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Cyclic Adenosine 3',5'-Monophosphate Levels and Activities of Adenylate Cyclase and Cyclic Adenosine 3',5'-Monophosphate Phosphodiesterase in *Pseudomonas* and *Bacteroides*

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A modified Gilman assay was used to determine the concentrations of cyclic adenosine 3',5'-monophosphate (cAMP) in rapidly filtered cells and in the culture filtrates of *Pseudomonas aeruginosa*, *Escherichia coli* K-12, and *Bacteroides fragilis*. In *P. aeruginosa* cultures, levels of cAMP in the filtrate increased with the culture absorbance (3.5 to 19.8 $\times 10^{-9}$ M) but did not vary significantly with the carbon source used to support growth. Intracellular concentrations (0.8 to 3.2 $\times 10^{-5}$ M) were substantially higher and did not vary appreciably during growth or with carbon source. Sodium cAMP (5 mM) failed to reverse the catabolite repression of inducible glucose-6-phosphate dehydrogenase (EC 1.1.1.49) synthesis caused by the addition of 10 mM succinate. Exogenous cAMP also had no discernible effect on the catabolite repression control of inducible mannitol dehydrogenase (EC 1.1.1.67). *P. aeruginosa* was found to contain both soluble cAMP phosphodiesterase (EC 3.1.4.17) and membrane-associated adenylate cyclase (EC 4.6.1.1) activity, and these were compared to the activities detected in crude extracts of *E. coli*. *B. fragilis* crude cell extracts contain neither of these enzyme activities, and little or no cAMP was detected in cells or culture filtrates of this anaerobic bacterium.

Several lines of evidence have indicated that cyclic adenosine 3',5'-monophosphate (cAMP) is involved in transient and catabolite repression control of inducible enzyme synthesis in *Escherichia coli* and other members of the family *Enterobacteriaceae* (26). Most of these organisms have a facultative anaerobic (fermentative) physiology, many have clustered arrangements of catabolic genes which are regulated as inducible operons, and most possess the phosphoenolpyruvate-sugar phosphotransferase system (PEP-PTS) for the group translocation of hexoses. Components of the PEP-PTS have been shown to be required for cAMP to exert its regulatory effect in bacteria (1, 27, 29, 39).

Members of the genus *Pseudomonas* differ sharply from these organisms. *Pseudomonas* is an obligate aerobe with an oxidative (respiratory) physiology, its genes appear to be more widely distributed on the genome, and the control of linked genes, which is poorly understood, appears more complex than that inherent in operons (7, 13). Further, the presence of the PEP-PTS has not been demonstrated in these bacteria (30, 33). Also, in contrast to the facultative anaerobes, *Pseudomonas* utilizes organic acids preferentially to glucose and other carbohydrates. In the presence of succinate or other organic acids, the synthesis of inducible carbohydrate catabolic enzymes is strongly repressed (17, 23, 38). Cultures of *P. aeruginosa* have been reported to contain both intracellular and extracellular cAMP (36; P. B. Hylemon and P. V. Phibbs, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P198, p. 174), but there is little information on the role of cAMP in the regulation of synthesis of inducible catabolic enzymes in this organism. However, the presence of exogenous cAMP has been reported to overcome the glucose repression of synthesis of an inducible cholinesterase in this species (8).

To delineate a possible role for cAMP in catabolite repression control in *Pseudomonas aeruginosa*, we measured both intracellular and extracellular levels of cAMP throughout growth on a variety of carbon sources and determined the ability of exogenous cAMP to overcome catabolite repression by organic acids of selected inducible catabolic enzymes in this

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organism. For comparative purposes, we have also examined the intra- and extracellular cAMP concentration and activities of related enzymes in *Bacteroides fragilis*, an obligately anaerobic gram-negative rod.

