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Adenosine 3',5'-Cyclic Monophosphate in *Vibrio cholerae*

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The extracellular concentration of cyclic adenosine 3',5'-monophosphate (AMP) of three different strains of *Vibrio cholerae* growing in syncase medium were measured. Cyclic AMP secreted by *V. cholerae* 569B varied widely with different carbon sources. Mutant 13, which produced little or no toxin, released half the amount of cyclic AMP as the wild type. The release of less cyclic AMP into the medium by mutant 13 may be accounted for by the lower activity of adenylate cyclase observed. High glucose (3%) in the culture medium reduced the concentration of cyclic AMP both in wild type and mutant 13. Reduction of cyclic AMP levels at high concentrations of glucose (3%) occurred without change of adenylate cyclase activity. The release of enterotoxin to the medium varied with carbon sources but was independent of conditions which reduced the cyclic AMP both within the cell and the medium. Neither adenylate cyclase activity nor toxin production was reduced by an increased concentration of glucose in wild-type *V. cholerae*, whereas cyclic AMP levels were reduced by sixfold. A lower activity of the adenylate cyclase was observed in a mutant of *V. cholerae* which produced no detectable toxin. Thus, a correlation exists between toxin production and adenylate cyclase activity in *V. cholerae*.

Although cyclic adenosine 3',5'-monophosphate (AMP) has been reported in several bacterial species (18), we are not aware of a description of such a system in *Vibrio cholerae*. *V. cholerae*, a motile gram-negative bacterium, may be of particular interest with respect to its cyclic nucleotides since the acute diarrhea of clinical cholera is mediated by an enterotoxin produced by this organism which exerts its effect both on intestinal and nonintestinal tissues by stimulating adenylate cyclase activity (16). In this paper, we describe a cyclic AMP system in *Vibrio cholerae* and some of its relations to the elaboration of enterotoxin.

MATERIALS AND METHODS

Cyclic AMP, adenosine 5'-triphosphate (ATP), 5'-AMP, 5'-nucleotidase Grade II, phosphoenolpyruvate, pyruvate kinase, 2-mercaptoethanol, and bovine serum albumin were obtained from Sigma Biochemical Co. Radioisotopes ([α-^{32}P]ATP cyclic 3',5'-[^{3}H]AMP, and 5'-[^{14}C]AMP) were purchased from New England Nuclear Corp. Protein kinase and protein kinase inhibitor were prepared in our laboratory by the method of Gilman (4). The cation exchange resin AG-50-X2, 200 to 400 mesh, hydrogen form, was supplied by Bio-Rad Laboratories. 2-Ethoxyethanol and 2-(2-methoxyethoxy)-ethanol were obtained from J. T. Baker Chemical Co. Collagenase was obtained from Worthington Biochemical Corp. Glycerokinase and α-glycerol phosphate dehydrogenase were purchased from Calbiochem. Glucostat reagent was purchased from Worthington Co.

The bacterial strains used were *V. cholerae* Inaba 569B and Ogawa 395, both of which produce toxin, and a mutant of Inaba 569B, IM-13, which produces little or no toxin (kindly provided by R. A. Finkelstein, University of Texas Southwestern Medical School, Dallas, Tex.). (In a personal communication, R. A. Finkelstein informed us that culture filtrates of M-13 have the characteristic elongating effect on Chinese hamster ovary cells as does purified cholera enterotoxin and crude culture filtrates of wild-type Inaba 569B, but only at 10,000 times the concentration of the latter. Thus M-13 may be capable of elaborating as small amount of toxin or a similar substance.)

The cells were grown in a 1-liter low-form flask with 200 ml of syncase medium (4) with different sources of carbon indicated in individual experiments. The culture was shaken (200 shakes per min) at 37 C. Resting organisms were obtained by inoculation of one loopful of organisms from a stock culture on nutrient agar and incubated for 18 h in syncase medium containing 0.25% sucrose as described above. The cells were harvested by centrifugation (12,000 $\times$ *g*, 10 min) at 4 C in a Sorvall model RC-2 centrifuge, washed once with the same sterile medium, resuspended to a density of 0.10 to 0.12 optical density at 650 nm and incubated at 37 C for 5 h with shaking in a New Brunswick reciprocating water bath shaker. The density of cells was measured in a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer at 650 nm.

