pyrophosphatase activities were assayed by measuring the release of P_i from their corresponding substrates. The reaction mixture contained, in a final volume of 0.2 ml, 10 μmoles of Tris-HCl, pH 8.5, 4 μmoles of $MgSO_4$, 1 μmole of ATP or pyrophosphate, and enzyme fraction. The mixtures were incubated at 34° for 30 min, and the reaction was terminated by the addition of 0.2 ml of 10% trichloroacetic acid. The precipitate was removed by centrifugation and P_i was determined by the method of Fiske and SubbaRow.[12] One unit of enzyme is defined as that amount that causes the production of 1 μmole of P_i under the assay conditions. Specific activity is expressed in units of enzyme per mg of protein.

Preparation of rabbit-brain cyAMP phosphodiesterase: Rabbit-brain cyAMP phosphodiesterase was prepared by the procedure of Drummond and Perrot-Yee.[13]

Growth of E. coli cells: Crooke's strain of *E. coli* (ATCC 8739) was grown as described by Makman and Sutherland.[1] All the experiments reported here were done with extracts from this organism unless otherwise stated. However, *E. coli* strains K-12 and Q-13 appear to contain similar amounts of enzyme. *E. coli* K-12 was purchased from Miles Laboratories and Q-13 was grown in a medium described by Nathans and Lipmann[14] for *E. coli* B. The cells were kept frozen at −20° until needed.

Purification of adenyl cyclase: Since Makman and Sutherland[1] had shown that *E. coli* accumulates large quantities of cyAMP when incubated in phosphate buffer in the absence of any carbon source, the cyclase was expected to be the most active in such cells. However, we found that cyclase activity in crude extracts is independent of resuspension in the absence of carbon source. After disruption of the cells, it was found to be slightly greater than 50% in the cytoplasmic fraction (S_I), the rest being in the membrane fraction (P_I), from which it can be extracted easily with high specific activity (S_{II}). This is shown in Table 1 for the Q-13 strain of *E. coli*. Similar results were obtained for Crooke's and K-12 strains.

TABLE 1. *Distribution of activity of adenyl cyclase from Q-13 strain in cytoplasmic and particulate fractions.*

Fraction	Volume (ml)	Protein conc. (mg/ml)	Total protein (mg)	Specific activity	Total units
Crude extract	95	36	3400	517	17.6×10^5
S_I	80	28	2240	314	7.1×10^5
P_I	34	32	1100	563	6.2×10^5
S_{II}	19	11	200	890	1.8×10^5
P_{II}	27	30	810	640	5.2×10^5

The crude extract of Q-13 was prepared by grinding 50 gm of frozen cells with 50 gm of alumina followed by extraction with a buffer containing 0.01 *M* Tris-HCl, pH 8.0, and 1 mM dithiothreitol. DNase was added and the mixture centrifuged at 10,000 × *g* for 10 min to remove unbroken cells and alumina. The crude extract, consisting of the supernatant fraction together with some loosely pelleted materials, was further centrifuged at 30,000 × *g* for 40 min. The supernatant (S_I) was removed and saved. The pellet (P_I) was washed with the buffer and recentrifuged at 30,000 × *g* for 40 min. The supernatant of the washing is referred to as S_{II} and the pellet, resuspended in the buffer, as P_{II}. All fractions were assayed for cyclase activity under the standard assay conditions.

Purification of the enzyme was carried out at 0–4° unless otherwise stated. Cell-free extracts were prepared by grinding 100 gm of the frozen Crooke's strain cells with 100 gm of alumina in a precooled mortar. They were then extracted with cold Tris-HCl buffer containing 0.01 *M* Tris-HCl, pH 8.0, 0.01 *M* $MgSO_4$, 0.25 mM EDTA, 1 mM dithiothreitol, and 10% sucrose (buffer *T*). DNase was added and the mixture centrifuged

at 10,000 × *g* for 20 min. The supernatant was removed and further centrifuged at 30,000 × *g* for 40 min (S-30 fraction).

The S-30 fraction was brought to 1% with streptomycin sulfate. After 1 hr, the precipitate was removed by centrifugation. Solid $(NH_4)_2SO_4$ was slowly added, with stirring, to the supernatant fraction to 35% saturation, and left for 1 hr. After centrifugation, the precipitate was dissolved in buffer *T* and was dialyzed against the same buffer for 3–4 hr. The solution was centrifuged at 150,000 × *g* for 2.5 hr to remove ribosomes and membrane fragments. The supernatant was saved and the pellet resuspended in buffer *T* without Mg^{++} and EDTA, and was again centrifuged at 150,000 × *g* for 2.5 hr. The supernatant portions were combined (S-150 fraction) and fractionated with ethanol.