MATERIALS AND METHODS

Microorganisms and culture conditions. Stock cultures of *P. aeruginosa* strains PAO (31) and OSU-64 (30) and *E. coli* K-12 strain X 289 (5) were maintained on nutrient agar slants incubated at room temperature (22 to 25°C). *B. fragilis* ATCC 25285 was maintained in chopped-meat medium, according to Holdeman and Moore (12). Both *P. aeruginosa* and *E. coli* were cultured in the same basal salts medium containing the appropriate carbon source(s) as described previously (14). *P. aeruginosa* was also grown in the medium described by Lessie and Vander Wyk (18) supplemented with the appropriate carbon source(s). A 5% (vol/vol) inoculum of *E. coli* or *P. aeruginosa* cells from a late-exponential-phase starter culture was used to initiate growth in experiments involving these two bacteria. Cells were cultured in 300-ml Nephelo culture flasks (Bellco, Vineland, N.J.) containing 50 ml of medium shaking in an Eberbach water-bath shaker (120 cycles per min, 37°C). *B. fragilis* was cultivated in a chemically defined medium as described previously (15). Bacterial growth was measured with a Klett-Summerson colorimeter equipped with a number 66 filter.

Preparation of samples for measurement of intra- and extracellular levels of cAMP. A procedure closely based on that of Buettner et al. (3) was used. For *P. aeruginosa* and *B. fragilis*, an appropriate volume of culture (equivalent to about 1 mg of cell protein) was rapidly collected on two membrane filters (Millipore Corp., DAWG 02500; 0.65-μm porosity, 25 mm in diameter); the culture medium which passed through the filters was collected in a test tube inside the vacuum flask. The filters were immediately immersed in 2 ml of hot 0.1 N HCl and kept at 95°C for 10 min. Sampling was completed within 30 s. After cooling, the filters were rinsed with distilled water and discarded. The extract, plus washings, was lyophilized and resuspended in 0.20 ml of 50 mM sodium acetate buffer (pH 4.5). Samples (20 μl) were then assayed in triplicate for cAMP. The culture filtrate was also kept at 95°C for 10 min. After cooling, 1.0 ml of the filtrate was lyophilized and resuspended in 0.20 ml of 50 mM acetate buffer. Samples (20 μl) were then assayed in triplicate for cAMP.

For *E. coli* K-12, 2 ml of culture was filtered and the cells were treated as described above. The culture filtrates were kept at 95°C for 10 min and after cooling were diluted 1:5 in fresh growth medium. Twenty-microliter samples of this dilution were used for the assay of cAMP.

cAMP assay. The protein-binding assay for cAMP of Gilman (9) was used with modifications. For use in the assay, a cAMP-binding protein was isolated in our laboratory from beef kidney according to the method of Cheung (4). The assay mixture contained 20 μl of sample or standard, 10 μl of [^{3}H]cAMP (0.5 pmol), 10 μl of bovine serum albumin (25 μg) in 50 mM sodium acetate buffer (pH 4.5), and 20 μl of binding protein (16 μg) in 50 mM acetate buffer. After 60 min in an ice-water bath, 1 ml of 20 mM potassium phosphate buffer (pH 6.0) was added to the reaction mixture. After 4 min of incubation, the mixture was quantitatively transferred to a membrane filter (HAWP 02412, Millipore Corp.; 0.45-μm porosity, 23 mm in diameter) and washed with 3 ml of phosphate buffer. The filter was then placed in a scintillation vial and dried. Radioactivity was determined in Aquasol. Alternatively, the dried filters were dissolved in 1.5 ml of monoethylene glycol and 6 ml of scintillation fluid {5 g of PPO [2,5-diphenyloxazole], 0.125 g of POPOP [1,4-bis-(5-phenyloxazolyl)benzene] in 1 liter of toluene} was then added. Radioactivity was assayed using a Beckman LS 350 liquid scintillation spectrometer.

Samples and standard were assayed in triplicate and a new standard curve (0 to 3.5 pmol of unlabeled cAMP) was prepared for each assay. A typical standard curve is shown in Fig. 1. When extracellular cAMP was to be assayed, the standards were prepared in 50 mM sodium acetate buffer (pH 4.5) containing appropriate concentrations of the growth medium. Since it was determined experimentally that a Millipore filter retained a maximum of 40 μl of growth medium, it was unnecessary to correct values obtained from intracellular cAMP for the amount of cAMP in the medium remaining on the filters.