Since cyclic AMP is quite stable in boiling aqueous

solutions and in dilute acid, we used the same extraction method as described by Makman and Sutherland (12). A minimum of 0.3 g (wet weight) of organism was measured after harvesting the cells by centrifugation at 12,000 × *g* for 10 min at 4 C. Without washing, the packed cells were immediately suspended in 10 volumes of cold 0.05 N HCl. The suspension was heated in a boiling water bath for 3 min, cooled, and centrifuged for 10 min. The supernatant fraction was then adjusted to pH 4 with 0.1 N NaOH. Portions of this fraction were diluted promptly in 50 mM acetate buffer (pH 4) and assayed for cyclic AMP in duplicate. Intracellular levels of cyclic AMP may undergo some changes even during a brief centrifugation. Accordingly, when cyclic AMP in the medium was to be measured, they were collected in a similar manner. Duplicate samples (2 ml) were removed every hour. One sample was centrifuged at 12,000 × *g* for 10 min to remove the cells. The supernatant fluid was placed in a boiling water bath for 3 min, and then cooled, and the pH was adjusted to 4 with 1 N HCl. The other sample (total) was placed in a boiling water bath prior to centrifugation and then processed as described. Samples were assayed for cyclic AMP concentration. The contribution to the total cyclic AMP by the cells was not large enough to be detected.

The alternative method which has been described of harvesting cells by membrane (Millipore Corp.) filtration (1), although somewhat reducing the time needed for collection, interposes the abnormal variable of exposing cells to surface effects which in the case of *V. cholerae* may be more damaging than centrifugation, since this organism does not survive when removed from an aqueous environment and may be damaged by exposures too short to cause cell death (3).

Cyclic AMP was measured by the procedure described by Gilman (6). The standard reaction mixture contained 50 mM sodium acetate-acetic acid (pH 4.0), cyclic [^{3}H]AMP (4.0 pmol per 0.02 μCi per tube), protein kinase (2 μg/tube), and protein kinase inhibitor (45 μg/tube), and the sample to be assayed in a final volume of 0.2 ml. After a 70-min incubation at 0 C, the mixtures were diluted to 1 ml with cold 20 mM phosphate buffer (pH 6.0); 4 to 5 minutes later the content of the assay tube was transferred quantitatively to a membrane filter (Millipore Corp.) by rinsing the tube with 10 ml of phosphate buffer. The Millipore disk was then placed in a counting vial containing 2 ml of cellosolve and methyl cellosolve in equal proportions and 15 ml of scintillation mixture [125 g of naphthalene, 7.5 g of 2,5-diphenyloxazole, 0.38 g of 1,4-bis-(5-phenyloxazolyl)-benzene and 1 liter of Dioxane], and radioactivity was measured in a Packard liquid scintillation counter. Control assays were carried out without protein kinase.

Extracts were prepared from bacteria which had been washed twice and suspended in 50 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.5). The cell suspension was sonically treated for 30 min with a Biosonic-Ultrasonic probe (Bronwell Scientific, Rochester, N.Y.) with cooling. Adenylate cyclase was determined directly on the sonicated material. We followed the same extraction method as described by Peterkofsky et al. (14).

