DIVERSE DIRECTIONAL CHANGES OF cGMP RELATIVE TO cAMP IN E. COLI

Jose E. Gonzalez and Alan Peterkofsky

Laboratory of Biochemical Genetics, National Heart and Lung Institute
Bethesda, Maryland 20014

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Abstract. The relationships of the changes of cAMP and cGMP concentrations in E. coli varied as a function of experimental conditions. (1) Cells starved for carbon source for a short time period had high cAMP and low cGMP concentrations. Addition of carbon source (succinate, glucose or α-methyl glucoside) led to a decrease in cAMP and an increase in cGMP (bi-directional change). (2) Washed cells starved for glucose for long time periods had low cAMP levels which did not change on glucose addition. Addition of succinate or glucose to such cells led to a transient increase in cGMP levels (uncoupled change). The cGMP concentration peaked at 15 minutes or 1 hour after glucose or succinate addition, respectively. (3) Sham shift-up experiments (addition of α-methyl glucoside to cultures growing in succinate) in E. coli 1100 and CA 8000 showed decreases in cGMP levels in both strains; however, cAMP levels increased in the former (bi-directional change) and decreased in the latter (unidirectional change).

Introduction. Recently Bernlohr et al (1) used an enzyme cycling procedure to detect the presence of cGMP in E. coli and described the changes in its levels under various conditions. Using a radioimmunoassay modified to detect as little as 5 femtomoles of cGMP, we confirmed the presence of cGMP in E. coli. Bernlohr et al (1) suggested that cAMP levels tend to vary in a direction opposite to those of cGMP; our studies indicate that the cyclic nucleotide levels can, depending on experimental conditions, vary bi-directional, unidirectionally, or in an uncoupled fashion.

Methods. Cyclic nucleotide concentrations were determined in cells as follows: aliquots of cell suspension were rapidly filtered on Millipore filters, not washed, then extracted with hot formic acid as described (2). This procedure has been utilized for quantitative extraction of cAMP from E. coli; we assume that cGMP would behave similarly. Culture medium which passed through the Millipore filters was also collected and assayed to provide a correction factor for the amount of cyclic nucleotide trapped on the filter (2). The cell extracts were lyophilized, dissolved in buffer and assayed for cGMP as described in Figure 1. Before assay, samples were succinylated by a modification of the method of Cailla et al (3). Succinylation of cGMP increased the sensitivity of the assay approximately 100-fold (Figure 1). The authenticity of cGMP in various samples was verified in several ways. Treatment of samples with cyclic nucleotide phosphodiesterase (Sigma) before succinylation resulted in loss of reactivity. Additionally, omission of the succinylation step led to substantially reduced reactivity

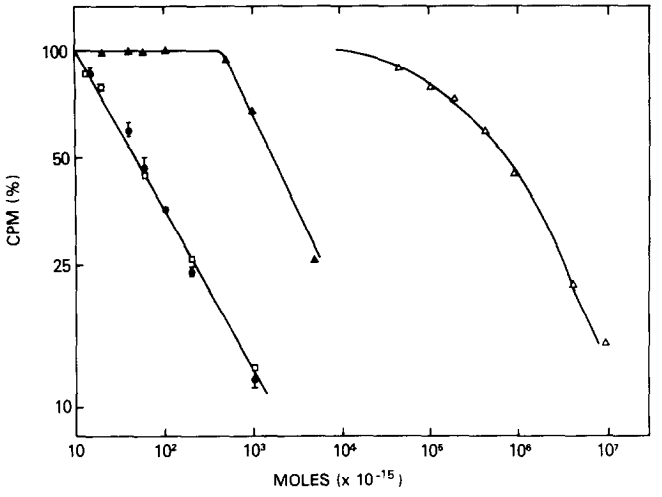

Fig. 1 - Succinyl-cGMP Standard and Cross Reactivity Curves.

Concentrations (in femtomoles) of the indicated compound are plotted in the abscissa. The ratio between I^{125} cGMP antigen bound in the presence of the indicated concentrations of unlabeled compound divided by the I^{125} cGMP antigen (5 femtomoles) bound in the absence of competing unlabeled compound has been multiplied by one hundred and plotted as % cpm in the ordinate. Radioactivity bound in the absence of competing unlabeled compound (approximately 50% of total I^{125} cGMP antigen added; 5 femtomoles; 3500 cpm) is set at 100%. For succinylation of samples, reaction tubes contained 1 mg of succinic anhydride (initially solubilized in acetone, then evaporated), 50 µl of sample or standard and 25 µl of a solution of triethylamine diluted 2:23 with H_2O. The succinylation incubation was at 37° for 20 mins. The remainder of the procedure was as described in the Collaborative Research radioimmunoassay kit with the following exceptions: imidazole buffer (.05 M, pH 7.0) was substituted for acetate buffer; 10 femtomoles of the derivative of cGMP were used in an overnight incubation; the antigen-antibody complex was trapped on a Millipore filter (HA-25) (6) which was counted by scintillation counting.

