ACCUMULATION OF CYCLIC GUANOSINE 3':5'-MONOPHOSPHATE IN THE CULTURE MEDIUM OF GROWING CELLS OF *Escherichia coli*

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Summary: In an exponentially growing culture of *E. coli*, the concentration of cyclic guanosine 3':5'-monophosphate (cyclic GMP) was found to increase in parallel with the bacterial growth. As the cells approach the stationary phase of growth, the increment of cyclic GMP also ceases progressively to reach to a plateau. When cells are separated from the medium by centrifugation, almost all of the cyclic GMP is recovered in the culture supernatant. The amount of cyclic GMP accumulated is proportional to the number of cells present in the culture. These results suggest that a constant number of cyclic GMP molecules is synthesized each generation of *E. coli*, and is excreted from the cells to accumulate into the medium.

The occurrence of cyclic guanosine 3':5'-monophosphate (cyclic GMP) has been reported in a wide variety of organisms (1), and it has been suggested that it functions also, like cyclic AMP, as a chemical mediator in regulating a number of cellular processes (see ref. 1 for a review). In several eukaryotic cells, a transient elevation of the intracellular level of cyclic GMP was observed at an early stage of induction of cell proliferation (2,3), suggesting a role for the nucleotide in promoting cell growth.

On the other hand, very little is known about the function of cyclic GMP in *E. coli*. Bernlohr *et al.* (4) reported that the level of cyclic GMP in *E. coli* cells is elevated at an early exponential phase of growth and decreases at the stationary phase. However, it is difficult to draw a definite conclusion from their

experiments on the relationship between cyclic GMP and cell growth, since the intracellular levels of the nucleotide declined before the middle of the exponential phase (4). In addition, the concentration of cyclic GMP in E. coli cells was found to be extremely low, being approximately one to two orders of magnitude lower than that in mammalian cells. Recently, Macchia et al. (5) have demonstrated the presence of guanylate cyclase in E. coli, distinct from adenylate cyclase, and purified the enzyme to an almost homogeneous state. However, the physiological function of the nucleotide in this microorganism still remains obscure.

In a previous report (6), we have described a novel procedure for the determination of cyclic GMP which is highly accurate and specific. Using this new assay procedure, we have followed the concentration, both intracellular and extracellular, of cyclic GMP during the growth of E. coli cells. The results to be described in this paper demonstrate that there is an accumulation of a considerable amount of cyclic GMP in the culture medium, proportional to the density of the cells in the culture. The possible involvement of cyclic GMP in regulation of the growth of E. coli cells is discussed.

Materials and Methods

Strain, Medium, and Culture Conditions —— E. coli K 12 strain CP 78 (leu^-, thr^-, his^-, arg^-, thi^-, rel^+) was used in this study. The cells were cultured at 37° with shaking in a casamino acids (0.2 %)-glucose (0.4 %)-M9 medium supplemented with amino acid requirements (each 20 µg/ml) and thiamine (2 µg/ml).

Preparation of Samples for the Assay of Cyclic GMP —— For the determination of the total i. e., intracellular and extracellular, amount of cyclic GMP, 2 ml of 60 % (v/v) perchloric acid was directly added to 50 ml of the bacterial culture. The mixture was rapidly chilled in an ice-bath, and centrifuged for 10 min at 10,000 x g. The precipitate was discarded and to the supernatant were added 8,000 cpm of [^{3}H]cyclic GMP (14 mCi/µmole) as a recovery marker, and 1 ml of 5 % Norit A. After stirring, the Norit was collected by centrifugation and packed onto a Celite column (0.8 x 1 cm). The nucleotides were eluted with 0.3 N NH_4OH in 50 % ethanol, diluted with water, and adsorbed to a column of Dowex 1-X2 (0.6 x 1.2 cm). The column was eluted stepwise first with 4 ml of 0.5 N formic acid and then with 4 ml of 4 N formic acid. The

latter fraction containing cyclic GMP was lyophilized, and the residue was dissolved in 1.5 ml of 20 mM ammonium formate (pH 6.5). The solution was passed through an alumina column (0.6 x 1.2 cm) and the column was washed with 3.5 ml of the same solution. The effluent and the washing were combined, and lyophilized to dryness. The residue was dissolved in 300 µl of distilled water and used as the sample for the determination of cyclic GMP.

When intracellular and extracellular concentrations of cyclic GMP are to be determined separately, 50 ml of the culture was rapidly chilled and centrifuged for 5 min at 10,000 x g. The extracellular content of cyclic GMP was determined with the supernatant fraction in the same way as above. For the determination of the intracellular level of cyclic GMP, the pellets were homogenized in 3 ml of ice-cold 5 % trichloroacetic acid. After centrifugation, the supernatant was extracted with ethyl ether and the water layer was subjected to the Dowex 1-X2 and alumina columns as described above.