The enzyme activity was determined by measuring the formation of radioactive cyclic AMP from radioactive ATP. The reaction mixtures contained 25 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.5), 10 mM $MgCl_2$, 1.5 mM [α-^{32}P]ATP (2 μCi/tube), 5 mM theophylline, 5 mM phosphoenolpyruvate, and 50 μg/ml of pyruvate kinase and enzyme, in a final volume of 50 μliters. The reaction was carried out at 37 C. After 10 min of incubation, 0.5 ml of recovery mixture (containing 50 μg of cyclic AMP, 100 μg of ATP, and 0.01 μCi of cyclic [^{3}H]AMP) was added, and the samples were immediately placed in a boiling water bath for 3 min. The reaction blank was incubated without enzyme for 10 min at 37 C, enzyme was added immediately after the recovery mixture, and the tubes were placed in the boiling water bath for 3 min. Cyclic AMP formed during incubation was determined by the methods of Krishna et al. (10). After cooling at room temperature, the complete mixture was then applied to a column (0.5 by 3 cm) of Dowex 50W-X2, and the nucleotides were eluted with water. The first 2 ml of effluent was discarded, while the next 2 ml of effluent containing most of the cyclic AMP was collected and subjected to $Ba(OH)_2$ and $ZnSO_4$ precipitation. Two milliliters of the supernatant fraction was placed in a counting vial containing 15 ml of scintillation fluid (1 liter of toluene containing 0.25 g of 1,4-bis-(5-phenyloxazolyl)-benzene and 8.25 g of 2,5-diphenyloxazole and 500 ml of Triton X) and counted in Packard Tri-Carb liquid scintillation counter. The formation of cyclic AMP was proportional to the concentration of crude protein up to 1.2 mg/ml added at 37 C for 10 min.

Glucose was analyzed with Glucostat reagent obtained from Worthington Biochemical Corp. Samples of the culture were taken out periodically, and the cell debris was removed by centrifugation at 12,000 × *g* for 20 min at 4 C. The supernatant fluid was saved and kept frozen until glucose was measured.

Protein was determined by the method of Lowry et al. (11) with bovine plasma albumin used as a standard.

Samples of cultures were removed periodically and centrifuged at 12,000 × *g* for 20 min at 4 C. Supernatant was saved for toxin assay. The activity of toxin was determined by its stimulation of cyclic AMP dependent lipase in rat epididymal fat cells measured by glycerol release to the medium as described by Greenough et al. (7).

Epididymal fat pads from male rats weighing 120 to 150 g were used. Fat cells were isolated by the method of Rodbell (17). After treatment with collagenase, cells were washed three times with Krebs-Ringer phosphate buffer (pH 7.4) containing bovine albumin, and were resuspended at a concentration of 0.25 g/ml with same buffer. Samples of this suspension were incubated with culture supernatant fluid for 3 h at 37 C. Release of glycerol was measured by an enzymatic method (13).

RESULTS

The extracellular concentration of cyclic AMP produced by different strains of *V. cholerae* was measured (Table 1). The station-

TABLE 1. *Extracellular concentration of cyclic AMP produced by different strains of V. cholerae after 5 h of incubation*[a]

Strain	No. of expts	Culture media	A_{650}[b]	Cyclic AMP[c] (pmol/ml of media)
Inaba 569B	5	Syncase with 0.25% sucrose	2.0	620 (555–705)
Ogawa 395	3	Same	1.8	680 (630–710)
Inaba 569B M-13 mutant	3	Same	2.2	320 (300–350)

[a] Overnight cultures of three strains of *V. cholerae* were centrifuged and suspended in syncase medium containing 0.25% sucrose. Samples were incubated for 5 h and processed as described in the text.

[b] A_{650}, Absorbance at 650 nm.

[c] The mean value for cyclic AMP is shown, with the range indicated in parentheses.

ary cultures of both wild-type Inaba 569B and Ogawa 395 in syncase medium with 0.25% sucrose accumulated comparable concentrations of cyclic AMP. However, the concentration of cyclic AMP formed by the mutant M-13 Inaba 569B was approximately half of that formed by the wild type. Syncase media with sucrose as a carbon source was used in these experiments since it is a standard medium for toxin production in *V. cholerae* (4).