Symbols used were: ☐ , authentic succinyl-cGMP; ●, cGMP samples subjected to succinylation as described above (± standard error at the mean for 6 determinations); ▲, cGMP, not subjected to succinylation; △, GTP or 5'-GMP, subjected to succinylation.

(see Figure 1). The efficiency of succinylation in experimental samples was always verified by inclusion of known amounts of authentic cGMP in a separate set of duplicates. Intracellular cyclic nucleotide concentrations were calculated as described (2) using 10^{-12} cm^3 as the volume of a single cell.

Evidence that we specifically measured cGMP in our samples rather than other naturally occurring compounds was accumulated. No displacement of counts was observed with up to 100 pmoles of cAMP or succinylated cAMP; this level of cAMP is never present in samples assayed for cGMP. The level of GTP reported to be present in E. coli (4) suggested that it might be another source of interference in our samples. This possibility was eliminated. The hot formic acid extraction used in sample preparation converts all GTP to 5'-GMP. We found that treatment of authentic 5'-GMP with our cyclic nucleotide

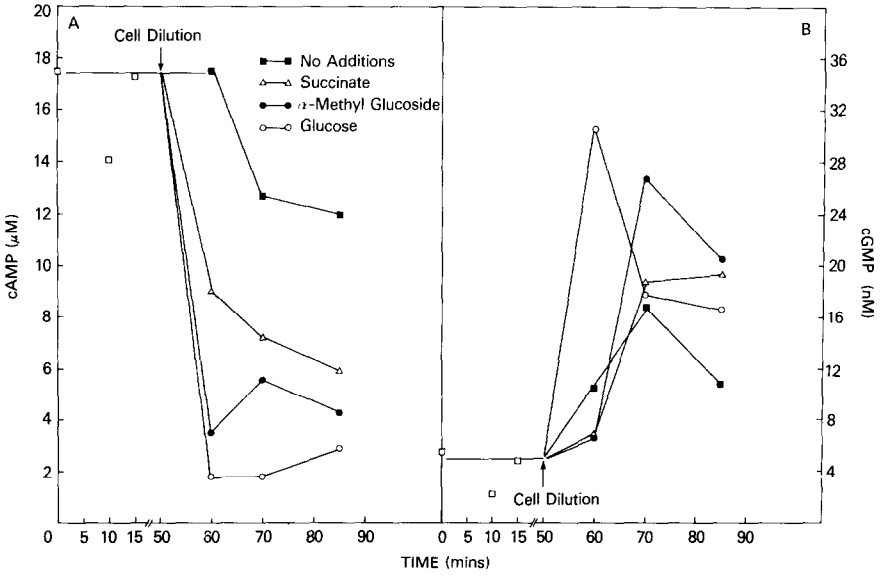

Fig. 2 - Cyclic Nucleotide Levels in E. coli CA 8000 After Addition of Carbon Sources to Short-Term Glucose-Starved Cells.

Cells were grown on salts medium (7) supplemented with glucose (3 mM) and thiamine (10 μg/ml). Fifty minutes after growth stopped (A_{650} = .74), the culture was diluted 7.5-fold into fresh salts medium and the indicated additions were made. □, before dilution; ■, after dilution into salts medium; Δ, dilution into salts medium and 50 mM sodium succinate; ●, dilution into salts medium and 20 mM α-methyl glucoside; O, dilution into salts medium and 30 mM glucose.

Panel A, cAMP; panel B, cGMP.

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phosphodiesterase preparation did not change its reactivity in the radioimmunoassay. In contrast, our samples lost most of their capacity to react with immune serum after treatment with the cyclic nucleotide phosphodiesterase.

Cyclic AMP assays were performed with radioimmunoassay kits supplied by Schwarz/Mann.

