CYCLIC 3'5'-AMP: STIMULATION OF β-GALACTOSIDASE AND TRYPTOPHANASE INDUCTION IN E. COLI

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Cyclic 3',5'-adenosine monophosphate (cyclic 3',5'-AMP) participates in the regulation of many metabolic pathways in animal tissues (Sutherland and Rall, 1960). Cyclic 3',5'-AMP is also found in Escherichia coli, where its function is unknown (Makman and Sutherland, 1965). This paper reports that cyclic 3',5'-AMP increases the differential rate of synthesis of two inducible enzymes, β-galactosidase and tryptophanase. When the synthesis of these enzymes is repressed by glucose and pyruvate respectively, cyclic 3',5'-AMP restores their synthesis to normal levels. Cyclic 3',5'-AMP fails to stimulate the rate of synthesis of the repressible enzyme alkaline phosphatase.

Materials and Methods--Isopropyl-β-D-thiogalactoside (IPTG), o-nitrophenyl-β-D-galactoside (ONPG) and L-tryptophan were purchased from Mann Research Laboratories, cyclic 3',5'-AMP (phosphate free) was from Calbiochem and L-leucine-^{14}C (u.l., 250mcuries/mmole) and ^{3}H-uracil (u.l., 3 curies/mmole) from New England Nuclear Corp. Chloramphenicol was a product of Parke, Davis and Company. P-nitrophenyl phosphate was obtained from Sigma.

Bacteria and Media: Crooke's strain of E. coli, ATCC 8739, was grown in medium AG, a medium containing 14.0 g K_2HPO_3, 6.0 g

KH_2PO_4, 2.0g $(NH_4)_2$, 0.2 g $MgSO_4$ and 5 ml of glycerol per liter (Makman and Sutherland, 1965). All experiments were done at 37°.

EDTA treatment: E. coli were treated with EDTA by a modification of the method of Leive (1965). Bacteria were grown at 37° with shaking in medium AG to a density of about 1 x 10^9 cells/ml. They were then centrifuged, washed once at room temperature with 0.12 M Tris (pH 8) containing 0.5% glycerol and 10^{-3} M sodium phosphate, and resuspended in this buffer at a density of about 5 x 10^9/ml. This suspension was incubated at 37° for 2 minutes with 10^{-3} M EDTA, and then diluted into 10 volumes pre-warmed medium AG. Experiments were begun within a few minutes after dilution. In each experiment the final bacterial density was about 4 x 10^8/ml. For studies of alkaline phosphatase cells grown in medium AG were washed and treated with EDTA in phosphate free tris buffer and then incubated in medium AG in which phosphate was replaced by 0.08M Tris-HCl (pH 7.5) and 0.04M KCl.

β-galactosidase was induced by incubation of E. coli in the presence of $5 \cdot 10^{-4}$M IPTG, and was assayed by the method of Pardee, Jacob and Monod (1959) except that the assay was done at 23°C. One unit of β-galactosidase catalyzes the hydrolysis of 1 μmole ONPG/minute under these conditions. Cyclic 3',5'-AMP had no effect on the β-galactosidase assay. Tryptophanase was induced by incubation of E. coli with $2.5 \cdot 10^{-4}$ M L-tryptophan, and was assayed by the method of Bilezikian et al (1967). One unit of enzyme activity is the amount that will produce 1 mμmole of indole/minute under these conditions. Alkaline phosphatase was measured in toluene treated cells by the method of Garen and Levinthal (1960). One unit of enzyme is the amount that will hydrolyze 1 mμmole of p-nitrophenyl phosphate per hour under these conditions. Cyclic 3',5'-AMP at 10^{-3}M stimulates the activity of alkaline phosphatase by 25% and inhibits tryptophanase by 15%. All of our results have been corrected for these effects.

The incorporation of ^{14}C-leucine into protein and of ^{3}H-uracil into RNA was measured by a filter paper disc method (Byfield and Scherbaum, 1966).

Results--We chose to investigate the effect of cyclic 3',5'-AMP on enzyme synthesis by adding it to cultures of E. coli in which the synthesis of β-galactosidase was induced by IPTG, or in which tryptophanase synthesis was induced by tryptophan. Cyclic 3',5'-AMP was without effect when added to normal growing E. coli. It seemed possible that the failure of cyclic 3',5'-AMP to act might be due to its exclusion from normal cells, and therefore we studied the effect of cyclic 3',5'-AMP on Tris-EDTA treated cells, which are permeable to Actinomycin D (Leive, 1965) and various nucleoside triphosphates (Buttin and Kornberg, 1966). In Tris-EDTA treated cells, cyclic 3',5'-AMP stimulated IPTG-induced β-galactosidase production (Table 1), and tryptophan induced tryptophanase production (Table 2). The magnitude of the stimulation was variable; β-galactosidase was usually increased by 40-100%, and tryptophanase production by 100-800%. Cyclic 3',5'-AMP did not produce a detectable stimulation of the synthesis of these enzymes in the absence of inducers. The effect of cyclic 3',5'-AMP required protein synthesis, since no effect was detected in cells in which protein synthesis was inhibited by chloramphenicol. The effect of cyclic 3',5'-AMP was not due to restoration of cellular functions lost during Tris-EDTA treatment, since the treated cells synthesized tryptophanase and incorporated leucine-C^{14} into protein almost as well as untreated cells, and cyclic 3',5'-AMP increased enzyme synthesis above that of untreated cells. Further, although cyclic 3',5'-AMP increased the synthesis of β-galactosidase and tryptophanase, it did not increase the incorporation of leucine-C^{14} into total cellular protein (Tables 1 and 2) or the incorporation of uracil-H^{3} into RNA. Therefore, cyclic 3',5'-AMP increased the differential rate of synthesis of these two enzymes.