Intracellular concentration of cAMP in terms of molarity was calculated according to Buettner et al. (3) for *E. coli* K-12. In the case of *P. aeruginosa*, protein content was determined from a calibration curve of turbidity versus cell protein concentration. Since protein comprises about 50% of the dry weight and since the intracellular water content of *P. aeruginosa* is 2.7 μl/mg (dry weight) (10), intracellu-

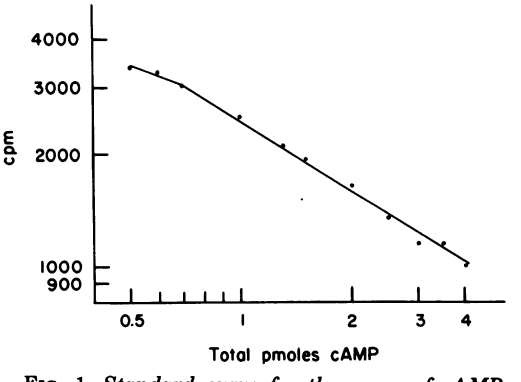

FIG. 1. *Standard curve for the assay of cAMP. The assay mixture contained: 50 mM acetate buffer (pH 4.5); 0 to 3.5 pmol of cAMP; 0.5 pmol of [^{3}H]cAMP (22,500 cpm/pmol); 25 μg of bovine serum albumin; and 16 μg of cAMP-binding protein prepared from beef kidney according to the method of Cheung (4).*

lar concentrations in terms of molarity could be calculated. A similar procedure was used for *B. fragilis*.

Preparation of cell extracts. Cell extracts for use in enzyme assays were prepared as previously described (14), except for studies on the differential rates of synthesis of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) and mannitol dehydrogenase (D-mannitol:NAD^+ 2-oxidoreductase, EC 1.1.1.67). In those cases, cells of *P. aeruginosa* were harvested by centrifugation at 12,000 × *g* for 10 min at 4°C. After washing once with 50 mM potassium phosphate buffer (pH 7.0), the cells were resuspended in 3 ml of ice-cold 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0) containing 10 mM 2-mercaptoethanol and broken by sonic oscillation (Bronwill Biosonik IV) in three 10-s periods alternating with 1 min periods of cooling in ice. The crude cell lysate was centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant fluid was assayed for glucose-6-phosphate dehydrogenase or mannitol dehydrogenase activity. Cell extracts for use in assays for cyclic 3',5'-nucleotide phosphodiesterase (EC 3.1.4.17) and adenylate cyclase (EC 4.6.1.1) were prepared as described previously (15).

Enzyme assays. Glucose-6-phosphate dehydrogenase activity was assayed as previously described (14). The activity of 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (phospho-2-keto-3-deoxy-gluconate aldolase, EC 4.1.2.14) was measured by the method of Meloche and Wood (21). For the determination of mannitol dehydrogenase activity, the reaction mixture included: 50 mM Tris-hydrochloride (pH 8.0), 10 mM 2-mercaptoethanol, 42 mM mannitol, 0.3 mM NAD^+, and cell extract in a final volume of 1.0 ml. All enzyme assays were initiated by the addition of the substrate and were performed at room temperature (22°C). The initial rates of reduction of the pyridine nucleotides were monitored with a Gilford 2400-S recording spectrophotometer.