Results. Cells of E. coli CA 8000 (5), subjected to a short term starvation for glucose were assayed for intracellular cAMP (Figure 2A) and cGMP (Figure 2B), before and after dilution into media containing various carbon sources. Addition of glucose or α-methyl glucoside led to a marked decrease in cAMP in less than 10 minutes. Succinate addition resulted in a slower decrease in cellular cAMP. Dilution into fresh medium without carbon source led to approximately a 30% decline in cellular cAMP over a 40 minute period (Figure 2A). Under these conditions, the effects of carbon source addition on cGMP levels were roughly

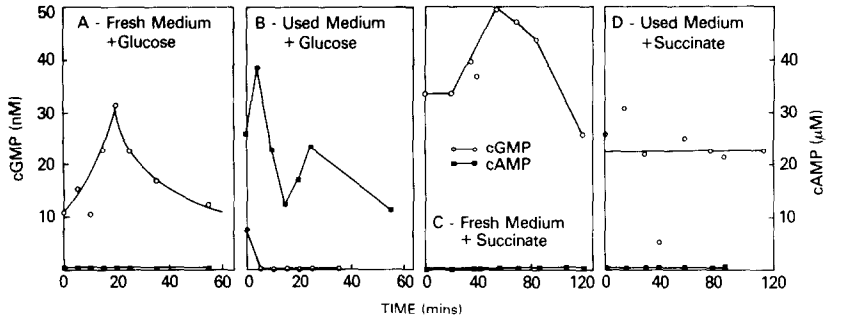

Fig. 3 - Effect of Glucose or Succinate on cAMP and cGMP Levels in E. coli CA 8000 suspended in Fresh or Used Medium.

E. coli CA 8000 was grown overnight in salts medium (7) containing thiamine (10 μg/ml) and glucose (30 mM) or succinate (30 mM). For resuspension of cells in fresh medium (panels A and C), the cultures were centrifuged, and the cells washed with minimal medium. The cells were diluted to $A_{650} \approx .1$ in medium containing glucose (6 mM, panel A) or succinate (20 mM, panel C). Used medium was prepared by centrifugation of the cultures grown in glucose (panel B) or succinate (panel D). For the experiments with used medium, the overnight cultures of glucose or succinate-grown cells were directly diluted into the used media supplemented with glucose or succinate as above. Used glucose medium had a pH of 5.9. The generation times were: A, 98 mins; B, 98 mins; C, 225 mins; D, 300 mins. At the designated times, samples (15 ml) were collected on 47 mm Millipore filters and processed for cAMP and cGMP determinations as described in Methods.

opposite to that of cAMP (Figure 2B). Dilution into media with or without added carbon sources resulted in higher cGMP concentrations. The presence of glucose was responsible for a rapid rise followed by a decline in cGMP concentrations. A similar pattern but with somewhat different time course was seen on the addition of α-methyl glucoside or succinate (Figure 2B).

Cells of E. coli CA 8000 grown overnight on glucose (Figure 3A and B) or succinate (Figure 3C and D) were suspended in fresh or used medium supplemented with the same carbon sources, then monitored for cAMP and cGMP levels. The transient increase in cGMP concentration showed a clear peak at about 20 minutes (Figure 3A). Washed cells with low initial cAMP levels showed essentially no change in cellular cAMP during this time period. Therefore, under these conditions, there was no bi-directional relationship between cAMP and cGMP.

When cells were diluted into used medium supplemented with glucose the increase in cGMP levels was prevented (Figure 3B). In contrast, there was a

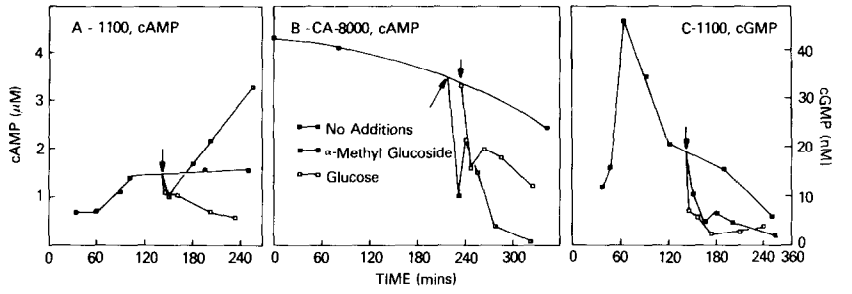

Fig. 4 - Effect of Glucose or α-methyl Glucoside Addition to Cells Growing on Succinate on cAMP and cGMP Levels.

Cultures of E. coli 1100 (8) (panels A and C) or CA 8000 (panel B) were grown in salts medium plus succinate (30 mM). At the designated times, glucose or α-methyl glucoside (20 mM) were added. Samples (15 ml) were processed for cAMP and cGMP levels (see Methods). The pattern for cGMP in strain CA 8000 was similar to that shown for 1100 (data not shown). The A_{650} initially and the times of glucose or α-methyl glucoside addition were for 1100 - .24 and .37; for CA 8000 - .16 and .60. E. coli 1100 is a **spontaneous mutant** derived from Hfr 3000 (same as CA 8000) which ferments β-glucosides (8). Probably as a result of this mutation, E. coli 1100 will utilize α-methyl glucoside as a carbon source, while E. coli CA 8000 will not.

slow decrease in cellular cAMP. Again, there was no bi-directional regulation of cAMP and cGMP levels.

