Adenosine 3':5'-Cyclic Monophosphate and Catabolite Repression in *Escherichia coli*

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1. Both permanent and transient catabolite repression of β -galactosidase synthesis in *Escherichia coli* are abolished by 5 mM-3':5'-cyclic-AMP when elicited by glucose, but not when caused by a mixture of glucose, glucose 6-phosphate, gluconate and casein hydrolysate (casamino acids). 2. Glucose uptake is slightly increased by 3':5'-cyclic-AMP. 3. No significant effects of the nucleotide were found on the synthesis of protein and RNA, either in exponential growth on one substrate, or during a growth shift from glycerol to glycerol plus glucose. 4. Marked changes in the soluble-protein profiles of cells growing in glycerol and glucose were caused by the presence of 3':5'-cyclic-AMP. 5. Measurements of $^{14}\text{CO}_2$ release from specifically-labelled glucose showed that 3':5'-cyclic-AMP greatly stimulated glycolytic activity while having a minor depressing effect on the metabolic flow through the pentose phosphate cycle. 6. The concentrations of several metabolic intermediates, particularly fructose 1,6-diphosphate, were greatly affected by the presence of 3':5'-cyclic-AMP. 7. Several metabolites partially relieved glucose repression of β -galactosidase synthesis in EDTA-treated cells; three out of five of these metabolites reversed the effect more effectively than did 3':5'-cyclic-AMP. 8. The evidence for and against a direct role for 3':5'-cyclic-AMP is discussed. It is concluded that the evidence for indirect action is at least as strong as that for direct action.

Recent reports (Perlman & Pastan, 1968*a,b*; Pastan & Perlman, 1968; Jacquet & Kepes, 1969; Ullmann & Monod, 1968) have shown that catabolite repression of β -galactosidase synthesis in *Escherichia coli* is prevented by the presence of 1-5 mM-3':5'-cyclic-AMP. It was suggested that relief of repression resulted from a direct involvement of 3':5'-cyclic-AMP on either the transcription or the translation of the lactose genes.

Goldenbaum & Dobrogosz (1968) found that repression by glucose was indeed blocked, but that glucose 6-phosphate and mannitol remained effective repressing substrates in the presence of 1 mM-3':5'-cyclic-AMP. They concluded that the action of the nucleotide was on the formation of glucose 6-phosphate from glucose, and not directly on the synthesis of β -galactosidase *per se*. K. Paigen & B. Williams (personal communication) suggested that 3':5'-cyclic-AMP either may be the effector for catabolite repression, or may affect an enzyme involved in effector mechanism.

We have studied the effect of 3':5'-cyclic-AMP on several aspects of the physiology of *E. coli* with a view to resolving the question of its direct or

indirect action. Our results have indicated that 3':5'-cyclic-AMP has widespread effects on carbohydrate metabolism in this bacterium. It remains a possibility that 3':5'-cyclic-AMP acts directly in catabolite repression, but there is ample reason for concluding that this is probably not the case, and that relief of repression results from secondary effects on intermediary cell metabolism.

EXPERIMENTAL

Bacterial strains. The following strains of *Escherichia coli* were used in this study; their relevant genotypes and sources are indicated: 300 U ($i^+o^+z^+y^-$) from J. Monod; CA8001 ($i_L^+p_L^-o^+z^+y^+$) and XA7010 ($F^-i_{del}^+o_{del}^+z_{del}^+y^+$) from J. R. Beckwith. An additional strain (VP19) was constructed by inserting the episome $F'pro_A^+lac^+$ into strain XA7010, using selection on lactose-minimal medium.

Growth conditions. Medium 63, supplemented with the desired carbon source (0.2%, w/v) was used as the standard minimal medium. Cultures were shaken at 37°C, and growth was followed by measuring E_{650} (Moses & Prevost, 1966).

EDTA-treated cells. Cells growing exponentially in minimal medium were collected on a membrane filter,

washed, and resuspended in 0.12M-tris-HCl buffer, pH 7.6; the temperature was maintained at 37°C throughout these manipulations. EDTA (1mM) was then added; 1 min later the cells were diluted with 9 vol. of medium containing the desired carbon source (Jacquet & Kepes, 1969).

