CYCLIC 3';5' ADENOSINE MONOPHOSPHATE-PHOSPHODIESTERASE AND THE RELEASE OF CATABOLITE REPRESSION OF β-GALACTOSIDASE BY EXOGENOUS CYCLIC 3';5' ADENOSINE MONOPHOSPHATE IN *ESCHERICHIA COLI*

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SUMMARY: The level of cAMP-phosphodiesterase was much lower in cells of *E. coli* K-12 grown in glucose-containing medium than in glycerol-containing medium. When in addition to glucose the growth medium contained also cAMP, the level of this enzyme increased to that observed in the glycerol-grown cells or even higher. This enzyme reduced the efficiency of exogenous cAMP in releasing catabolite repression.

Several observations (1-4) have led to the conclusion that catabolite repression is a result of the depletion of the intracellular cyclic 3';5' adenosine monophosphate (cAMP). The degradation of this nucleotide by cAMP-phosphodiesterase (5-7) is one of the mechanisms which could be suggested for the lowering of the cAMP level inside the cells. But this enzyme has been found only in some strains of *E. coli*, whereas it was present in small amounts or even completely absent in others (8). Since strains lacking cAMP-phosphodiesterase are sensitive to catabolite repression as well as strains containing high level of this enzyme, its role in lowering the intracellular concentration of cAMP to produce catabolite repression is uncertain (8). The rapid release of the intracellular cAMP to the medium, occurring when glucose is added (1), suggests that the low concentration of cAMP, present inside the cells under conditions of catabolite repression, results primarily from its excretion rather than from the action of the cAMP-splitting enzyme.

The present study suggests that, though the role of cAMP-phosphodiesterase in the depletion of the intracellular cAMP during catabolite repression is in

doubt, this enzyme, when it exists in a high level, affects the efficiency of exogenous cAMP in releasing catabolite repression. It also shows that this enzyme is repressed during the incubation of the cells with glucose, and that this repression is prevented by addition of cAMP.

MATERIALS AND METHODS: The strains used here were E. coli K-12 Hfr.H. requiring thiamin, and E. coli B. They were grown up to the logarithmic phase of growth in a medium, described previously (9), to which glycerol (0.2% w/v), or glucose (1% w/v) were added as carbon source.

For crude extract preparation, the cells were harvested by cold centrifugation, washed twice with Tris-HCl buffer (40 mM, pH 8.45) and resuspended in this buffer, with a turbidity of 0.7-0.9 O.D. (at 540 mμ). After adding 3 mM EDTA, 200 μg/ml lysosyme (Calbiochem) and 50 μg/ml deoxyribonuclease (Worthington Biochemicals Corp.) the suspension was vigorously agitated at 37°C until the turbidity stopped to decline. The lysate was centrifuged for 15 minutes at 36,000 g and the clear supernatant was used for the assay of cAMP-phosphodiesterase. In some experiments the cells were washed and lysed in the presence of 100 μg/ml chloramphenicol, in order to prevent any possible synthesis of cAMP-phosphodiesterase during these processes. Mg^{++} (50 mM) was added to all extracts.

The hydrolysis of cAMP to 5'-AMP by this enzyme was estimated by measuring the inorganic phosphate (Pi) liberated from 5'-AMP by the action of 5'-nucleotidase (British Drug Houses). Before using it for the assay, the crude extract was incubated with 5'-nucleotidase for at least 30 minutes at 37°C, in order to liberate Pi from all the sources available in the extract. The amount of 5'-nucleotidase was adjusted so that its final concentration in the reaction mixture was 0.1 mg/ml. The reaction was started by adding the mixture of the extract with 5'-nucleotidase to test tubes containing cAMP dissolved in Tris-HCl buffer, giving a final concentration of 1 mM. The total volume of the reaction mixture was 1 ml. The reaction was terminated by adding 0.1 ml 55% (w/v) TCA. Pi was estimated by the method described by Sumner (10), modified for samples of 0.5 ml. The enzyme activity was estimated by the

difference in the amount of Pi detected in reaction mixtures to which TCA was added after a measured time of incubation at 37°C, and the amount of Pi in parallel mixtures to which TCA was added at zero time of incubation. Each assay was performed in triplicate, the average of which was used for these calculations. The amount of Pi liberated by a direct action of 5'-nucleotidase on cAMP was very small, and was neglected in routine assays. The rate of cAMP degradation was found in control experiments to be linear only for the first 18-20 minutes. For this reason the assays of cAMP-phosphodiesterase were performed by incubating the reaction mixtures for 15-17 minutes.