Because glucose is known to lower the intracellular concentration of cyclic AMP in *Escherichia coli* (12, 14), we also studied the effect of low and high glucose concentrations on cyclic AMP content of the organism and its media. The time course of growth, glucose utilization, and cyclic AMP secretion into medium by *V. cholerae* in low (0.25%) and high-glucose (3%) medium are shown in Fig. 1. In low-glucose medium, growth was maximal at 4 h. At that time, glucose had disappeared from medium and a marked rise of cyclic AMP concentration was observed. In medium with a high glucose content, glucose utilization and cyclic AMP secretion pattern was quite different. There were substantial amounts of glucose present long after cessation of growth. However, the cyclic AMP content of the medium was much less (110 pmol/ml) than that observed in similar cells grown in low-glucose medium (680 pmol/ml). This observation was very similar to those reported for *E. coli* (14).

The level of cyclic AMP in cells during the logarithmic phase of growth in syncase medium with 0.25% glucose was much higher than that found in similar cells grown in 3% glucose (Table 2). In a low-glucose medium after 2 h of growth (logarithmic stage), 6.2 pmol of cyclic AMP per mg of cells was found compared to 0.81 pmol of cyclic AMP per mg of cells grown in high-glucose medium. After 5 h (stationary phase), the lowering of cyclic AMP content inside the cells grown in low-glucose medium was correlated with the rise of cyclic AMP concentration into the medium. However, when cells were incubated with 3% glucose, the low level of cyclic AMP inside the cells was not associated with any increase in cyclic AMP released into the medium. (In these studies, the contribution of trapped medium in the sedimented cells was not measured; however, this correction is small [2 to 3%] in membrane-filtered cells [1], and would have tended to increase the differences observed. If it is assumed that under conditions of high glucose concentration all of the activity in the pellet were due to trapped medium, the maximum correction for this would have been 0.0033 to 0.0038 ml per mg of wet weight.)

Since a high glucose concentration suppressed cyclic AMP formation, we investigated whether enterotoxin formation was also dependent (like cyclic AMP) on the carbon sources present in the medium (Fig. 2). A high concentration of sucrose (like glucose) suppressed the secretion of cyclic AMP into the medium, whereas in similar experiments with lactose, galactose, and glycerol, there was no difference in the accumulation of cyclic AMP over a concentration range from 0.25% to 3%. The production of toxin was greatest in the presence of glucose compared to other carbon sources.

When the pH of the medium was maintained between 7.6 and 8.0 (2), the production of enterotoxin was the same at glucose concentrations of both 0.25 and 3.0%. Since the amount of cyclic AMP released to the medium and present in the cell was markedly reduced by 3.0% glucose, it appears that toxin release is not directly dependent on the cyclic AMP level of *V. cholerae*. It is interesting to note, however, that the maximum concentration of toxin formed extracellularly varied with the carbon sources (Fig. 2). There was 67% less toxin formed at both low and high concentrations of galactose and glycerol when compared to the level of toxin formed in presence of glucose.

