

## Glucose Inhibition of Adenylate Cyclase in Intact Cells of *Escherichia coli* B

(glucose effect/catabolite repression)

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Communicated by Marshall Nirenberg, January 21, 1974

**ABSTRACT** Previous studies in *E. coli* B have demonstrated an inverse correlation between the presence of glucose in the medium and the accumulation of cyclic AMP in the medium. This observation could not be explained by the action of glucose as a repressor of adenylate cyclase (EC 4.6.1.1) synthesis, as a stabilizer of cyclic AMP phosphodiesterase (EC 3.1.4.17) activity, or as a direct inhibitor of adenylate cyclase activity in cell-free preparations. The recent development of an *in vivo* assay for adenylate cyclase has provided a basis for further exploring the inhibitory action of glucose in intact cells. With this assay it has been possible to show that, while glucose does not affect adenylate cyclase *in vitro*, it rapidly inhibits the enzyme activity in intact cells. Extensive metabolism of glucose is not required, since  $\alpha$ -methylglucoside also inhibits adenylate cyclase *in vivo*. When cells are grown on glucose as carbon source, some sugars (mannose, glucosamine) substitute for glucose as adenylate cyclase inhibitors while others (e.g., fructose) do not. Dose-response studies indicate that low concentrations of glucose lead to essentially complete inhibition of adenylate cyclase activity while only moderately decreasing intracellular cyclic AMP concentrations. The evidence presented suggests that the decreased cellular cyclic AMP levels resulting from glucose addition can be accounted for by inhibition of adenylate cyclase without any significant effect on cyclic AMP phosphodiesterase or the transport of cyclic AMP from the cells to the medium.

The thorough studies carried out primarily by Pastan and his collaborators (1) have established a key role for cyclic AMP (cAMP) in metabolic control in *Escherichia coli*. It has now been amply demonstrated that this cyclic nucleotide is required for transcription of the genes for several induced enzymes. These studies, together with the earlier report by Makman and Sutherland (2) on the glucose-dependent changes of cAMP levels in *E. coli*, have provided the necessary link in explaining the role of glucose in the phenomenon of catabolite repression. It has been suggested (1, 2) that glucose promotes the release of cAMP from cells and, in addition, inhibits the synthesis of the cyclic nucleotide.

Our previous study on the metabolism of cAMP in *E. coli* (3) indicated that glucose exercised inhibitory control over the total amount of cAMP in a culture. *In vitro* experiments could provide no support for explanations for this phenomenon based on repression of adenylate cyclase (EC 4.6.1.1) synthesis or activation of cAMP phosphodiesterase (EC 3.1.4.17) activity. Our recent description of a method for measuring cAMP synthesis in intact cells (4) has made it possible to examine the effect of glucose on adenylate cyclase *in vivo*. The present study demonstrates that, while glucose has no effect on adenylate cyclase in cell-free extracts, it is an effective inhibitor of the enzyme *in vivo*. An abstract of some of this work has been presented (5).

### METHODS

The conditions for growth of *E. coli* B and the methodology for assay of adenylate cyclase *in vivo* and of ATP have been described (4).

**cAMP Assays.** For determination of intracellular cAMP, aliquots (10 ml) of culture were filtered on Millipore membranes (HAWP 02500, HA 0.45  $\mu$ m; 25-mm diameter) and the trapped cells, with no washing, were immersed in 2 ml of 0.2 N HCOOH. The cell suspensions were immersed in a boiling-water bath for 5 min; then the Millipore filters were removed and the samples were lyophilized. The dried samples were dissolved in 2 ml of 50 mM acetate buffer (pH 4), centrifuged to remove debris, and assayed for cAMP by the Gilman procedure (6). For determination of extracellular cAMP, the culture fluid derived from Millipore filtration of the cells was collected, then immersed in a boiling H<sub>2</sub>O bath for 5 min. After the pH was adjusted to 4 with 1 N HCl, the samples were assayed for cAMP by the Gilman procedure (6). Various tests were performed to determine the amount of extracellular cAMP trapped on the filters containing the collected cells. Determinations of the weight of culture medium or radioactivity derived from added [<sup>3</sup>H]cAMP that adhered to a filter after filtration of 10 ml of culture medium indicated that about 0.07 ml of extracellular fluid was associated with the filters. Studies with <sup>3</sup>H<sub>2</sub>O indicated that the 10 ml of packed cells trapped an additional 0.01 ml of fluid. Therefore, each determination of intracellular cAMP was corrected for contamination by extracellular cAMP by subtracting an amount corresponding to 0.08 ml of the extracellular cAMP from the value obtained for the cells collected on a filter. We are indebted to Dr. Ira Pastan for pointing out the necessity for such a correction factor. The calculation of intracellular cAMP concentration requires a value for cell volume. For this purpose, we calibrated a spectrophotometer for  $A_{650}$  against cell number, determined by plate counts. The volume of a single cell was taken as  $10^{-12}$  cm<sup>3</sup>. An  $A_{650}$  value of 1 is equivalent to  $1.75 \times 10^9$  cells per ml.