Protein concentration of the extracts was assayed by the method of Lowry et al. (11).

In experiments with live organism we used exponentially growing cells which were washed once with cold medium lacking any carbon source, and resuspended in this medium with a turbidity adjusted to 0.12 O.D.

Induction and assay of β-galactosidase were as described previously (9).

Total protein synthesis, in live cells, was followed by the incorporation of ^{14}C-L-leucine as described elsewhere (12).

RESULTS AND DISCUSSION: Brana and Chytil (6) reported that cAMP-phosphodiesterase from E. coli was insensitive to the inhibitory effect of methyl

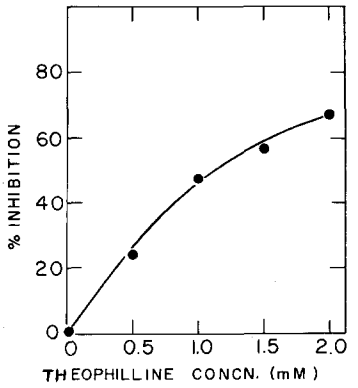

Fig.1. Inhibition of cAMP-phosphodiesterase by theophilline. The reaction was started by adding 0.9 ml of a preincubated mixture of extract of E. coli K-12 and 5'-nucleotidase into test tubes containing 0.05 ml cAMP (final concentration 1 mM) and 0.05 ml of various concentrations of theophilline.

xanthin derivatives observed in cAMP-phosphodiesterase from animal sources (13). But this was based on experiments using caffeine, the weakest inhibitor among the methyl xanthine derivatives, even for the enzyme from animal sources (13). We tested the effect of theophilline, and as can be seen in Fig. 1, the enzyme in the extract of E. coli K-12 was inhibited by this compound as well. A concentration of 1 mM of theophilline inhibited about 50% of the enzyme activity. We used this inhibitor to clarify whether cAMP-phosphodiesterase had an effect on the efficiency of exogenous cAMP. Cells of E. coli K-12 pregrown in glycerol-containing medium were induced to form β-galactosidase and allowed to incorporate ^{14}C-leucine in glucose-containing medium, and the effect of theophilline (1 mM) on the differential rate of β-galactosidase formation was tested in the presence and the absence of cAMP (3 mM). In a control experiment this concentration of theophilline was found to inhibit an average