Enzyme induction and assay. The synthesis of β -galactosidase was induced with 0.5 or 1mM-IPTG.* Samples for measurement of the enzyme were handled as described by Palmer & Moses (1968), modified by the replacement of toluene treatment by hexadecyltrimethylammonium bromide (22 μ g/ml) during the enzyme assay (Tyler & Magasanik, 1969).

Protein and RNA synthesis. These were measured by the accumulation of trichloroacetic acid-precipitable radioactivity after the addition of L-[G- 14 C]phenylalanine and [3 H]uracil to the cultures. Preparation of the samples for radioactivity measurements used the techniques described by Moses & Prevost (1966).

Glucose uptake. This was measured radiochemically by the removal of 14 C from the medium after the addition of [G- 14 C]glucose to the culture (Prevost & Moses, 1967).

Labelling of intermediary metabolites. Cells growing in low-phosphate medium (Prevost & Moses, 1967) were labelled by incubation with $\text{KH}_2^{32}\text{PO}_4$. Samples of the culture were taken into 4 vol. of methanol 10, 25, 40 and 60 min after the introduction of ^{32}P . Portions of the cell suspension in methanol were analysed by paper chromatography (Prevost & Moses, 1967), and radioactivity was determined with an automatic chromatogram spot counter (Moses & Lonberg-Holm, 1963).

Release of respiratory CO_2 . The release of $^{14}\text{CO}_2$ from cells supplied with [1- 14 C]glucose or [6- 14 C]glucose was measured by a simplified adaptation of the radiorespirometric method (Wang *et al.* 1958). Cells were grown in medium 63 containing glucose, and adjusted with HCl to pH 6.2. Samples (10 ml) of the cultures during exponential growth were placed in 25 ml Erlenmeyer flasks which were agitated on a reciprocal shaker at 37°C. The flasks were flushed with air at about 20 ml/min per flask. The stream of effluent air was led through a fine tube into a bubbler tube (1.2 cm internal diam.) containing 5 ml of NCS Solubilizing Reagent model 190620 for absorption of CO_2 . After addition of labelled glucose to the cultures, $^{14}\text{CO}_2$ was collected for 15 min intervals from each flask. Portions (4 ml) of the NCS Reagent containing $^{14}\text{CO}_2$ were mixed with 14 ml of scintillation fluid [2,5-diphenyloxazole, 4.5 g/l; 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 0.56 g/l; dissolved in toluene], and ^{14}C was measured in a Packard Tri-Carb Scintillation Counter, with external standardization. A preliminary experiment showed that a second trap in series with the first was superfluous; at least 99.9% of the $^{14}\text{CO}_2$ in the effluent stream was trapped in the first tube.

Soluble-protein profiles. Soluble-protein profiles of cells growing in various media were determined by electrophoresis on polyacrylamide gels (Moses & Wild, 1969). Typically, 20 ml of cell culture, containing about 90 μ g of bacterial protein/ml, was mixed with 1 ml of chloramphenicol (1 mg/ml) and chilled to 0°C. The cells were

harvested by centrifugation and washed in a buffer free from bivalent metal cations. They were lysed with EDTA and lysozyme as described by Godson (1967); the final volume of the lysed cell preparation was 0.14 ml. Cell debris, ribosomes, etc. were removed by centrifugation for 2 h at about 45000g. Solid sucrose and Bromothymol Blue were added to each protein solution; samples (10–25 μ l) were then subjected to electrophoresis in slabs of polyacrylamide gel. The protein bands were subsequently stained with Amido Black 10B. After electrophoretic removal of excess of stain the colour intensities of the bands were measured with a Gelscan scanner connected to a Servogor recorder (Gelman Instrument Co., Ann Arbor, Mich., U.S.A.).