***In Vitro* Adenylate Cyclase Assay.** The adenylate cyclase assay was modified from the previous procedure (3). Fifty milliliters of a stationary phase culture ( $A_{650} = 1.57$ ) was centrifuged, and the pelleted cells were resuspended in 2 ml of 10 mM Tris·HCl (pH 7.5). The cell suspension was sonicated in a test tube at peak power in a Raytheon Sonicator (10 kilocycles) for 30 min with cooling, then dialyzed for 2 hr against 10 mM Tris buffer (pH 7.5). The protein concentration of the dialyzed sonicate was 16 mg/ml. Assays were carried out on the sonicates without removal of the cell debris. Incubations

TABLE 1. Specificity for inhibition of adenylate cyclase *in vivo*

Additions	pmol of cAMP formed/2 min per 10 ml	% of control activity
<b>Exp. I*</b>		
None	828	—
Glucose	80	10
Gluconic acid	866	105
Glucuronic acid	674	81
$\alpha$ -Methylglucoside	84	10
<b>Exp. II†</b>		
None	1106	—
Glucose	49	4
Mannose	294	26
Glucosamine	284	25
Acetylglucosamine	359	32
Fructose	2501	226
Mannitol	1553	140
Glycerol	858	77
Lactose	836	75
Ribose	974	88

\* The conditions for this experiment were: 10-ml aliquots of stationary cells grown in minimal medium supplemented with 0.3% glucose were exposed to [ $^3$ H]adenosine for 1 min. At that point, the designated additions were made (final concentration = 1 mM) and a large excess of unlabeled adenosine (30  $\mu$ moles) was added. Incubation at 37° was continued for an additional 3 min before the reactions were terminated for assay of [ $^3$ H]cAMP.

† The designated compounds (final concentration = 3 mM) were added at the same time as [ $^3$ H]adenosine. Incubation at 37° was continued for 2 min, then the reactions were terminated. [ $^3$ H]cAMP was determined as described (4).

(0.5 ml) contained, in  $\mu$ moles: Tris·HCl, pH 7.5, 50; MgCl<sub>2</sub>, 10; ATP, 0.25; and enzyme, 0.2–0.4 mg of protein. Incubations (at 30°) were carried out for 0, 20, 40, and 60 min, then terminated by heating the tubes in a boiling H<sub>2</sub>O bath for 3 min. After removal of debris by centrifugation, the samples were assayed for cAMP, using a cAMP radioimmunoassay kit (Schwarz-Mann). The procedure described in the kit (7) was modified as described by Weinryb *et al.* (8). In this method, the antibody-cAMP complex was trapped on Millipore filters.

**In Vitro cAMP Phosphodiesterase Assay.** The enzyme was prepared from stationary phase cells of *E. coli* B grown in minimal medium (9) supplemented with 0.3% (w/v) glucose. Washed cells were sonicated for 30 min at 0° in a Raytheon sonicator at full power. Assays contained, in a total volume of 0.5 ml: Tris·HCl, pH 8.5, 10  $\mu$ moles; MgCl<sub>2</sub>, 0.1  $\mu$ mole; [ $^3$ H]cAMP, 0.25  $\mu$ moles containing about 450,000 cpm; snake venom, 0.05 mg. Reactions were initiated by the addition of 0.01–0.2 mg of enzyme protein and incubated at 37° for 15 min. Boiled enzyme was used as a control. Reactions were terminated by boiling, then were processed by diluting with 0.2 ml of H<sub>2</sub>O, applying the samples to columns (0.5  $\times$  4-cm) of Dowex-3  $\times$  4, 100–200 mesh, and developing with H<sub>2</sub>O. The first 2 ml of H<sub>2</sub>O was collected in a vial and counted with 15 ml of Triton-toluene-Liquiflor counting fluid. The use of Dowex-3 for collecting the adenosine formed in the coupled assay of cAMP phosphodiesterase and 5'-nucleotidase (snake venom) is advantageous. Adenosine is nonspecifically retarded by Dowex-1, less so by Dowex-2, but hardly at all by Dowex-3.