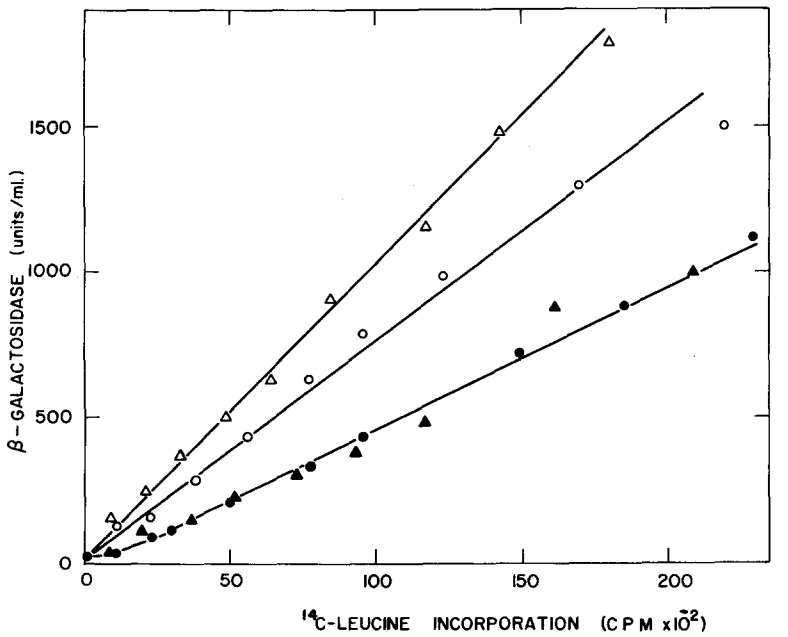

Fig.2. Effect of theophilline on differential rate of β-galactosidase synthesis in presence and absence of cAMP in glycerol-pregrown E. coli K-12. The glycerol-pregrown cells were induced to form β-galactosidase and to incorporate ^{14}C-leucine in glucose containing medium. At time zero, the culture was divided into four equal portions, receiving (●—●) no further additions, (▲—▲) 1 mM theophilline, (o—o) 3 mM cAMP and (Δ—Δ) 1 mM theophilline + 3 mM cAMP. Samples for β-galactosidase and ^{14}C-leucine incorporation assays were taken each 10 min.

of 12% of β-galactosidase activity; all the assays of β-galactosidase activity in cultures containing theophilline were therefore corrected accordingly. The results, presented in Fig.2, show that theophilline almost doubled the efficiency of the exogenous cAMP in stimulating the differential rate of β-galactosidase synthesis, whereas in the absence of the exogenous cAMP, it had no effect.

In agreement with other reports (8), we found an insignificant hydrolysis of cAMP by extracts of E. coli B. In order to verify that the effect of theophilline observed in E. coli K-12 was due to the inhibition of cAMP-phosphodiesterase, we tested its effect in E. coli B. Fig. 3 shows that in this strain, theophilline had no effect on the differential rate of β-galactosidase synthesis, irrespective of whether cAMP was present or not. It shows also that the exogenous cAMP approximately doubled the differential rate of β-galactosidase formation in this organism, while in E. coli K-12, such an efficiency

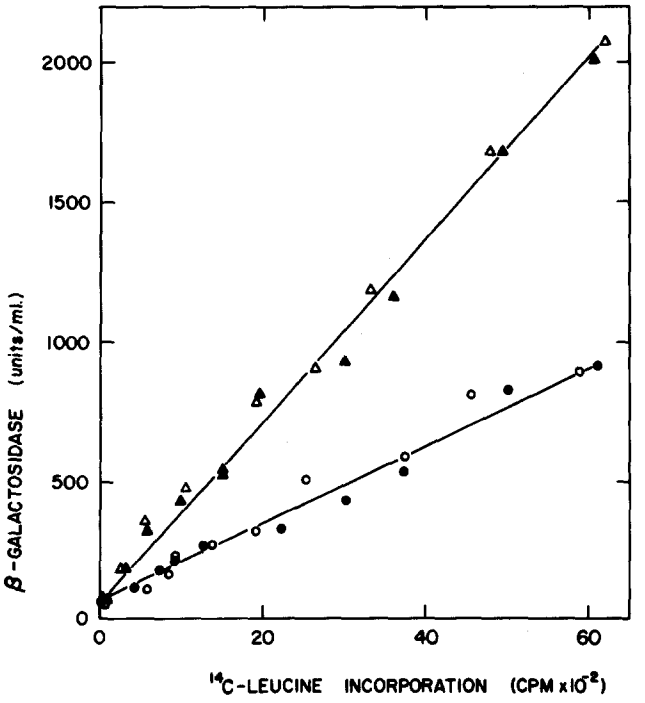

Fig.3. Effect of theophilline on differential rate of β-galactosidase synthesis in presence and absence of cAMP in glycerol-pregrown E. coli B. Experimental details are the same as for Fig.2. Symbols are: (●—●) no additions, (o—o) 1 mM theophilline, (▲—▲) 3 mM cAMP, (△—△) theophilline + cAMP.

of the exogenous cAMP was achieved only with the aid of theophilline (see Fig.2) These two experiments indicated that cAMP-phosphodiesterase indeed reduced the efficiency of the exogenous cAMP in stimulating β-galactosidase synthesis.