Chemicals and radiochemicals. Labelled materials were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. NCS Solubilizing Reagent was from Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A. Other chemicals were obtained as follows: lysozyme, PRPP, fructose 1,6-diphosphate, 3':5'-cyclic-AMP, ONPG, IPTG, glucose 6-phosphate, NADP $^+$, NADPH, NAD $^+$ and NADH were from Calbiochem, Los Angeles, Calif., U.S.A.; ribose 5-phosphate was from Sigma Chemical Co., St Louis, Mo., U.S.A.; chloramphenicol was from Parke, Davis and Co., Detroit, Mich., U.S.A.; hexadecyltrimethylammonium bromide was from Matheson, Coleman and Bell, Cincinnati, Ohio, U.S.A.; Cyanogum-41 was from E. C. Apparatus Corp., Philadelphia, Pa., U.S.A.; Amido Black 10B was from E. Merck A.-G., Darmstadt, Germany.

RESULTS

Effect of 3':5'-cyclic-AMP on β -galactosidase synthesis. β -Galactosidase synthesis in wild-type cells growing in glycerol-minimal medium was stimulated by 5mM-3':5'-cyclic-AMP (Fig. 1). Neither transient nor permanent repression was elicited by the addition of 10mM-glucose.

If more severe repression was caused by the simultaneous addition of several repressing components, the protection afforded by 3':5'-cyclic-AMP was incomplete, in confirmation of the results reported by Goldenbaum & Dobrogosz (1968). Supplementing the medium with glucose (10mM) plus glucose 6-phosphate (10mM) plus sodium gluconate (10mM) plus casein hydrolysate (1 mg/ml) resulted in a degree of repression in wild-type cells greater than could be overcome by 5mM-3':5'-cyclic-AMP (Fig. 2). It should be noted, nevertheless, that in the presence of 3':5'-cyclic-AMP, repression was slow to develop. It was only just beginning to become significant by the time recovery from transient repression was observed in the absence of the nucleotide. Transient repression was thus completely abolished in the presence of 3':5'-cyclic-AMP, though permanent repression was nearly as pronounced as in its absence.

Repression by the mixed substrates was less marked in the presence of 20mM-3':5'-cyclic-AMP. The relative differential rates of β -galactosidase synthesis in glycerol medium were as follows:

* Abbreviations: IPTG, isopropyl β -D-thiogalactopyranoside; ONPG, o-nitrophenyl galactopyranoside; PRPP, 5-phosphorylribose 1-pyrophosphate.

Fig. 1. Effect of 3':5'-cyclic-AMP on glucose repression of β -galactosidase synthesis in strain VP19 growing in glycerol-minimal medium and induced with 0.5 mM-IPTG. Glucose (10 mM) was added as shown by the arrows. Mass-doubling times before and after glucose addition were 90 min and 79 min respectively in each case. In the control, transient repression lasted for 35 min. ●, Control; ○, plus 5 mM-3':5'-cyclic-AMP.

Fig. 2. Effect of 3':5'-cyclic-AMP on repression by several substrates of β -galactosidase synthesis in strain VP19 growing in glycerol-minimal medium and induced with 1 mM-IPTG. The arrow indicates the simultaneous addition of glucose (10 mM), glucose 6-phosphate (10 mM), sodium gluconate (10 mM) and casamino acids (1 mg/ml). Mass-doubling times before and after additions were 92 min and 47 min respectively in each case. In the control, transient repression lasted for 17 min. ●, Control; ○, plus 5 mM-3':5'-cyclic-AMP.

no additions, 1.00; plus mixed substrates, 0.18; plus mixed substrates with 5 mM-3':5'-cyclic-AMP, 0.27; plus mixed substrates with 20 mM-3':5'-cyclic-AMP, 0.66.

Silverstone, Magasanik, Reznikoff, Miller & Beckwith (1969) found that strain CA 8001, carrying the L1 deletion of the *lac* promoter gene, was unaffected by 3':5'-cyclic-AMP and was not repressed by glucose 6-phosphate (concentration not stated). We have found the behaviour of this strain to be anomalous. In some experiments, the rate of β -galactosidase synthesis was increased by 3':5'-cyclic-AMP in glycerol-grown cells. Further, the induced rate of β -galactosidase synthesis was increased about sixfold by the addition of the mixed substrates described above; this increase was less pronounced in the presence of 3':5'-cyclic-AMP (Fig. 3). The growth characteristics of strain CA 8001 are typical both in glycerol-minimal medium and in the highly supplemented medium. We do not understand the behaviour of this strain, and are unable to assess the role of the promoter gene in catabolite repression as it has been proposed by Silverstone *et al.* (1969).