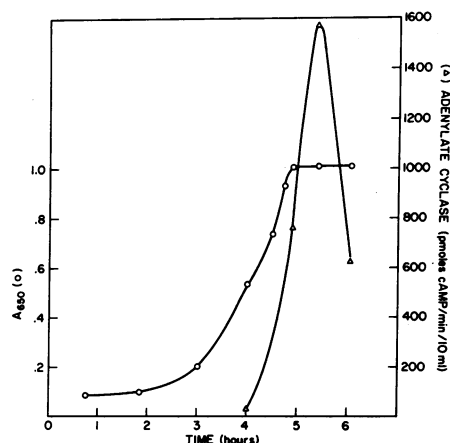


FIG. 1. Effect of growth stage on adenylate cyclase activity. A culture of *E. coli* B was grown in salts medium (9) supplemented with glucose (0.1%). Growth at 37° was monitored at 650 nm. At the designated times, 10-ml aliquots of the culture were pulse-labeled with [ $^3$ H]adenosine for the determination of adenylate cyclase *in vivo*, as described (4).

## RESULTS

**Growth Phase-Dependent Expression of Adenylate Cyclase Activity.** We recently described a method for measuring adenylate cyclase activity in intact cells. The procedure is based on pulse-labeling with [ $^3$ H]adenosine, followed by the monitoring of cellular ATP specific activity and the isolation of labeled cAMP (4). Our previous study (4) showed that there was substantially more detectable *in vivo* adenylate cyclase activity in stationary phase cells than in logarithmic phase cells. This was the basis for our conclusion that adenylate cyclase was under a regulatory control that was correlated with the disappearance of glucose from the culture medium. A more detailed study of the change of adenylate cyclase activity during the course of growth is depicted in Fig. 1. At logarithmic phase there was no detectable adenylate cyclase activity, but as soon as growth stopped due to glucose depletion from the medium, there was a measurable level of enzyme activity. Over the next 30 min, the adenylate cyclase activity approximately doubled. At 1 hr after termination of growth the adenylate cyclase activity decayed to the level characteristic of early stationary phase. Most of the experiments reported here were aimed at establishing that glucose inhibits adenylate cyclase in intact cells. For this reason, we performed tests under conditions where the enzyme was most active *in vivo*, between 1/2 and 1 hr after termination of growth.

**In Vivo Inhibition of Adenylate Cyclase by Glucose.** *E. coli* cells grown in medium supplemented with 0.3% glucose showed substantial *in vivo* adenylate cyclase activity when assayed at stationary phase. However, if glucose (1–3 mM) was included in the assay mixture, the activity was virtually abolished (Table 1). It was the primary purpose of this study to characterize the mechanism by which glucose lowered the amount of labeled cAMP made by intact cells from [ $^3$ H]adenosine. Since the inhibition by glucose was rapid, occurring during the course of the 2- to 3-min assay, it seemed unlikely that glucose was effecting a change in the amount of an enzyme involved in cAMP metabolism. The assay measures the [ $^3$ H]cAMP found in both the cells and the suspending medium. Therefore, the glucose inhibition seen under these