The activity of cyclic 3',5'-nucleotide phosphodiesterase (EC 3.1.4.17) was assayed as described by Thompson and Appleman (37), and the reaction mixture was modified according to Nielsen et al. (24). The standard reaction mixture contained in a total volume of 0.4 ml: 40 mM Tris-hydrochloride (pH 7.0), 2.5 mM dithiothreitol, 5 mM $MgCl_2$, 10 μM $FeSO_4$, 0.5 mM unlabeled cAMP, [^{3}H]cAMP (22 Ci/mmol, approximately 140,000 cpm per reaction mixture), and cell extract. The initial step of the assay was carried out at 37°C for 5 min. The reaction was terminated by heating for 3 min at 95°C under conditions that prevented evaporation. After cooling the reaction mixture to 37°C, 0.1 mg of snake venom (*Ophiophagus hannah*) was added and the mixture was incubated for 15 min. The reaction was terminated by adding 1.0 ml of a 1:3 (wt/vol) aqueous slurry of Bio-Rad resin (AG 2-X8, 200 to 400 mesh). After centrifugation at 1,500 × *g* for 5 min, 0.5 ml of supernatant fluid was placed in a scintillation vial, 8 ml of scintillation fluid (4 g of Soli-mix, 333 ml of Triton X-100, 667 ml of toluene) was added, and the radioactivity was determined. Boiled crude cell extract samples were used as controls. The specific activities of cAMP phosphodiesterase were determined at a protein concentration where the initial velocities were linear with respect to protein concentration and time. About 6 to 9% of the radioactivity present in the commercial [^{3}H]cAMP was not removed by the anionic resin. Ascending paper chromatography of [^{3}H]cAMP on Whatman 3MM paper with a solvent system of ethanol-1 M ammonium acetate (75:30) (6) indicated that most of the unbound label comigrated with a commercial adenosine standard. Hence, the necessity of using boiled enzyme controls for background activities must be emphasized.

Adenylate cyclase (EC 4.6.1.1) activity was assayed as described by Khandelwal and Hamilton (16), using [^{3}H]adenosine 3'-triphosphate (ATP). An ATP-regenerating system consisting of 5 mM phosphoenolpyruvate and 1.62 IU of pyruvate kinase was used. [^{3}H]cAMP formed in the reaction mixture was isolated by paper chromatography according to the method of Drummond and Duncan (6). Unlabeled cAMP, to a final concentration of 5 mM, was added as a carrier to each reaction mixture.

Protein was determined by the method of Lowry et al. (19) using crystalline bovine serum albumin as the standard.

Materials. [8-^{3}H]cAMP (16 or 23 Ci/mmol) was purchased from Schwarz/Mann (Orangeburg, N.Y.). New England Nuclear Corp. (Boston, Mass.) supplied [^{3}H]cAMP (22 Ci/mmol), [^{3}H]ATP (20 to 40 Ci/mmol), and Aquasol. Soli-mix was purchased from ICN. NAD^+, $NADP^+$, and cAMP were obtained from P-L Biochemicals (Milwaukee, Wis.). Sigma Chemical Co. (St. Louis, Mo.) provided glucose-6-phosphate and snake venom (*Ophiophagus hannah*). Pyruvate kinase was supplied by Calbiochem (Los Angeles, Calif.). All other chemicals were obtained commercially and were of the highest purity available.

RESULTS

Intracellular and extracellular levels of cAMP during growth of *P. aeruginosa* on a variety of carbon sources. *P. aeruginosa* PAO was cultured on a variety of carbon sources, and the cAMP levels in the cells and in the culture filtrate were measured throughout growth. The results from a typical experiment, in which 20 mM glucose was used as the carbon source, are depicted in Fig. 2A. It should be noted that the intracellular concentrations of cAMP are graphed on a 10^{-5} M scale, whereas the extracellular concentrations are plotted on a 10^{-9} M scale. The intracellular levels of cAMP remained relatively unchanged throughout the growth cycle when glucose served as the sole carbon and energy source. In contrast, intracellular cAMP in *E. coli* has been shown to rise markedly in the early stationary phase of growth (2, 3, 20), with increases from 5-fold (2, 20) to 30-fold (3) reported. The filtrate levels of cAMP for glucose-grown cultures of *P.*