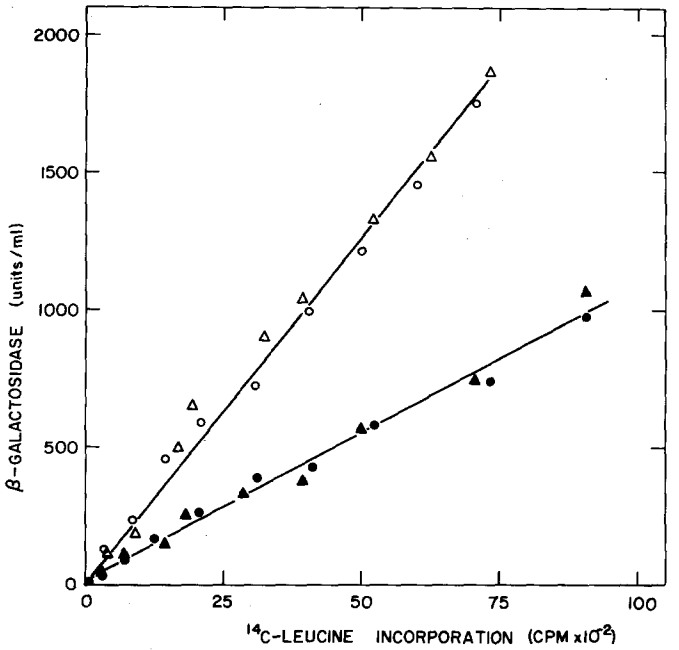

Fig.4. Effect of theophilline on differential rate of β-galactosidase synthesis in presence and absence of cAMP in glucose-pregrown E. coli K-12. Experimental details and symbols are the same as for Fig.2.

The experiment presented in Fig.4 shows that in E. coli K-12 pregrown in medium containing glucose instead of glycerol, theophilline had no effect on β-galactosidase synthesis with or without exogenous cAMP. These results suggest that, in contrast to glycerol-pregrown cells of E. coli K-12, glucose-pregrown cells of this organism contained cAMP-phosphodiesterase in much smaller amounts, if at all. This was strongly supported by comparison of the specific activity of cAMP-phosphodiesterase (expressed as mμmole Pi liberated per minute per mg extract protein) in extracts of E. coli K-12 cells pregrown in glycerol-containing medium, and of those pregrown in glucose-containing medium. Table 1 summarizes the results of 5 independent experiments, and it can be seen that the specific activity of cAMP-phosphodiesterase in extracts

TABLE 1

Comparision of specific activity of cAMP-phosphodiesterase in extracts of *E. coli* K-12 cells grown in glycerol, glucose and glucose + cAMP

Cells grown on:	% Specific activity
Glycerol	100
Glucose	38.2 ± 5.1
Glucose + cAMP	117.3 ± 13.2

Exponentially growing cells were harvested and washed with Tris buffer containing 100 µg/ml chloramphenicol, resuspended in this buffer and lysed as described in Materials and Methods. Specific activity of cAMP-phosphodiesterase in the lysate was determined as mµmole inorganic phosphate liberated per minute per mg of extract-protein. The results are given as percent (with standard deviations), taking the specific activity of glycerol-grown cell extract as 100%.

of glucose pregrown cells was 38.2 ± 5.1% of that observed in extracts of glycerol-pregrown cells. A mixture of 1:1 (v/v) of these two types of extracts showed an activity approximately equal to the arithmetic average of the separate activities of the two extracts indicating that the low activity in the extract of glucose-pregrown cells did not result from the presence of an inhibitor in the extract, but from a smaller amount of cAMP-phosphodiesterase. In 3 independent experiments (Table 1) we also determined the specific activity of this enzyme in extracts of cells pregrown in medium containing glucose and cAMP (3 mM), and found that cAMP restored the enzyme content to a level even higher than that of glycerol-pregrown cells. These results suggest one of two alternatives: a) that cAMP-phosphodiesterase is a catabolite repressible enzyme, so that its formation is repressed by glucose and restored by exogenous cAMP, or b) that it is an inducible enzyme, and cAMP is its inducer and that the repression of its synthesis by glucose results from the removal of its inducer, whereas exogenous cAMP does the reverse.