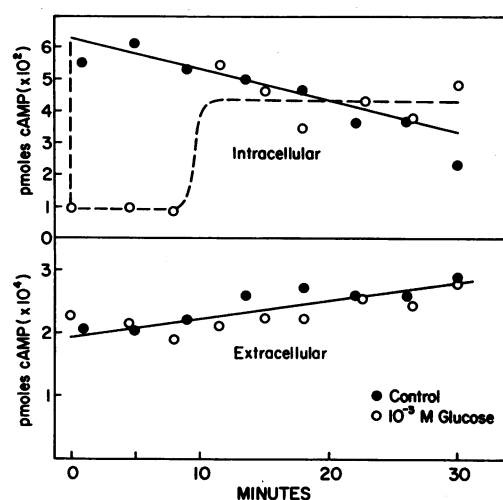


Fig. 2. Effect of glucose on intracellular and extracellular cAMP concentrations. A culture of *E. coli* B was grown in minimal salts medium (9) supplemented with glucose (0.1%). About 1 hr after the culture reached stationary phase ( $A_{650} = 0.74$ ), aliquots of the cell suspension were incubated with shaking in the presence and absence of glucose (1 mM). Aliquots (10 ml) were removed at the times shown and rapidly filtered. Intracellular and extracellular cAMP levels were determined. The data are presented as the total pmoles of cAMP in a 10-ml aliquot of culture suspension distributed in the cells (*top panel*) and the medium (*bottom panel*). The data for intracellular cAMP have been corrected for 150–220 pmoles of cAMP derived from the medium that was trapped on the filters. Values on *ordinates* should be multiplied by  $10^4$  and  $10^2$ , as indicated.

conditions cannot be explained on the basis of a stimulation of the exit of intracellular cAMP to the medium (2), resulting in lower intracellular cAMP levels.

A survey of the specificity of the sugar-mediated inhibition of adenylate cyclase (Table 1, Exp. I) indicated that the sugar acids, gluconic acid and glucuronic acid, are not inhibitors, while the "nonmetabolizable" analog of glucose,  $\alpha$ -methylglucoside (10), is a potent inhibitor. A glucose determination by the Glucostat method (3) of the  $\alpha$ -methylglucoside indicated that there was less than 1% contamination by glucose. That level of glucose (see Table 2) is insufficient to account for the observed inhibition. The observation that  $\alpha$ -methylglucoside is an inhibitor indicates that extensive metabolism of glucose is not required for the inhibition observed. Evidence has been presented (11) that  $\alpha$ -methylglucoside is taken up and phosphorylated by *E. coli*, but that it is not further metabolized. Exp. II constituted a survey of various other sugars as adenylate cyclase inhibitors. In this series, only mannose, glucosamine, and acetylglucosamine gave substantial inhibition, while fructose, mannitol, glycerol, lactose, and ribose showed either slight inhibition or stimulation.

*Glucose Can Inhibit Adenylate Cyclase More Severely Than It Effects a Lowering of Intracellular cAMP Concentration.* The characteristics of the *in vivo* adenylate cyclase assay indicate that the exposure of cells to a pulse of [ $^3$ H]adenosine results in a rapid conversion of labeled ATP to cAMP in a linear time-course for a few minutes. The rate of incorporation of radioactivity into cAMP levels off after a few minutes, probably as a result of equilibration of the newly synthesized [ $^3$ H]cAMP with the pool of cellular cAMP, which is "turning over" (4).

TABLE 2. Effect of glucose concentration on *in vivo* adenylate cyclase and intracellular cAMP concentrations in *E. coli* strains

Glucose concentration (M)	<i>In vivo</i> adenylate cyclase	Intracellular cAMP	
	pmoles/min per 10-ml culture	pmoles/10-ml culture	$M \times 10^{-5}$ *
<i>E. coli</i> B			
0	1575	511	3.6
$10^{-6}$	1347	532	3.8
$10^{-5}$	1816	497	3.5
$10^{-4}$	99	259	1.8
$10^{-3}$	77	184	1.3
<i>Crookes strain</i>			
0	237	215	1.4
$10^{-6}$	257	166	1.1
$10^{-5}$	149	166	1.1
$10^{-4}$	9	163	1.0
$10^{-3}$	17	140	0.9

A culture of *E. coli* was grown at 37° in minimal salts medium (9) supplemented with 0.1% glucose. Aeration of the culture was continued for 30–50 min after the cells stopped growing [ $A_{650} = 1.05$  (*E. coli* B); 0.742 (Crookes strain)]. At that time, 10-ml aliquots of the culture were tested for adenylate cyclase activity in intact cells as described (4). Where indicated, the designated concentrations of glucose were added at the same time as the [ $^3$ H]adenosine pulse. Duration of the [ $^3$ H]adenosine pulse was 1 min. For determination of intracellular cAMP concentrations, cultures were grown as above to stationary phase [ $A_{650} = 0.79$  (*E. coli* B); 0.89 (Crookes strain)]. Aliquots (10 ml) of the cells were incubated at 37° with shaking. One minute after the designated concentrations of glucose were added, the cells were processed for determination of intracellular cAMP concentration (see *Methods*). The amount of cAMP adsorbed to filters from the extracellular fluid in the various samples ranged from 130–220 pmoles (*E. coli* B) and 120–230 pmoles (Crookes strain). The values shown for intracellular cAMP have been corrected for the contamination by extracellular cAMP.