The picture with succinate addition to cells in fresh medium (Figure 3C) was similar to that for glucose except that the transient increase in cGMP showed a peak at about 60 minutes. As was the case with glucose grown cells, used succinate medium suppressed the transient increase in cGMP (Figure 3D). Succinate grown cells had characteristically low cAMP levels with or without washing. Therefore, succinate grown cells did not show bi-directional changes in levels of the two cyclic nucleotides under these conditions.

A further indication for independent regulation of cAMP and cGMP levels was seen in studies involving "shift-up" from succinate to either glucose or α-methyl glucoside. While two different strains showed identical response in cGMP levels to glucose or α-methyl glucoside addition (Figure 4C) the responses in cAMP levels differed. Addition of glucose to strain 1100 (Figure 4A) led to a decrease in cAMP; on the other hand, α-methyl glucoside addition led to an increase in cAMP. In contrast, addition of either glucose or α-methyl

TABLE I
Observed Changes in Cyclic Nucleotide Pools
Under Various Conditions

Direction of Change		
cAMP	cGMP	Condition
↑	↓	succinate to α-methyl glucoside shift (Fig. 4A, C)
↑	-*	40-100 mins. after succinate to α-methyl glucoside shift (Fig. 4A, C)
↓	↓	succinate to glucose shift (Fig. 4A, B, C)
↓	↑	suspension in fresh medium (Fig. 2)
-	↑	0-20 mins. after glucose addition (Fig. 3A)
-	↓	20-60 mins. after glucose addition (Fig. 3A)
↓	-	suspension in used medium (Fig. 3B)

* indicates no change

glucoside to E. coli CA 8000 (Figure 4B) resulted in lowering of cAMP levels. Therefore, depending on the bacterial strain and the addition, "shift-up" experiments showed changes in cAMP and cGMP that were or were not bi-directional.

Discussion. The various studies presented here indicate that there is no uniform correlation between the direction of change of cAMP and cGMP under various metabolic conditions. Table I presents a summary of the fluctuations of the cyclic nucleotides demonstrated in these experiments. Further evidence that there is no interdependence between cAMP and cGMP comes from the observation that a pattern of cGMP accumulation similar to that seen in wild-type strains (Figure 3A) is also seen in an adenylate cyclase deletion (5) (data not shown).

The suggestion has been made that an increase in growth rate is correlated with an increase in cGMP concentration (1). However, we have observed (Figure 4C) that a culture growing on succinate (generation time, 180 minutes) grows more rapidly after glucose addition (generation time, 72 minutes), but shows a marked decrease in cellular cGMP. Furthermore, succinate grown cells have

longer doubling times (Figure 3C) than glucose grown cells (Figure 3A), but nevertheless develop higher maximum levels of cGMP. Finally, the growth rate of cells is not affected when intracellular cGMP is no longer detectable (Figure 3B). These results suggest that cellular cGMP concentrations are not proportional to growth rate in E. coli and that the presence of cGMP may not even be required for growth.

The experiments reported here do not elucidate the mechanism by which cGMP levels are regulated. Any model for regulation of cGMP concentration must take into consideration the observation that there is a transient increase followed by a decrease in cyclic nucleotide level. The fact that used medium prevents the transient accumulation of cGMP (Figure 3B) suggests that a metabolite may account for the decline in this cyclic nucleotide. The observation (Figure 2B) that dilution of a stationary culture into fresh medium results in increased cGMP concentration is consistent with the idea that an inhibitor of cGMP accumulation is formed metabolically. The increase in cGMP level due to the addition of carbon source (Figure 2B) probably does not require extensive metabolism of that carbon source, since α-methyl glucoside is effective. This compound is phosphorylated during transport into cells but not further metabolized. Therefore, some aspect of the transport process or the presence of a phosphorylated sugar may be involved in the observed increase in cGMP levels.

The function of cGMP in E. coli is not clear. It is noteworthy that during a complete course of growth through stationary phase, the transient increase of cGMP occurs only once (data not shown). The increased concentration of cGMP early after addition of carbon sources (Figure 3A and C) suggests that it may be involved in adaptation to growth after a period of starvation. The possibility that this increase in cGMP concentration depends on a period of synchronous growth is under investigation.

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