Effect of 3':5'-cyclic-AMP on glucose uptake. The amount of glucose removed from the medium per unit increase of cell mass was increased about 15% by the presence of the nucleotide.

Effect of 3':5'-cyclic-AMP on cell growth and on the synthesis of RNA and protein. No consistently significant effects of the nucleotide have been found on either the mass doubling times or on the rates of incorporation of L-[G- 14 C]phenylalanine or [3 H]-uracil into the macromolecules (Fig. 4).

Effect of 3':5'-cyclic-AMP on soluble protein profiles. de Crombrughe, Perlman, Varmus & Pastan (1969) have found that the synthesis of several inducible enzymes is increased by 3':5'-cyclic-AMP. An expected consequence of this is that the relative proportions of various proteins in growing cells would be altered by the presence of the nucleotide.

The soluble-protein profiles of cells growing in glycerol, glucose, or glucose plus glycerol, with and without 3':5'-cyclic-AMP, were investigated. From the profiles shown in Fig. 5 it is clear that the growth substrate was responsible for many characteristics of the pattern; similar observations have

Fig. 3. Effect of 3':5'-cyclic-AMP on stimulation of β -galactosidase synthesis in strain CA8001 growing in glycerol-minimal medium and induced with 1 mM-IPTG. Supplementation was as for Fig. 2. Mass-doubling times before and after additions: control, 98 min and 37 min respectively; plus 3':5'-cyclic-AMP, 94 min and 48 min respectively. ●, Control; ○, plus 5 mM-3':5'-cyclic-AMP.

been made by Moses & Wild (1969). Consistent changes in the patterns resulted from the presence of 3':5'-cyclic-AMP in the growth medium (Fig. 5). Prominent among these were the enhancement of peaks M and P in all growth media tested, and of peak Y in glucose-containing media; peak AG was decreased by the presence of 3':5'-cyclic-AMP in glycerol-minimal medium. The effects on the balance of protein synthesis exerted by 3':5'-cyclic-AMP were thus sufficiently distinct to be observed even in these complex profiles.

These changes in the proportions of soluble proteins would be expected to result in a redistribution in the balance of metabolic activities. The detailed consequences of this cannot at the moment be assessed, but it is likely to cause changes in the intracellular concentrations of a number of intermediary metabolites, some of which may be effector molecules. We conclude that the actions of 3':5'-cyclic-AMP are widespread, and that caution must be exercised before ascribing any of its effects to direct interaction.

Effect of 3':5'-cyclic-AMP on $^{14}\text{CO}_2$ release from labelled glucose. It is known that 3':5'-cyclic-AMP activates phosphofructokinase in some animal tissues (Stone & Mansour, 1967). If this also occurs in *E. coli*, metabolism of glucose through glycolysis would probably be favoured in comparison with

Fig. 4. Effect of 3':5'-cyclic-AMP on macromolecule synthesis during a growth shift. Strain VP19, in glycerol-minimal medium, was supplied with L-[G- ^{14}C]phenylalanine and [^3H]uracil. Samples were taken at intervals for determination of trichloroacetic acid-precipitable radioactivity. Glucose (10 mM) was added as shown by the arrow. (a) [^3H]Uracil incorporation; (b) L-[G- ^{14}C]phenylalanine incorporation. ●, Control; ○, plus 5 mM-3':5'-cyclic-AMP.

Fig. 5. Soluble-protein profiles of strain VP19 in minimal media supplemented as follows: (a) glycerol; (b) glycerol plus 5 mM-3':5'-cyclic-AMP; (c) glucose; (d) glucose plus 5 mM-3':5'-cyclic-AMP; (e) glycerol plus glucose; (f) glycerol plus glucose plus 5 mM-3':5'-cyclic-AMP.

oxidation via the pentose phosphate cycle. The specific rates of release of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{glucose}$ and $[6\text{-}^{14}\text{C}]\text{glucose}$ may be used to study the relative utilization of the two pathways, since the Entner-Doudoroff pathway does not operate in *E. coli* (Wang *et al.* 1958).