\* Values should be multiplied by  $10^{-5}$ , as indicated.

A possible mechanism whereby the accumulation of label in [ $^3$ H]cAMP could be decreased by glucose would be a stimulation of cAMP phosphodiesterase. Under these conditions, the cellular cAMP pool would decrease substantially and the amount of radioactivity accommodated by the pool would also decrease. The experiments shown in Table 2 provide evidence against such a mechanism. The glucose dose-response studies showed that  $10^{-4}$  M glucose led to essentially complete inhibition of the adenylate cyclase in *E. coli* B while the intracellular cAMP concentration decreased only 50% during the 1-min period of the experiment. It is noteworthy that in *E. coli* B the inhibition of adenylate cyclase by glucose occurred over a narrow dose-response range, progressing from no inhibition at  $10^{-5}$  M to virtually complete inhibition at  $10^{-4}$  M. Concentrations of glucose in the range of  $10^{-5}$  M might be expected to be metabolized by cells during a 1-min incubation. Therefore, it is possible that the effective inhibitory concentration of glucose is much lower than  $10^{-4}$  M, but that glucose utilization overcomes the inhibition. The glucose inhibition profile was somewhat different in the Crookes strain, where there was a more gradual dose-response effect of glucose on the adenylate

TABLE 3. Effect of sugars on *in vitro* adenylate cyclase and cAMP phosphodiesterase

Additions ( $10^{-3}$ M final concentration)	Adenylate cyclase (pmoles of cAMP formed/hr per mg of protein)	cAMP phospho- diesterase (pmoles of cAMP degraded/hr per mg of protein)
None	187	13,150
Glucose	230	10,020
Glucose-6-phosphate	191	13,420
$\alpha$ -Methylglucoside	—	13,010

The preparation of cell-free sonicates and incubation conditions for adenylate cyclase and cAMP phosphodiesterase assays are described in *Methods*. The adenylate cyclase rates shown here were calculated from the best line drawn through a time study carried out for 0, 20, 40, and 60 min. Rates for cAMP phosphodiesterase assays were calculated from the linear portion of an activity curve using several concentrations of protein varying from 0.01 to 0.2 mg, with a constant incubation time of 15 min.

cyclase activity; there was approximately 50% inhibition of the adenylate cyclase activity at  $10^{-5}$  M glucose.

The Crookes strain of *E. coli* was reported to be defective in cAMP phosphodiesterase (12). Nevertheless, this strain exhibited a pattern of inhibition by glucose of [ $^3$ H]cAMP formation from [ $^3$ H]adenosine similar to that shown by *E. coli* B. This study provides further evidence that the inhibitory effect of glucose on cAMP formation is not due to a stimulation of cAMP phosphodiesterase or any other process that directly decreases the cellular cAMP pool (e.g., export of cAMP to the medium).

*Glucose Does Not Stimulate the Accumulation of Extracellular cAMP.* The data of Table 2 indicate that, in *E. coli* B, the inhibition of adenylate cyclase could not be explained on the basis of a glucose-mediated exit reaction. The study depicted in Fig. 2 further explored such a possibility by following the kinetics of cAMP formation in a control and glucose-treated culture. Stationary phase cells of *E. coli* B were exposed to 1 mM glucose. At the designated time intervals, up to 30 min, aliquots (10 ml) were processed for the determination of intracellular and extracellular cAMP concentrations (see *Methods*). Within the limits of the time required for processing samples (2 min), glucose addition led to approximately an 80% decrease in the intracellular cAMP concentration. After approximately 10 min, parallel with the utilization of the added glucose, the intracellular cAMP level rose again to a value near that of the control culture. While there was a clear effect of glucose on intracellular cAMP levels, there was no significant glucose-dependent change of the extracellular cAMP level. The data of this study provided no support for the existence of a glucose-stimulated cAMP exit reaction (however, see *Discussion*).