Precooled absolute ethanol (−20°) was added slowly, with constant stirring, to the S-150 fraction to 30% of the final volume. The solution was then stirred for 15 min at −5° and centrifuged. The precipitate was dissolved in a buffer containing 0.01 *M* Tris-HCl, pH 8.0, and 5 mM $MgSO_4$, and brought to 18% ethanol and centrifuged. The precipitate was dissolved in buffer *T* without Mg^{++} and EDTA, and was dialyzed against this buffer for 3–4 hr. The enzyme solution was kept frozen at liquid nitrogen temperature. All tests were done with this enzyme fraction.

Materials.—ATP-α-P^{32} was obtained from International Chemical and Nuclear Corp. ATP-H^3 was purchased from either New England Nuclear or International Chemical. Eastman chromagram (6065) cellulose thin-layer sheets with fluorescent indicator were purchased from Distillation Products Industries, and polyethylenimine-impregnated cellulose sheets (MN-polygram Cel 300 polyethylenimine) from Brinkmann Instruments, Inc.; cyAMP was obtained from Schwarz BioResearch, and *Crotalus atrox* venom from Sigma Chemical Co.

Results.—Purification of adenyl cyclase: The degree of purification of adenyl cyclase (about 100-fold) is shown in Table 2. The increase in specific activity

TABLE 2. *Purification of adenyl cyclase of Crooke's strain of E. coli.*

	Total proteins (mg)	Specific activity (units/mg)	Purification
S-30	3560	110	1
1% Streptomycin sulfate	3480	94	1
$(NH_4)_2SO_4$, 35%	200	290	2.6
S-150	95	2,800	25
Ethanol, 18%	6.2	12,400	110

in some steps of the purification was greater than had been expected from the mere removal of inert protein, and should be due to the presence of inhibitors or interfering enzymes. Since the enzyme was found to be relatively unstable, sucrose was included in all purification steps. Prolonged dialysis was also avoided since it led to considerable loss of enzyme activity. The activities of a few interfering enzymes were followed in all the purification steps. As is shown in Table 3, the final preparation was free of cyAMP phosphodiesterase activity, but retained most of its ATPase and some pyrophosphatase activity.

Properties of adenyl cyclase: In contrast to the soluble enzyme of *Brevibacterium liquefaciens*, the *E. coli* enzyme is not stimulated by pyruvate but, rather, is inhibited by concentrations above 3 mM. The time course of the reaction is shown in Figure 1. The rate of the reaction decreases after incubation for an hour, owing to enzyme inactivation, since the addition of fresh enzyme causes a return to the initial rate. The formation of cyAMP was found to be

TABLE 3. *Interfering enzymes at different stages of purification of adenyl cyclase of Crooke's strain of E. coli.*

	Specific Activity (μmoles/mg protein)		
	cyAMP phosphodiesterase	ATPase	Pyrophosphatase
S-30	0.45	1.2	138
Streptomycin sulfate, 1%	0.12	0.7	110
$(NH_4)_2SO_4$, 35%	0	2.1	37
S-150	0	0.6	56
Ethanol, 18%	0	0.8	3.9

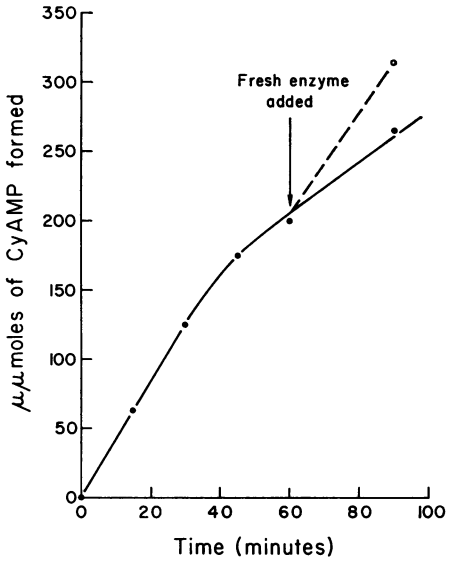

FIG. 1.—Time course of the reaction catalyzed by adenyl cyclase. The reaction mixture contained 2.5 μmoles of Tris-HCl, pH 8.5, 20 mμmoles of ATP-H^3 (50 cpm/μμmole), 1 μmole of $MgSO_4$, and 8 μg of enzyme protein, in a final volume of 50 μl. It was incubated at 34° and samples were removed at the indicated time intervals for determination of cyAMP-H^3 formed.

proportional to the amount of enzyme added (Fig. 2), and the pH optimum of the reaction in Tris-HCl buffer to be 9–9.5 (Fig. 3). The enzyme had an absolute requirement for Mg^{++}. The rate of the reaction was nearly proportional with Mg^{++} concentrations to 5 mM, reaching a maximum at 8 mM but showing no inhibition at concentrations up to 20 mM. K^+, Na^+, and NH_4^+ at a concentration of 10 mM did not affect the reaction.