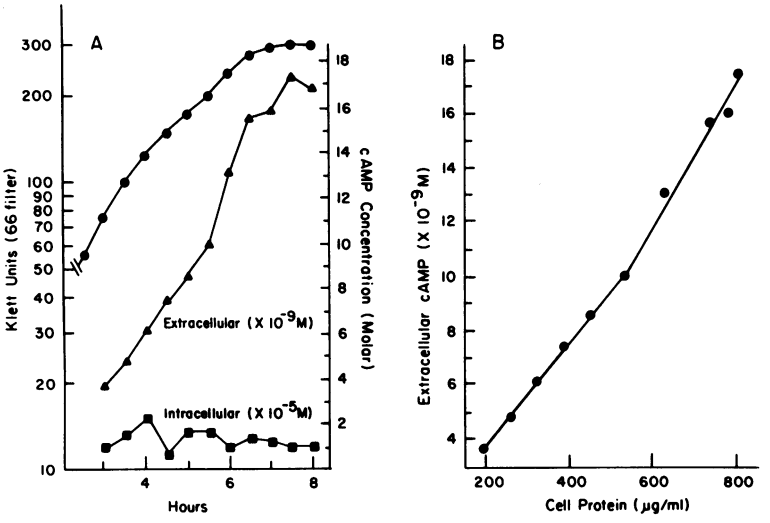

FIG. 2. *Intracellular and extracellular concentrations of cAMP in cultures of Pseudomonas aeruginosa PAO during growth in basal salts medium plus 20 mM glucose. cAMP concentrations were determined throughout growth as described in Materials and Methods. (A) Symbols: growth (●), intracellular cAMP (■), and extracellular cAMP (▲). (B) Relationship between extracellular cAMP concentration and the concentration of total cell protein in the culture.*

aeruginosa increased approximately fourfold in proportion to growth (Fig. 2). This extracellular cAMP was found to increase linearly with total cell protein during log phase (Fig. 2B). However, during the transition from late log to early stationary phase, the culture filtrate cAMP level increased more markedly (Fig. 2). A similar relationship between total cell protein and extracellular cAMP concentration has been reported for Crookes strain of *E. coli*, but the filtrate levels of cAMP in this organism rose much more markedly, at least 25-fold during growth (22).

Intracellular and extracellular concentrations of cAMP in cultures of *P. aeruginosa* PAO at comparable stages of the growth cycle with various carbon sources are shown in Tables 1 and 2, respectively. Cellular cAMP levels remained relatively unchanged throughout the growth cycles, and values for cells grown on each of the substrates were similar (Table 1). Extracellular cAMP levels increased in proportion to cell mass for each substrate, except acetate, and there is no marked difference in values for the various carbon sources, although the levels in the succinate culture filtrates are somewhat higher (Table 2). Neither the intracellular nor the extracellular concentration of cAMP was significantly altered by the addition of carbon sources that cause strong catabolite repression to cells that were initially growing on a less preferred carbon source.

Catabolite repression control of inducible enzyme synthesis in *P. aeruginosa*. Succinate and certain other organic acids have been reported to exert strong catabolite repression of synthesis of the inducible enzymes for catabolism of glucose and other carbohydrates in *Pseudomonas* (14, 17, 23). The repression of induced synthesis of glucose-6-phosphate dehydrogenase and KDPG aldolase, key enzymes for carbohydrate metabolism in *Pseudomonas*, was evaluated by measuring the differential rates of formation of these enzymes after the addition of succinate to cells that were growing exponentially on glucose. There is an immediate and strong decrease in the differential rate of synthesis of glucose-6-phosphate dehydrogenase upon the addition of either 5 mM or 10 mM succinate (Fig. 3A). The duration of this strong repression was dependent on the amount of succinate that was added to the growth medium. When 20 mM succinate (or more) was added, repression was even stronger and induced rates of enzyme synthesis did not resume during the course of the experiment (data not shown). The differential rate of synthesis of KDPG aldolase also was decreased immediately upon the addition of 10 mM succinate (Fig. 3B) as was that of glucokinase (data not shown). The strength and duration of catabolite repression of KDPG aldolase and glucokinase formation also were found to be dependent on the amount of succinate that was added to the growing culture.

Effect of cAMP on catabolite repression control of inducible enzyme synthesis in *P. aeruginosa*. Catabolite repression of induced

TABLE 1. *Effect of carbon source and growth stage on intracellular cAMP concentrations in Pseudomonas aeruginosa*[a]

Carbon source (mM) for growth	Generation time (min)	Intracellular cAMP concn (10^{-5} M) at:			
		Early log phase	Midlog phase	Late log phase	Early stationary phase
Succinate 40	74	0.98	0.97	1.62	1.22
Glucose 20	78	0.89	2.02	1.26	1.26
Lactate 40	79	1.32	1.84	2.70	2.19
Acetate 50	82	1.08	1.46	1.04	1.68
Gluconate 20	97	1.12	1.31	1.90	2.69
Glycerol 40	192	1.40	0.86	1.51	3.16

[a] All procedures are described in Materials and Methods.