*Glucose Inhibits the Rate of cAMP Formation.* A direct demonstration that glucose inhibits the rate of cAMP synthesis is shown in Fig. 3. The data of Table 2 indicated that the Crookes strain of *E. coli* showed approximately 50% inhibition of [ $^3$ H]cAMP formation by  $10^{-5}$  M glucose. A kinetic study of the effect of glucose on adenylate cyclase was performed under these conditions.

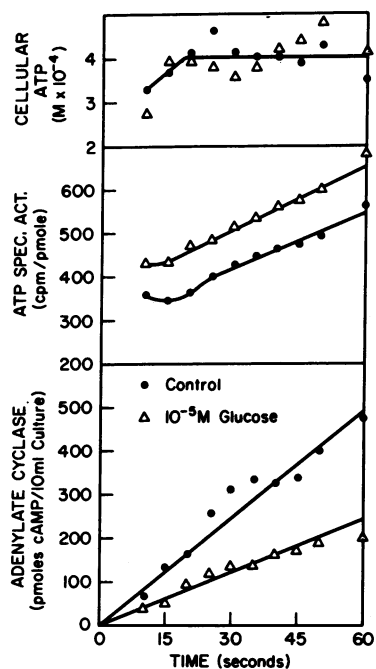


FIG. 3. Effect of glucose on the kinetics of the adenylate cyclase reaction in *E. coli*. A culture of *E. coli* (Crookes strain) was grown at  $37^\circ$  with shaking in minimal salts medium (9) supplemented with 0.1% glucose. Thirty minutes after the cells reached their maximum density ( $A_{650} = 0.71$ ), aliquots (10 ml) of cell suspension were pulse-labeled with [ $^3$ H]adenosine as described (4). Glucose ( $10^{-5}$  M) was added to one set of incubation mixtures immediately before the [ $^3$ H]adenosine. Reactions were terminated at the indicated times, and samples were assayed for [ $^3$ H]cAMP as well as for intracellular ATP and the specific activity of ATP, as described (4). Cellular ATP concentrations should be multiplied by  $10^{-4}$ .

The *top* and *middle panels* of the figure show a series of controls. The *top panel* of Fig. 3 shows that during the time of the study, glucose did not significantly affect the cellular concentration of ATP; the *middle panel* shows that the rate of labeling of the ATP pool by adenosine was slightly enhanced by glucose addition. Therefore, the inhibitory effect of glucose cannot be ascribed to an effect on the rate of entry of label into ATP.

It is clear from the data (Fig. 3, *bottom*) that glucose-supplemented incubation mixtures show inhibition of the rate of formation of [ $^3$ H]cAMP throughout the time studied. Considering the nature of the pulse-labeling assay that leads to a time-dependent equilibration of the newly synthesized [ $^3$ H]cAMP with the pool of cellular cAMP, if the effect of glucose were on some process that directly affected the pool of cAMP (e.g., stimulation of cAMP phosphodiesterase or the exit reaction), the kinetics of inhibition by glucose would not be affected at the earliest time points. Therefore, the data strongly indicate that the primary locus of action of glucose is on the synthesis of cAMP catalyzed by adenylate cyclase.

*Glucose Does Not Inhibit Adenylate Cyclase or cAMP Phosphodiesterase In Vitro.* The finding (Fig. 3) that glucose addition led to a rapid inhibition of adenylate cyclase activity in intact cells suggested that a similar glucose-mediated inhibition of adenylate cyclase might be demonstrable *in vitro*. Table 3 shows, however, that neither glucose nor glucose-6-phosphate ( $10^{-3}$  M) inhibited adenylate cyclase in sonicates of

*E. coli* B. A similar study with cAMP phosphodiesterase showed no significant effect of glucose, glucose-6-phosphate, or  $\alpha$ -methylglucoside on that enzyme activity.

### DISCUSSION

Substantial evidence has now accumulated to indicate that the phenomenon of "glucose repression" or "catabolite repression" can be explained by a glucose-mediated depression of intracellular cAMP levels. Our interest in these studies has been to investigate the mechanism by which glucose exerts this effect. Various mechanisms by which glucose can effect a lowering of cellular cAMP levels have been suggested. The initial studies of Makman and Sutherland (2), with resting cells, were interpreted to indicate that glucose inhibits adenylate cyclase and also promotes the release of cAMP to the medium. Buettner *et al.* (13) suggested, from their recent data, that cAMP ordinarily goes from its synthetic site into cells but that glucose "changed the direction of flow" of cAMP to an outward direction. Mechanisms by which glucose stimulates the utilization of cAMP by cAMP phosphodiesterase or other utilization reactions are also plausible.