Assay Procedure for Cyclic GMP —— Cyclic GMP was assayed by the previously published procedure (6) with some modifications as follows. The reaction mixture contained, in a final volume of 80 µl, 50 mM Tris-HCl buffer (pH 7.6), 10 mM magnesium acetate, 10 mM 2-mercaptoethanol, 2 mM EDTA, 1 µg of phosphodiesterase, and a sample containing cyclic GMP to be assayed. After incubation for 30 min at 37°, the reaction was stopped by boiling for 3 min. The mixture was supplemented with 30 µl of a solution containing 50 mM Tris-HCl buffer (pH 7.6), 10 mM magnesium acetate, 10 mM 2-mercaptoethanol, 2 mM EDTA, 4 µg of GMP kinase and 10 pmoles of [γ-^{32}P] ATP (3-5 mCi/µmole) and incubated for 60 min at 0°. The mixture was again supplemented with 15 µl of a solution containing EF-Ts (14 µg/ml) and EF-Tu·GDP (0.17 mg/ml). After further incubation for 5 min at 0°, the reaction was terminated by the addition of 2 ml of a cold solution containing 10 mM Tris-HCl buffer (pH 7.6) and 10 mM magnesium acetate, and the mixture was poured onto a nitrocellulose membrane filter (0.45 µ pore size). The filter was washed three times with 10 mM Tris-HCl buffer (pH 7.6) and 10 mM magnesium acetate, dried, and counted in a liquid scintilation spectrometer. Other materials and methods were the same as described previously (6).

Results and Discussion

In the experiment shown in Fig. 1, an overnight culture was diluted about 15-fold with a fresh medium, and the cells were grown at 37° aerobically. As shown in the figure, the total concentration of cyclic GMP in the culture increased in parallel with the growth curve. When intra- and extracellular concentrations of cyclic GMP were determined separately, it was found that over 95 % of the total cyclic GMP is present in the culture supernatants both at exponential and stationary phases of growth (data not shown). The increase of cyclic GMP came to the halt as the culture reached its stationary phase. The value of

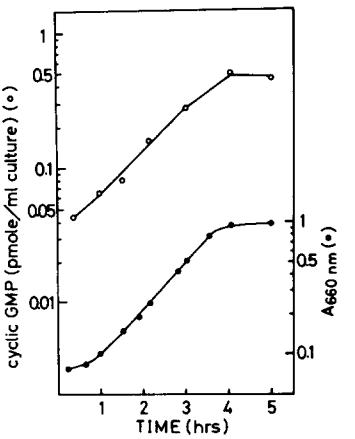

Fig. 1 About 1 liter of the medium was inoculated with one-fifteenth volume of overnight culture of E. coli CP 78. The medium and growth conditions used were as described in "Materials and methods." At times specified, 50-ml aliquots were withdrawn and assayed for their cyclic GMP content as described in the text. The values were corrected by subtracting the blank values (less than 0.05 pmole) obtained in the absence of phosphodiesterase. (○), the amount of cyclic GMP in the culture, and (●), the bacterial growth measured by turbidity at 660 nm using 18 mm tubes and Bausch and Lomb, Spectronic 20, colorimeter.

cyclic GMP at the plateau was approximately 0.5 pmole per ml of culture. No decrease of cyclic GMP in the culture medium was observed during further incubation for 4 hours at the stationary phase.

These results indicate that cyclic GMP is synthesized in E. coli cells during growth and is excreted into the medium. From the results in Fig. 1, and also from the determinations of the proteins in culture and the number of cells, it was calculated that about 1.2 pmoles of cyclic GMP was accumulated per mg of protein, or about 150 to 300 molecules of cyclic GMP per cell.

Since most of the cyclic GMP is present in the extracellular medium at any phase of growth, it is unlikely that the appearance of cyclic GMP in the medium is due to the lysis of nonviable cells. It is more likely that the synthesis and excretion of the nucleo-

tide are of physiological significance and possibly coupled with the cell proliferation. Although the excretion and accumulation of cyclic AMP in culture medium have been reported with E. coli (7,8), the amount of the nucleotide accumulated in the medium is not related to the cell growth. In another microorganism, Caulobacter crescentus, guanylate cyclase (9) as well as a cyclic GMP specific binding protein (10) have been found and partially purified. The effect of cyclic GMP on the regulation of stalk for mation of this bacteria has been reported (11).

The question arises as to whether growing E. coli cells are synthesizing cyclic GMP continuously or at certain stage of their cell cycle. Preliminary experiments carried out with synchronized cultures have revealed that cyclic GMP is synthesized not continuously, but at a definite phase of the cell cycle, most probably at some stage prior to the initiation of DNA replication. These results will be reported in a succeeding communication. To our knowledge, this is the first indication that cyclic GMP plays a role in controlling the cellular growth in prokaryotic cells. It is expected that the investigation on the mode of its action will throw more light on the regulatory mechanism of initiation of cell cycle and DNA replication.

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