TABLE 2. *Effect of carbon source and growth stage on extracellular cAMP concentrations in Pseudomonas aeruginosa*[a]

Carbon source (mM) for growth	Generation time (min)	Extracellular concn of cAMP (10^{-9} M) at:			
		Early log phase	Midlog phase	Late log phase	Early stationary phase
Succinate 40	74	8.6	10.2	19.5	19.8
Glucose 20	78	3.8	5.4	11.1	15.4
Lactate 40	79	4.4	5.7	12.5	15.4
Acetate 50	82	4.8	5.6	5.8	7.0
Gluconate 20	97	3.5	4.2	13.1	13.3
Glycerol 40	192	5.4	6.0	10.0	19.5

[a] All procedures are described in Materials and Methods.

enzyme synthesis by glucose in *E. coli* can be reversed by the exogenous addition of cAMP (26, 28, 40). To determine the effect of exogenous cAMP on catabolite repression control in *P. aeruginosa* PAO, 5 mM sodium cAMP was added with succinate to cultures that were initially growing on glucose. Exogenous cAMP failed to overcome the repression of glucose-6-phosphate dehydrogenase synthesis that occurred upon the addition of succinate (Fig. 4). Similarly, the addition of 5 mM sodium cAMP did not overcome the repression of mannitol dehydrogenase caused by the addition of either glucose or succinate to cultures that were initially growing on mannitol (Fig. 5A and B).

Comparative studies on cAMP concentrations and activities of related enzymes in selected gram-negative bacilli. *E. coli* and *P. aeruginosa* contained similar intracellular cAMP concentrations (Table 3). This material was shown to be labile to cAMP phosphodiesterase treatment. In contrast, the amount of cAMP determined in samples of filtered cells of *B. fragilis* was at the limit of detectability for the assay and was not degraded by cAMP phosphodiesterase (15). The extracellular concentration of cAMP was substantially higher for *E. coli* than for *P. aeruginosa*, whereas cAMP could not be detected in culture filtrates of *B. fragilis*. However, technical difficulties precluded the assay of concentrated filtrates of *B. fragilis* cultures since the presence of the concentrated medium interfered with the binding of cAMP to the protein and a reliable standard curve could not be obtained.

cAMP phosphodiesterase activity was readily measured in the soluble fraction of extracts of *E. coli* and *P. aeruginosa* (Table 3). For *P. aeruginosa*, the activity of this enzyme was constant, irrespective of the cultural conditions, and the specific activity was approximately 10-fold lower than that of *E. coli*. Activity could not be detected in extracts of *B. fragilis* under a number of assay conditions. For example, cell extracts of *E. coli* were mixed with extracts of *B. fragilis* without loss of the cAMP phosphodiesterase activity from *E. coli*. Furthermore, when 0.1 mM exogenous sodium cAMP was added to growing cultures of *B. fragilis* and samples were taken throughout the growth cycle (18 h), no detectable cAMP degradation occurred.

Crude cell extracts of these bacteria were assayed for adenylate cyclase activity. Evidence was obtained for the presence of this enzyme in *P. aeruginosa* and in the known positive control, *E. coli*, but activity could not be detected in *B. fragilis* (Table 3). All of the adenylate cyclase activity was found in subsequent determinations to be associated with the particulate fraction of cell extracts. These data are of only qualitative significance; quantita-