Cyclase was inhibited by NaF and PP_i. At 5 mM NaF and 2 mM $NaPP_i$ the inhibition was about 80 and 90 per cent, respectively. When both NaF and $NaPP_i$ were present in the reaction mixture, complete inhibition was observed (Table 4). The inhibition was not due to the lowering of the effective concentration of Mg^{++} by these ions since an excess of Mg^{++} was normally present and the addition of more Mg^{++} did not reverse the inhibition. The inhibition by NaF alone was not reversed by pyrophosphatase.

A reversal of the soluble cyclase reaction in *Brevibacterium liquefaciens* has been observed by Greengaard *et al.*[15] Pyrophosphate inhibition in our enzyme preparation seemed to indicate reversibility; however, attempts to reverse the cyclase reaction in the direction of ATP synthesis have so far been unsuccessful. No radioactive ATP could be detected when the enzyme was incubated with CyAMP-H^3 and PP_i, or $P^{32}P_i$ and nonradioactive cyAMP; neither was a

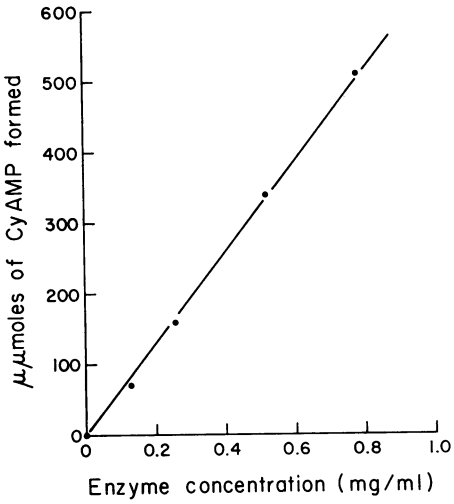

FIG. 2.—Effect of enzyme concentration on the rate of reaction. The reaction mixture contained 2.5 μmoles of Tris-HCl, pH 8.5, 15 mμmoles of ATP-H^3 (43 cpm/μμmole), 1 μmole of $MgSO_4$, and varying amounts of enzyme protein, in a final volume of 50 μl. Incubation was at 34° for 30 min, and the amount of cyAMP-H^3 formed was determined.

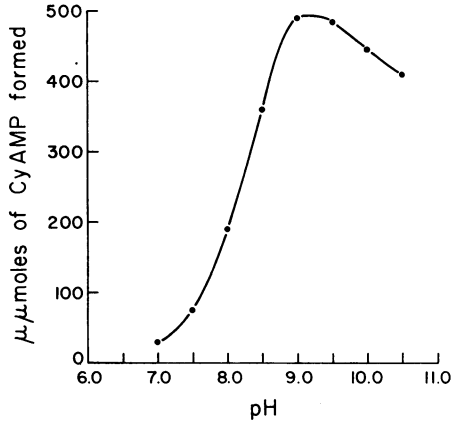

FIG. 3.—Effect of pH on the rate of reaction. The reaction mixture contained 2.5 μmoles of Tris-HCl, 15 mμmoles of ATP-H^3 (43 cpm/μμmole), 1 μmole of $MgSO_4$, and 26 μg of enzyme protein, in a final volume of 50 μl. Enzyme activities were determined under standard conditions.

formation of radioactive glucose 6-phosphate seen when hexokinase and glucose-C^{14} were present in a reaction mixture containing 5mM cyAMP and 5 mM PP_i.