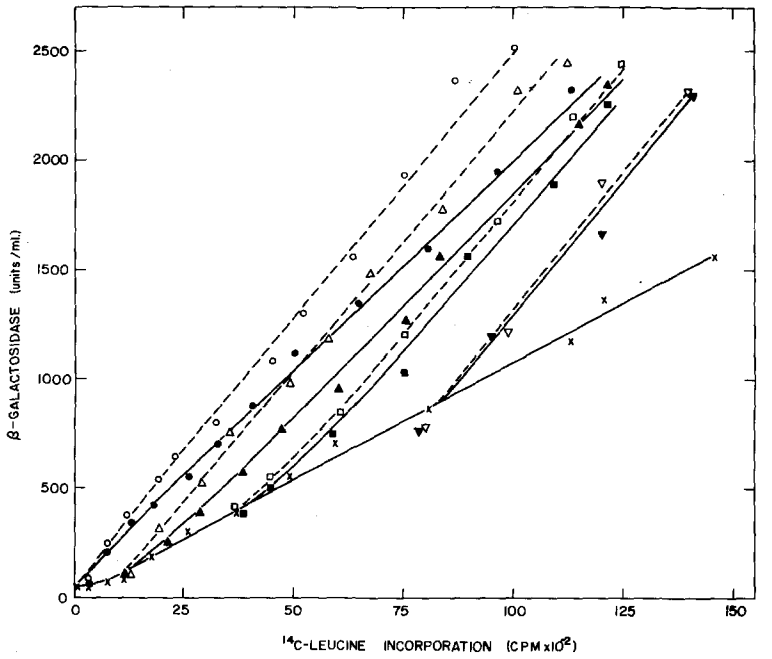

Fig.5. Effect of cAMP and theophilline added at various times on differential rate of β-galactosidase synthesis in glycerol-pregrown E. coli K-12. Glycerol-pregrown cells of E. coli K-12 were induced to form β-galactosidase and to incorporate ^{14}C-leucine in glucose-containing medium. At zero time the culture was divided into nine equal portions. One of them (x—x) serving as control received no further additions. The others received (●—●) cAMP and (o---o) cAMP + theophilline at 0 min; (▲—▲) cAMP and (Δ---Δ) cAMP + theophilline at 30 min; (■—■) cAMP and (□---□) cAMP + theophilline at 60 min; (▼—▼) cAMP and (▽---▽) cAMP + theophilline at 90 min. Samples for β-galactosidase and ^{14}C-leucine incorporation assays were taken each 10 min.

In the experiment described in Fig.5, glycerol-pregrown cells of E. coli K-12 were induced to form β-galactosidase in glucose-containing medium. At zero time the culture was divided into several portions, each receiving cAMP (3 mM) with or without theophilline (1 mM) at a different time. It was found that the later cAMP was added, the higher was the differential rate of β-galactosidase formation. This increasing effect of cAMP was not detected when it was added together with theophilline nor when it was added to E. coli B or E. coli K-12 which had been pregrown in a medium containing glucose instead of glycerol. This indicates that the time-dependent character of the cAMP effect in glycerol-pregrown cells of E. coli K-12 is a result of the repression of cAMP-phosphodiesterase during incubation of such cells with glucose.

REFERENCES

1. Makman, R.S. and Sutherland, E.W., J. Biol. Chem., 240: 1309 (1965).
2. Perlman, R.L. and Pastan, I., Biochem.Biophys.Res.Commun., 30:656 (1968).
3. De Crombrugghe, B., Perlman, R.L., Varmus, H.E. and Pastan, I., J.Biol.Chem., 244:5828 (1969).
4. Ulman, A. and Monod, J., Fed.Europ.Biochem.Soc.Letters, 2:57 (1968).
5. Monard, D., Janeček, J. and Rikenberg, H.V., Biochem.Biophys.Res.Commun., 35:584 (1969).
6. Brana, H. and Chytil, F., Folia Microbiol., 11:43 (1966).
7. Neu, H., J.Bacteriol. 95:1732 (1968).
8. Pastan, I. and Perlman, R., Science, 169:339 (1970).
9. Aboud, M. and Burger, M., Biochem.Biophys.Res.Commun., 38:1023 (1970).
10. Sumner, J.B., Science, 100:413 (1944).
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., J.Biol.Chem., 193:265 (1951).
12. Aboud, M. and Burger, M., Biochem.J. in press.
13. Butcher, R.W. and Sutherland, E.W., J.Biol.Chem., 237:1244 (1962).