Since the data of Table 1 showed that the

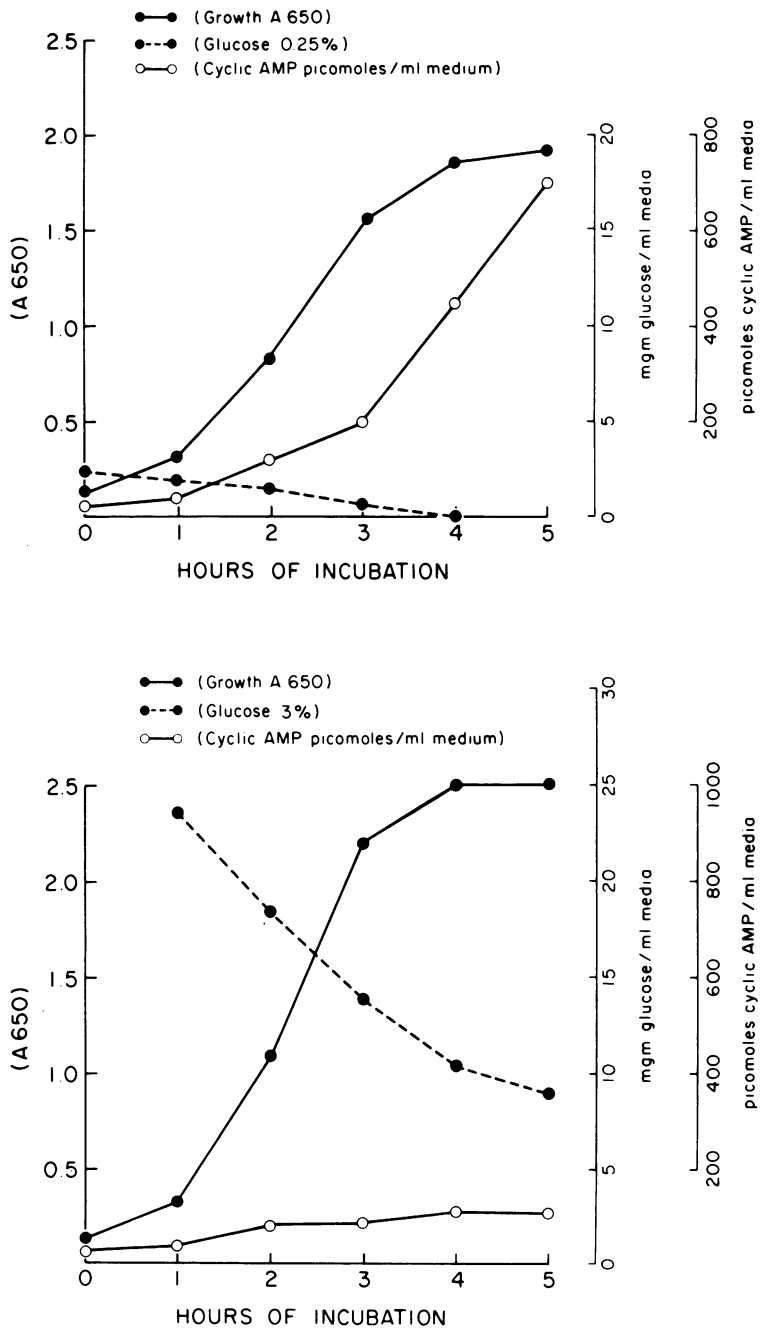

FIG. 1. *Effect of two different concentrations of glucose on growth, glucose utilization, and cyclic AMP accumulation by V. cholerae 569B.*

M-13 mutant secreted less cyclic AMP into the medium than that observed in wild 569B, we further studied this parameter with wild 569B (Table 3). A high concentration of glucose had the same suppressive effect on the mutant as it did in the wild type.

We measured the adenylate cyclase activity of wild-type Inaba 569B and the mutant M-13 and specifically selected a 3-h culture (logarithmic growth phase) because cyclic AMP content inside the cell is much higher than the stationary cells. Table 4 shows adenylate

cyclase activity of the organism grown with different concentrations of glucose. Extracts of wild-type cells obtained during the logarithmic state of growth showed similar adenylate cyclase activity in the presence of glucose irrespective of concentration. Extracts from mutant cells showed lower activity than that observed in the wild type.

DISCUSSION

The studies reported here indicate that different strains of *V. cholerae* have an active cyclic AMP system which seems very similar to that of other gram-negative bacteria which have been studied (8, 9, 14). There are no significant differences in extracellular cyclic AMP levels between the wild-type 569B and the Ogawa 395, both of which produce toxin and were isolated from patients with a full cholera syndrome. But the concentration of cyclic AMP formed by the mutant M-13 is only about half of that formed by wild type.