Strain VP19 was grown exponentially in glucose-minimal medium adjusted to pH 6.2. Of four parallel cultures, two received 5 mM-3':5'-

cyclic-AMP 90 min before the addition of labelled glucose; no addition was made to the other pair. At the start of the experimental period one flask from each pair was supplied with 4 μCi of $[1\text{-}^{14}\text{C}]\text{glucose}$ and the other with a similar quantity of $[6\text{-}^{14}\text{C}]\text{glucose}$. The resulting specific radioactivity was 0.46 $\mu\text{Ci/mol}$ of glucose in each case. Respiratory $^{14}\text{CO}_2$ was trapped for successive 15 min intervals over a total period of 3 h. Growth,

measured by E_{650} , was followed throughout in parallel cultures; cultures with 3':5'-cyclic-AMP grew more slowly than those without (0.74 doublings/h and 0.83 doublings/h, respectively).

Fig. 6 shows that early in the experiment 3':5'-cyclic-AMP markedly stimulated the release of $^{14}\text{CO}_2$ from [6- ^{14}C]glucose, and, to a lesser extent, decreased its rate of evolution from [1- ^{14}C]glucose.

Fig. 6. Effect of 3':5'-cyclic-AMP on release of $^{14}\text{CO}_2$ from labelled glucose. Strain VP19, growing in glucose-minimal medium, received [1- ^{14}C]glucose or [6- ^{14}C]glucose. $^{14}\text{CO}_2$ was trapped for a series of 15 min intervals; growth was measured by E_{650} . (a) Control; (b) plus 5mm-3':5'-cyclic-AMP. ●, ○, $^{14}\text{CO}_2$ from [1- ^{14}C]glucose; ▲, △, $^{14}\text{CO}_2$ from [6- ^{14}C]glucose.

Both effects decreased with time; some 4.5 h after the introduction of the nucleotide the pattern of respiratory CO_2 release in the cultures with 3':5'-cyclic-AMP was approaching that of the untreated ones. In the absence of the nucleotide the proportion of CO_2 released as a result of glycolytic activity increased with increasing cell density in the culture. This probably reflected an increasing limitation of O_2 availability as the culture became more dense.

The effect of 3':5'-cyclic-AMP on glucose dissimilation was mostly to increase the flow through glycolysis, though some decrease in metabolism through the pentose phosphate pathway was also observed. The total release of $^{14}\text{CO}_2$ was higher in the presence of 3':5'-cyclic-AMP, a result consistent with the finding that the net consumption of glucose was also increased by this substance. The presence of the nucleotide in cultures of growing cells thus appeared to shift the balance of glucose metabolism away from the pentose phosphate cycle and towards glycolysis. Such a result is not unexpected in view of its known action in stimulating phosphofructokinase.

Effect of 3':5'-cyclic-AMP on concentrations of phosphorylated intermediary metabolites. The concentrations of a number of phosphorylated intermediates were measured at various periods after the

Table 1. Incorporation of [^{32}P] P_i into intermediary metabolites

Strain VP19 was grown exponentially in low-phosphate (0.75 mM- P_i) minimal medium containing glycerol or glucose, with or without 5 mM-3':5'-cyclic-AMP. $\text{KH}_2^{32}\text{PO}_4$ was added to give a specific radioactivity of 400 $\mu\text{Ci}/\mu\text{mol}$. The labelling in intermediary metabolites was determined after 1 h of incubation at 37°C, by using paper chromatography. Values are recorded as $10^{-3} \times \text{d.p.m.}/\mu\text{g}$ of bacterial protein.