Table 1

Exp.	Glucose (M)	Cyclic 3',5'-AMP	β-galactosidase Production U/ml/min	Protein Synthesis cpm/ml/min	Differential Rate (U/cpm)x10^3
1.	-	-	0.50	330	1.5
	-	10^{-3}	0.92	342	2.7
2.	-	-	0.75	298	2.5
	-	10^{-3}	0.97	294	3.4
	10^{-1}	-	0.30	370	0.8
	10^{-1}	10^{-3}	0.82	342	2.4
	10^{-2}	-	0.28	338	0.8
	10^{-2}	10^{-3}	0.84	398	2.0

Table 1: Stimulation of β-galactosidase synthesis, and reversal of glucose repression by cyclic 3',5'-AMP. Each flask contained 5 ml of cells in medium AG, $5x10^{-4}$M IPTG, $4x10^{-4}$M leucine and 2μc of ^{14}C-leucine. Aliquots were removed after 10 and 20 min of incubation. The difference between the 10 and 20 min samples was used to calculate the rates of enzyme and protein synthesis since the rates were approximately linear over that period.

Cyclic 3',5'-AMP stimulated tryptophanase production at concentrations as low as $2.5x10^{-5}$M, with a half-maximal response at about 10^{-4}M. Cyclic 3',5'-AMP stimulated β-galactosidase synthesis at concentrations as low as 10^{-4}M. These are 'physiological' concentrations, since cyclic 3',5'-AMP concentrations can be greater than 10^{-4}M in glucose-starved E. coli (Makman and Sutherland, 1965).

The stimulatory effect on β-galactosidase and tryptophanase synthesis was specific for cyclic 3',5'-AMP; 10^{-3}M ATP, ADP, 5'-AMP, 3'-AMP, and fructose 1,6-diphosphate were inactive. These findings, coupled with the observation that E. coli do not metabolize cyclic 3',5'-AMP, but regulate their cyclic 3',5'-AMP concentration by secreting it into the medium (Makman and Sutherland, 1965), indicate that cyclic 3',5'-AMP

Table 2

Pyruvate (M)	Glucose (M)	Cyclic 3',5'-AMP (M)	Tryptophanase production U/ml/min	Protein synthesis cpm/ml/min	Differential rate (U/cpm)x10^4
-	-	-	0.028	296	0.95
-	-	10^{-3}	0.083	296	2.80
10^{-2}	-	-	0.001	281	0.04
10^{-2}	-	10^{-3}	0.032	293	1.09
10^{-3}	-	-	0.013	298	0.44
10^{-3}	-	10^{-3}	0.045	312	1.45
-	10^{-2}	-	0.011	288	0.38
-	10^{-2}	10^{-3}	0.086	297	2.89
-	10^{-3}	-	0.015	290	0.52
-	10^{-3}	10^{-3}	0.072	300	2.39

Table 2: Stimulation of tryptophanase synthesis, and reversal of pyruvate and glucose repression by cyclic 3',5'-AMP. Each flask contained 3 ml of cells in medium AG, 2.5×10^{-4}M tryptophan, 4×10^{-4} leucine and 1.3μc of ^{14}C-leucine. Aliquots were removed at 10 and 20 minutes and the difference used to calculate the rates of enzyme and protein synthesis.

does not act by its conversion to other adenine-containing nucleotides or by stimulation of phosphofructokinase. High concentrations of inducer (5×10^{-4}M IPTG, 2.5×10^{-4}M tryptophan) were employed, so that the rate of enzyme synthesis would not be limited by the rate of inducer uptake (Herzenberg, 1959). Furthermore, since Tris-EDTA treated cells were employed, induction of a permease for IPTG or tryptophan would not be required for their entrance into cells.

The addition of glucose and pyruvate repress the synthesis of both β-galactosidase and tryptophanase. The differential rate of β-galactosidase synthesis was decreased by 68% by 0.01M and 0.1M glucose (Table 1). Cyclic 3',5'-AMP at 10^{-3} restored enzyme synthesis

to control levels. When ß-galactosidase synthesis was repressed by glucose, cyclic 3',5'-AMP produced a three-fold increase in the rate of enzyme synthesis. Pyruvate at 10^{-2}M repressed tryptophanase production by 95% (Table 2), and cyclic 3',5'-AMP at 10^{-3}M restored enzyme synthesis to control levels. Under these conditions, cyclic 3',5'-AMP increased tryptophanase synthesis about 27-fold. In the absence of cyclic 3',5'-AMP glucose repressed tryptophanase production by 60%. However, cyclic 3',5'-AMP stimulated tryptophanase production equally well in the presence or absence of glucose.