The effect of glucose on cyclic AMP was also very similar to *E. coli* (14). In the presence of 3% glucose, both the release and cellular concentration of cyclic AMP were reduced. Synthetic capacity is indicated by measurements of adenylate cyclase which appears to have greatest activity during the logarithmic phase of growth. The failure of 3% glucose to reduce adenylate

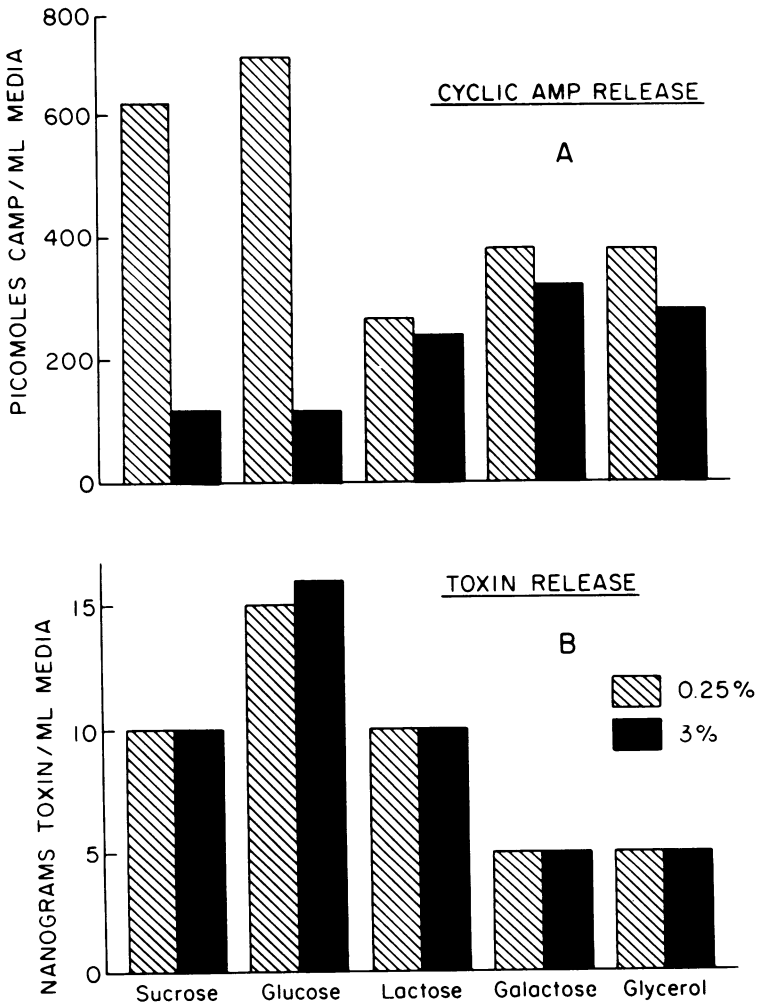

FIG. 2. *Effect of different carbon sources on cyclic AMP and toxin secretion by V. cholerae 569B. An overnight culture was centrifuged and suspended in syncase medium to a cell density of 0.12 absorbance at 650 nm. Carbon sources were added and incubated for 5 h. Samples were removed at 0 h and 5 h. Cyclic AMP concentration of 0-h samples were less than 20 pmol/ml and toxin concentration was zero.*

cyclase activity when such profound effects were seen on both intra- and extracellular levels of cyclic AMP requires explanation.

The phosphodiesterase activity of *V. cholerae* in two experiments (not illustrated here) was low and did not seem to be increased by 3% glucose. Recent reports on *E. coli* (15) showed that the measurement of adenylate cyclase activity in intact cells can explain the cyclic AMP levels in low and high glucose medium. However, we have no data on intact cells and cannot comment on this.

The total amount of cyclic AMP in the stationary phase was much higher in the medium than in the bacteria. The value for cyclic AMP concentration was 54 pmol/20 mg (wet weight) of the cells, the usual yield of 1 ml of culture. On the other hand, the total culture showed 816 pmol/ml of media. This indicates that *V. cholerae* secretes cyclic AMP into the media at all stages of growth, which is in agreement with our study in different phases of growth (Fig. 1).