Identification of enzymatic product: The reaction product was isolated from an incubation mixture of 0.25 ml containing 12.5 μmoles of Tris-HCl, pH 8.5, 5 μmoles of $MgSO_4$, 75 mμmoles of ATP-H^3 (43 cpm/μμmole), and 0.195 mg of enzyme protein. The mixture was incubated at 34° for 1 hr and heated in a boiling water bath for 3 min. After removal of the precipitate by centrifugation,

TABLE 4. *Effect of fluoride and inorganic pyrophosphate.*

	cyAMP-P^{32} formed (μμmoles)
No addition	410
+ NaF	84
+$NaPP_i$	28
+ NaF, $NaPP_i$	0

The reaction mixture contained 2.5 μmoles of Tris-HCl, pH 8.5, 1 μmole of $MgSO_4$, 15 mμmoles of ATP-α-P^{32}, 28 μg of protein, with 0.25 μmole of NaF and 0.1 μmole of $NaPP_i$ added or eliminated, in a final volume of 50 μl.

the supernatant fraction was chromatographed together with carrier cyAMP on cellulose thin-layer sheets using solvent *A*. It was eluted with 10 ml of water in the cold, and the eluate was filtered to remove contaminating cellulose, then dried down with a stream of air at 34° to about 0.2 ml.

The product was rechromatographed with the same solvent, and re-isolated in the same manner. Using thin-layer chromatography with four different solvents (cf. *Methods: A, B,* and *C* on cellulose, *D* on polyethylenimine-cellulose) it was found to have the same mobility as authentic cyAMP. The identity of the product was confirmed by treatment with rabbit-brain phosphodiesterase which cleaves cyAMP to 5′-AMP. The mixture consisting of 5 μmoles of Tris-HCl, pH 7.5, 2 μmoles of $MgSO_4$, tritiated enzyme product, 80 μg of phosphodiesterase, and 0.2 μmole of cold 5′-AMP in a final volume of 0.1 ml was incubated at 34° for 30 min. In one sample, 0.005 mg of *Crotalus atrox* venom was added to and cold 5′-AMP was omitted from the reaction mixture. The samples were chromatographed with the proper solvent systems. The results show that the enzymatic product was converted by phosphodiesterase to 5′-AMP (Fig. 4*b*) and further converted to adenosine by the 5′-nucleotidase of *Crotalus atrox* venom (Fig. 4*c*). Treatment with the venom alone had no effect. When treated similarly, commercial cyAMP behaved like the enzymatic product.

Discussion.—Considerable enzymatic activity was found to be associated with the 30,000 × *g* and 150,000 × *g* pellets. Most of the pellet activity can be removed by extraction with Tris-HCl buffer. This may indicate binding of the enzyme to the cell membrane. In contrast to the mammalian enzyme, the *E. coli* enzyme appears to be not very tightly bound there.

Hirata and Hayaishi[7] first described the isolation of a soluble adenyl cyclase from *Brevibacterium liquefaciens*. They found that pyruvate was essential for the enzyme reaction. The solubilized adenyl cyclase of *E. coli* differs by the absence of pyruvate stimulation. So far we have not found a stimulatory substance or a cofactor, other than Mg^{++}. A difference between these enzymes may be correlated by the different conditions under which accumulation of

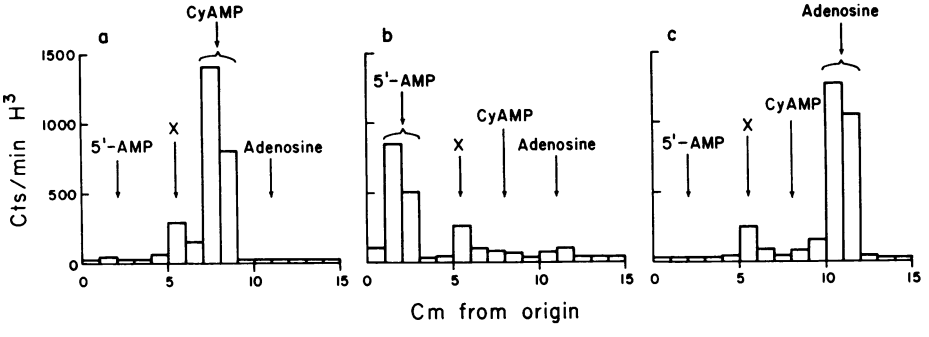

FIG. 4.—Identification of enzymatic product by specific degradation. The enzymatic product was isolated and treated with hydrolytic enzymes as described in the text. (*a*) Untreated, (*b*) treated with cyAMP phosphodiesterase, and (*c*) treated with cyAMP phosphodiesterase and 5′-nucleotidase from *Crotalus atrox* venom. Solvent *A* was used in the thin-layer chromatography. *X*, breakdown product in the process of isolation.

cyAMP is observed in the two organisms: with *Brevibacterium liquefaciens* when grown in the presence of DL-alanine,[6] with *E. coli* when deprived of carbon source.[1]

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