Both ß-galactosidase and tryptophanase and enzymes subject to glucose repression. We therefore have studied the synthesis of alkaline phosphatase, an enzyme not subject to glucose repression (McFall and Magasanik, 1959), but whose synthesis is repressed by inorganic phosphate. The rate of synthesis of alkaline phosphatase in derepressed cells was not stimulated by cyclic 3',5'-AMP (Table 3).

Table 3

Glucose (M)	Phosphate (M)	Cyclic 3',5'-AMP (M)	Alkaline Phosphatase Production U/ml/min	Tryptophanase Production (U/ml/min)x10^2
-	-	-	1.20	0.72
-	-	10^{-3}	1.14	4.32
10^{-1}	-	-	1.50	0.45
-	-	10^{-3}	1.20	4.14
-	10^{-3}	-	0	-
-	10^{-3}	10^{-3}	0	-

Table 3: Effect of cyclic 3',5'-AMP, glucose, and phosphate on alkaline phosphatase and tryptophanase synthesis. Each flask contained 10 ml of cells in medium AG in which the phosphate was replaced by 0.08M Tris, pH 7.5, and 0.04M KCl. Tryptophan ($2.5x10^{-4}$M) was added at 40 minutes. Aliquots were removed for enzyme assays at 50 and 60 minutes (10 and 20 minutes after the addition of tryptophan), and the difference used to calculate the rates of enzyme synthesis.

Further when alkaline phosphatase was repressed by 1 mM phosphate, cyclic 3',5'-AMP did not overcome this repression. Glucose failed to repress alkaline phosphatase synthesis and cyclic 3',5'-AMP also did not stimulate alkaline phosphatase synthesis when glucose was present. In most experiments a small inhibition of alkaline phosphatase synthesis was noted in the presence of 10^{-3}M cyclic 3',5'-AMP.

Discussion--Cyclic 3',5'-AMP stimulates the induction of β-galactosidase and tryptophanase in E. coli. Makman and Sutherland (1965) observed that when E. coli are grown on glucose and then suspended in a phosphate buffer without glucose, they accumulate large amounts of cyclic 3',5'-AMP. Under these conditions, the differential rate of synthesis of β-galactosidase and tryptophanase is maximal (Rickenberg and Lester, 1955; Magasanik, 1961). The addition of glucose to these cells results in a rapid fall in the intracellular concentration of the nucleotide (Makman and Sutherland , 1965), and a decrease in the differential rate of β-galactosidase and tryptophanase synthesis. Acetate, which causes a relatively small decrease in the intracellular concentration of cyclic 3',5'-AMP, does not repress β-galactosidase or tryptophanase synthesis. The high differential rate of synthesis of these enzymes in cells with a high content of cyclic 3',5'-AMP, the low rate in cells with little nucleotide, and our finding that cyclic 3',5'-AMP stimulates the induction of these enzymes even when repressed by glucose, suggest that cyclic 3',5'-AMP participates in the regulation of the synthesis of these enzymes. Further support for this hypothesis is the fact that the synthesis of alkaline phosphatase, an enzyme which is not repressed by glucose (McFall and Magasanik, 1960), is not stimulated by cyclic 3',5'-AMP.

The repression of β-galactosidase synthesis in cells induced with high concentrations of inducer can be separated into a permanent weak type of repression that occurs when glucose is present in the medium

(catabolite repression), and a severe transient repression that occurs when cells grown on one carbon source are exposed to a different carbon source (Tyler, Loomis and Magasanik, 1967). Low cyclic 3',5'-AMP concentrations may be in part responsible for one or both types of repression.

Cyclic 3',5'-AMP partly overcame the repression of tryptophanase synthesis by pyruvate. Thus pyruvate may also act, in part, by lowering intracellular cyclic 3',5'-AMP levels. However, the effect of pyruvate on the nucleotide concentrations has not been measured. Since pyruvate represses tryptophanase synthesis more effectively than β-galactosidase synthesis (McFall and Mandelstam 1963), factors other than cyclic 3',5'-AMP are probably involved in the pyruvate effect.

When E. coli are grown on acetate and then starved of a carbon source, they accumulate less cyclic 3',5'-AMP than do glucose-grown cells (Makman and Sutherland, 1965). Both acetate and glucose depress the nucleotide concentration in acetate-grown cells. We have not studied enzyme induction in acetate-grown cells, and so cannot correlate cyclic 3',5'-AMP concentrations and enzyme synthesis under these conditions.

Enzyme synthesis can be regulated at the level of m-RNA production (transcription) or protein synthesis (translation). Recently Khairallah and Pitot (1967) have reported that cyclic 3',5'-AMP stimulates polypeptide chain release from liver polysomes. Preliminary experiments show that in E. coli cyclic 3',5'-AMP also acts at the level of translation.

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