The release of enterotoxin to the medium varied widely with carbon sources but is independent of conditions which reduce cyclic AMP both within the cell and the medium. Wild-type cells (569B) incubated with glucose irrespective of its concentration have similar levels of adenylate cyclase activity and toxin production. Mutant M-13, which produces little or no toxin, also has approximately one-third of activity of adenylate cyclase found in the wild type. This observation suggests that a lower level of extracellular cyclic AMP in the mutant may be due to a lower activity of adenylate cyclase. Since

TABLE 2. *Cyclic AMP content of V. cholerae 569B in cells and medium under different growth conditions*[a]

No. of expts	Glucose (%)	Growth stage	pmol of cyclic AMP/mg (wet weight) organism[b]	pmol of cyclic AMP/ml of media[a]
3	0.25	Logarithmic	6.2 (5.8-6.5)	125 (110-135)
		Stationary	2.7 (2.6-2.8)	816 (775-875)
2	3	Logarithmic	0.81 (0.79, 0.83)	65 (55, 75)
		Stationary	0.42 (0.41, 0.43)	110 (100, 120)

[a] An overnight culture of *V. cholerae* 569B was centrifuged and suspended in syncase medium containing different concentrations of glucose with initial absorbance of 0.15 to 0.17 and was incubated for 5 h. Samples were removed at logarithmic (2 h) and stationary phase (5 h) and processed as described in the text.

[b] The mean value for cyclic AMP is shown, with the range indicated in parentheses.

TABLE 3. *Comparison of cyclic AMP formation by two strains of V. cholerae*[a]

No. of expts	Strain	Glucose (%)	A_{650}[b]	Cyclic AMP[c] (pmol/ml of media)
6	Inaba 569B	0.25	2.0	762 (650-875)
		3	2.5	110 (100-120)
3	Inaba 569B M-13 mutant	0.25	2.2	363 (360-370)
		3	2.0	63 (60-66)

[a] An overnight culture of *V. cholerae* wild-type and mutant 569B was centrifuged and suspended in syncase medium containing different concentration of glucose and then incubated for 5 h. Samples were removed at 5 h and processed as described in the text.

[b] A_{650}, Absorbance at 650 nm.

[c] The mean value for cyclic AMP is shown with the range indicated in parentheses.

TABLE 4. *Adenylate cyclase activity in homogenates of V. cholerae grown in glucose*[a]

No. of expts	Strain	Glucose in medium (%)	Growth stage	Adenylate cyclase (pmol per mg of protein per 10 min)
5	Inaba 569B	0.25	Logarithmic	240
		3	Logarithmic	280
		0.25	Post-logarithmic (18 h)	93
3	Inaba 569B M-13 mutant	0.25	Logarithmic	97
		3	Logarithmic	71

[a] Cultures of *V. cholerae* wild and mutant 569B were grown in syncase medium containing different concentrations of glucose to a cell density of 0.10. Cells were removed after 3 h, centrifuged, and suspended in 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.5) buffer and cells were lysed as described in the text. Results are means of indicated experiments.

toxin is rapidly produced in the presence of 3% glucose, it would appear that the level of cyclic AMP inside or outside the cells is not involved in the regulation of toxin secretion. However, there is a correlation between the production of toxin and adenylate cyclase activity. The mutant strain which produced little or no toxin also shows less activity of adenylate cyclase and a lesser production of cyclic AMP. This mutant does respond in the same way to repression by glucose as did the wild type. The growth pattern of the mutant M-13 is similar to that of wild-type Inaba 569B.

Since the exotoxin of *V. cholerae* specifically stimulates adenylate cyclase in nonbacterial cells, and the production of this toxin is at least circumstantially proportional to the activity of adenylate cyclase in both the wild-type Inaba 569B and mutant M-13 Inaba 569B, it will be important to investigate further a possible role of this toxin in cyclic AMP metabolism in *V. cholerae*, and in other bacteria which produce related enterotoxins.

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