The studies reported here argue strongly that the major, if not sole, mechanism that leads to the *instantaneous* regulation of cAMP levels by glucose is via the inhibition of adenylate cyclase. The development of an *in vivo* assay for adenylate cyclase (4) has been of great assistance in pursuit of these studies. Since the *in vivo* assay for adenylate cyclase measures the rate of entry of radioactivity from [<sup>3</sup>H]ATP into cAMP before the cellular pool is radiochemically equilibrated, we reasoned that it was possible to distinguish kinetically an effect of glucose on the synthesis or degradation of cAMP. If the initial effect of glucose were on the degradation of cAMP, leading to a lowered pool of cellular cAMP, we would anticipate no change in the initial rate of labeling of cAMP from [<sup>3</sup>H]ATP, and would expect to see an earlier time for equilibration of the pool. On the other hand, if the glucose effect were on the synthesis of cAMP, we would expect to see an immediate and continued effect on the rate of the adenylate cyclase reaction. The data of Fig. 3 strongly support the model that the mechanism of the glucose effect involves inhibition of the adenylate cyclase reaction rather than degradation or exit of cAMP. Effects of glucose on the entry of label from adenosine to ATP are also eliminated by the data of Fig. 3.

There is additional evidence against the model that glucose stimulates release of cAMP into the culture medium. The measurements that we have made using the *in vivo* assay for adenylate cyclase (4) have involved measurements of the amount of labeled cAMP formed in the mixture of *both* the cells and the medium. Nevertheless, we observed an inhibition by glucose of the appearance of labeled cAMP in the total of these fractions (Table 1). Thus, we find no support in these short-term studies for the mechanism suggested by Buettner *et al.* (13), whereby glucose stimulates the deposition of cAMP into the medium. Therefore, the data presented here suggest that the control of cAMP levels mediated by glucose may be solely due to its effect on the adenylate cyclase system.

The study shown in Fig. 2 deserves some further comment. Here we attempted, over a short period of time, to reproduce the type of effect reported by Buettner *et al.* (13) in which the decreased cellular cAMP levels caused by glucose addition could be accounted for by an increase in cAMP in the medium.

The data in our figure are expressed as the total amount of cAMP in the cells or medium derived from a 10-ml aliquot of cells. This mode of presentation makes it clear that there is roughly 50 times as much cAMP in the culture fluid as in the cells. The sensitivity of the assay methodology would not detect a change in the extracellular cAMP concentration corresponding to a complete release of intracellular cAMP to the medium. Therefore, in our hands, this type of experiment provides strong evidence neither for nor against a model involving glucose-stimulated release of extracellular cAMP.

The intracellular cAMP levels reported here (about 2 to 4  $\times 10^{-5}$  M; Table 2) are in the range previously cited by Pastan and Perlman (1) using a different analytical procedure, but substantially lower than those reported by Buettner *et al.* (13), who used a procedure similar to that reported here. In our studies, we found that sampling 2-ml aliquots of cell suspension gave unsatisfactory results, because as much as 80% of the cAMP measured could be accounted for by trapped extracellular cAMP (see *Methods*). Therefore, we routinely used 10-ml aliquots of cells for determination of intracellular cAMP levels, although the time required for filtration was almost 2 min. Aside from these methodology differences, the reasons for the different intracellular cAMP concentrations reported here and by Buettner *et al.* (13) are not clear.

It is noteworthy that while glucose addition leads to inhibition of adenylate cyclase *in vivo*, the sugar does not inhibit the enzyme activity *in vitro*. These data suggest that glucose inhibition of adenylate cyclase does not involve a direct interaction of glucose with the enzyme, but requires yet another factor. The preparation of cell-free extracts may either inactivate the factor or dislodge it from its position in an adenylate cyclase complex. The indications that *E. coli* adenylate cyclase is membrane-associated (14, 15) are compatible with this idea.

We appreciate the helpful comments on the manuscript by Drs. James Harwood, Ira Pastan, Robert Lazzarini, and Michael Schramm.

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