Medium ...	$10^{-3} \times \text{Incorporation (d.p.m.}/\mu\text{g of protein)}$			
	Glycerol	Glycerol+ 3':5'-cyclic-AMP	Glucose	Glucose+ 3':5'-cyclic-AMP
UTP+UDP	104	40	128	64
UDP-glucose	48	52	62	74
ATP	42	54	84	80
ADP	22	22	25	22
PRPP	4.8	4.8	5.6	6.0
Glucose 6-phosphate	16	5.2	30	5.2
Fructose 6-phosphate	16	16	20	20
Fructose 1,6-diphosphate	0	74	0	96
α -Glycerophosphate	80	100	28	32
Dihydroxyacetone phosphate	8.0	6.8	8.0	8.0
NADP ⁺ +NADPH	10	15	13	15
Unidentified sugar phosphate	12	8.8	20	19
Unidentified compound 1	18	18	18	18
Unidentified compound 2	0	12	0	152
Unidentified compound 3	0	9.6	0	9.6
Unidentified compound 4*	11	11	11	11
Unidentified compound 5*	28	43	46	52
Unidentified compound 6*	16	16	16	16

* These are probably nucleoside monophosphates.

introduction of $\text{KH}_2^{32}\text{PO}_4$ to cells grown in low phosphate medium containing glycerol or glucose, with and without 3':5'-cyclic-AMP. Table 1 records the amounts of incorporated ^{32}P in 18 compounds after 1 h of labelling. The results are presented as d.p.m., and are to be used only to compare the relative degrees of labelling among metabolites.

The labelling of several substances varies with the carbon source; UTP+UDP, UDP glucose, ATP, glucose 6-phosphate, α -glycerophosphate and an unidentified mononucleotide were the most prominent of these. The extent of labelling of some substances also varied as a result of the presence of 3':5'-cyclic-AMP. Among these, label in UTP+UDP and glucose 6-phosphate was decreased by the nucleotide, while that in fructose 1,6-diphosphate and in two unidentified phosphorylated compounds was dramatically increased.

The effect of 3':5'-cyclic-AMP on ^{32}P incorporation into fructose 1,6-diphosphate is consistent with its presumed stimulatory action on phosphofructo-

kinase, and with its effect on $^{14}\text{CO}_2$ release from [6- ^{14}C]glucose. To judge from both the release of $^{14}\text{CO}_2$ from [1- ^{14}C]glucose, and the extent of ^{32}P incorporation into PRPP (Table 1), its effect directly on the activity of the pentose phosphate cycle was relatively minor.

Ability of several intermediary metabolites to reverse catabolite repression. Jacquet & Kepes (1969) showed that cells shocked with 1 mM-EDTA for 1 min and then returned to growth medium remained sensitive to catabolite repression by glucose, and to its reversal by 3':5'-cyclic-AMP. EDTA-treated cells are believed (Leive, 1965) to be permeable to substances which are not normally able to enter them. The ability of a number of metabolites to elicit or reverse catabolite repression was investigated in such cells.

Strain 300 U, maintained for 18 subcultures in glucose-nutrient broth medium, was used for these experiments. Cells were grown for several generations in glycerol-minimal medium, treated with

Table 2. *Effects of intermediary metabolites on the induced synthesis of β -galactosidase in EDTA-treated cells*

Strain 300 U, grown in glycerol-minimal medium, was treated with EDTA as described in the Experimental section. The cells were resuspended in minimal medium containing either glycerol (22 mM) or glucose (5 mM) as indicated. IPTG (1 mM) was added; 3 min later samples of the culture were mixed with an equal volume of the same medium containing the test substances (10 mM). Samples for β -galactosidase determination were removed periodically during the next 18 min. Results are expressed as enzyme synthesized in 18 min compared with the control value for that experiment.

Expt. no.	Carbohydrate in suspension medium	Metabolite	β -Galactosidase synthesized in 18 min
1	Glycerol	None	100
		3':5'-Cyclic-AMP	980
		Fructose 1,6-diphosphate	180
		3':5'-Cyclic-AMP+fructose 1,6-diphosphate	1110
2	Glycerol	None	100
		3':5'-Cyclic-AMP	224
		PRPP	344
		3':5'-Cyclic-AMP+PRPP	615
3	Glycerol	None	100
		Glucose	10
		Fructose 1,6-diphosphate	90
		PRPP	123
4	Glycerol	None	100
		Glucose	18
		NADP ⁺	96
		NADPH	111
5	Glucose	None	100
		3':5'-Cyclic-AMP	170
		Fructose 1,6-diphosphate	217
		PRPP	136
6	Glucose	None	100
		NADP ⁺	222
		NADPH	134
		Ribose 5-phosphate	197

EDTA, diluted into minimal medium containing either glycerol or glucose (see the Experimental section) and incubated at 37°C.

IPTG (1mM) was added, and 3min later four 2ml samples were simultaneously removed from the culture and immediately mixed with 2ml of the same medium containing IPTG (1mM), the test substance at a concentration of 10mM, plus L-[G-¹⁴C]phenylalanine and [³H]uracil. Samples for measurements of enzyme activity and acid-precipitable radioactivity were taken at intervals during the following 20min.

None of the test substances altered the rates of incorporation of the labelled precursors for the period of the experiments. Table 2 shows that in glycerol medium only glucose caused significant repression of β -galactosidase synthesis. All other compounds were either without effect, or increased enzyme synthesis. When two metabolites were present simultaneously, their effects were additive. In glucose medium, repression was alleviated by all six compounds that were tested; 3':5'-cyclic-AMP was not the most effective antagonist of glucose repression.

DISCUSSION

A direct or indirect role for 3':5'-cyclic-AMP. de Crombrughe *et al.* (1969) have presented the arguments supporting a direct-role for 3':5'-cyclic-AMP in the synthesis of inducible enzymes. Summarized briefly, these are: (a) in certain mutants of the phosphoenolpyruvate phosphotransferase system unable to grow on glucose, or to accumulate glucose or α -methyl glucoside, both substances repress β -galactosidase synthesis, and this repression is overcome by 3':5'-cyclic-AMP (Pastan & Perlman, 1969). (b) At a concentration of 3':5'-cyclic-AMP which effectively reverses catabolite repression by glucose, the uptake of glucose is little affected (Pastan & Perlman, 1969). (c) Chambers & Zubay (1969) have reported that 3':5'-cyclic-AMP stimulates β -galactosidase synthesis in a cell-free system. (d) High concentrations of the nucleotide reverse repression exerted by glucose 6-phosphate, and by glucose plus gluconate, as well as by glucose alone (Perlman, de Crombrughe & Pastan, 1969).

In view of the observations we report in the present paper, a number of comments are appropriate: (i) The concentrations of many metabolic intermediates are affected by the growth substrate (Prevost & Moses, 1967), and by the presence of 3':5'-cyclic-AMP, as we have shown above. Before mutants of the phosphoenolpyruvate phosphotransferase system can be fully understood in terms of catabolite repression responses, it is important to know more about them. This applies particularly to their concentrations of intermediary

metabolites, and how these may be altered by the presence in the medium of glucose and α -methyl glucoside, even if the latter are not metabolized. It is further necessary to know how completely blocked is the metabolism of these substances. Even with this information, the possibility would remain that the local concentrations of metabolites at the sites of interaction between effector molecules and their targets are significantly different from those in the cell as a whole. (ii) Although we confirm that glucose uptake is not markedly affected by 3':5'-cyclic-AMP, we have found that the oxidation pattern is greatly altered. (iii) The evidence given by Chambers & Zubay (1969) is perhaps the most suggestive of all in support of a direct role for 3':5'-cyclic-AMP. However, their cell-free system contains very many components, some of which may themselves play an indirect part in regulation. It is difficult to be sure, even in their system, that the nucleotide is acting directly as an effector. (iv) We have found that 3':5'-cyclic-AMP is unable to overcome repression produced simultaneously by several substrates.

We should also consider the evidence against a direct role for 3':5'-cyclic-AMP. A very considerable shift in the flow of glucose carbon is caused by its presence. The Embden-Meyerhof pathway becomes much more important than the pentose phosphate cycle. This is reflected both in the concentrations of metabolic intermediates, and in the pattern of release of ¹⁴CO₂ from specifically-labelled glucose. Both may readily be understood from the known ability of 3':5'-cyclic-AMP to activate phosphofructokinase. We observed some of the consequences of this activation, and probably there are others that have escaped our attention. Also, earlier studies (Prevost & Moses, 1967) suggested that pentose phosphate cycle activity was necessary at least for transient repression. One of the main consequences of treatment with 3':5'-cyclic-AMP is a decrease in the relative importance of the pentose phosphate cycle as a metabolic pathway. The inhibitory effects of high concentrations of 3':5'-cyclic-AMP on RNA synthesis (de Crombrughe *et al.* 1969) may be due to a decrease in the availability of pentose precursors for nucleotide synthesis as a result of impaired pentose phosphate cycle activity.

The changes in metabolite concentrations are likely to arise concurrently from two sources. The direct activation of phosphofructokinase, and perhaps other enzymes, will rapidly change metabolite concentrations. The regulation of many enzymes probably depends on such metabolites, and in time, the protein composition of the cell will undergo an appreciable rearrangement. One might expect that this, in turn, will have further effects on metabolite concentrations, until a new equi-

brium is reached. Our studies on the soluble-protein profiles have shown that marked changes in the protein composition result from the presence of 3':5'-cyclic-AMP.

Finally, we mention two more significant findings: 3':5'-cyclic-AMP has a restricted ability to reverse catabolite repression, and it is not the only substance that can do so. Of five other phosphorylated intermediates tested for their ability to relieve glucose repression in EDTA-treated cells, three were at least as effective as 3':5'-cyclic-AMP. They are, however, more difficult to study than the latter because of the difficulty with which some of them enter cells not previously rendered permeable with EDTA. No specific conclusion can readily be drawn from such a result unless it is that the metabolic interrelations are complex and require further investigation.

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REFERENCES

- Chambers, D. A. & Zubay, G. (1969). *Proc. natn. Acad. Sci. U.S.A.* **63**, 118.
- de Crombrughe, B., Perlman, R. L., Varmus, H. E. & Pastan, I. (1969). *J. biol. Chem.* **244**, 5828.
- Godson, G. N. (1967). In *Methods in Enzymology*, vol. **12**, p. 503. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Goldenbaum, P. E. & Dobrogosz, W. J. (1968). *Biochem. biophys. Res. Commun.* **33**, 828.
- Jacquet, M. & Kepes, A. (1969). *Biochem. biophys. Res. Commun.* **36**, 84.
- Leive, L. (1965). *Biochem. biophys. Res. Commun.* **18**, 13.
- Moses, V. & Lonberg-Holm, K. K. (1963). *Analyt. Biochem.* **5**, 11.
- Moses, V. & Prevost, C. (1966). *Biochem. J.* **100**, 336.
- Moses, V. & Wild, D. G. (1969). *Folia microbiol., Praha*, **14**, 305.
- Palmer, J. & Moses, V. (1968). *Biochem. J.* **106**, 339.
- Pastan, I. & Perlman, R. L. (1968). *Proc. natn. Acad. Sci. U.S.A.* **61**, 1336.
- Pastan, I. & Perlman, R. L. (1969). *J. biol. Chem.* **244**, 5836.
- Perlman, R. L., de Crombrughe, B. & Pastan, I. (1969). *Nature, Lond.*, **223**, 810.
- Perlman, R. L. & Pastan, I. (1968a). *Biochem. biophys. Res. Commun.* **30**, 656.
- Perlman, R. L. & Pastan, I. (1968b). *J. biol. Chem.* **243**, 5420.
- Prevost, C. & Moses, V. (1967). *Biochem. J.* **103**, 349.
- Silverstone, A. E., Magasanik, B., Reznikoff, W. S., Miller, J. H. & Beckwith, J. R. (1969). *Nature, Lond.*, **221**, 1012.
- Stone, D. B. & Mansour, T. E. (1967). *Molec. Pharmac.* **3**, 161.
- Tyler, B. & Magasanik, B. (1969). *J. Bact.* **97**, 550.
- Ullmann, A. & Monod, J. (1968). *FEBS Lett.* **2**, 57.
- Wang, C. H., Stern, I., Gilmour, C. M., Klungsoyr, S., Reed, D. J., Bialy, J. J., Christensen, B. E. & Cheldelin, V. H. (1958). *J. Bact.* **76**, 207.