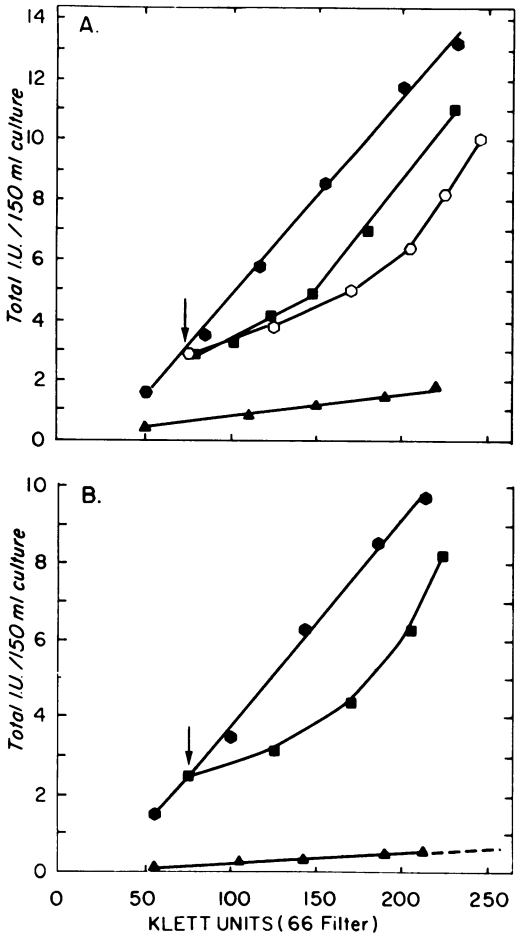

FIG. 3. *Catabolite repression of glucose-6-phosphate dehydrogenase and KDPG aldolase synthesis after the addition of succinate to cells of P. aeruginosa OSU-64 growing exponentially on 35 mM glucose. (A) Differential rate of synthesis of glucose-6-phosphate dehydrogenase in cells grown on 35 mM glucose (●), 40 mM succinate (▲), or upon the addition of 5 mM or 10 mM succinate plus 10 mM additional glucose (■) and (○), respectively. (B) Differential rate of synthesis of KDPG aldolase. Cultures and symbols as described for (A). Enzyme activities are expressed as total IU per 150 ml of culture as a function of culture density (Klett units with a number 66 filter). Additions of succinate plus more glucose were made when the culture densities reached 75 Klett units (arrows).*

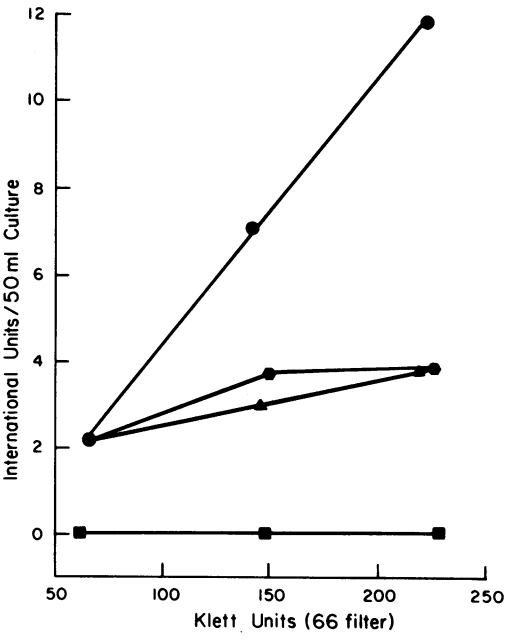

FIG. 4. *Differential rates of synthesis of glucose-6-phosphate dehydrogenase in P. aeruginosa PAO. Enzyme activities (IU per 50 ml of culture) plotted as a function of culture density (Klett units with a number 66 filter) are shown for cells grown on 20 mM glucose (●), 40 mM succinate (■), 20 mM glucose plus the addition of 10 mM succinate immediately after removal of the first sample (▲), and 20 mM glucose plus the addition of 10 mM succinate and 5 mM sodium cAMP immediately after removal of the first sample (⬢).*

tive determinations of this activity could not be made due to the presence of competing enzymes which rapidly degraded the substrate and the product. All cell extracts contained high levels of adenosine triphosphatase activity, and cAMP phosphodiesterase activity was a problem in cell extracts of *P. aeruginosa* and *E. coli*. Several attempts were made to improve the assay conditions, i.e., incorporation of an ATP-regenerating system and addition of unlabeled exogenous cAMP to quench cAMP phosphodiesterase activity. However, the values obtained for the specific activities of adenylate cyclase in crude extracts remained highly variable and were interpreted as having only qualitative significance.

DISCUSSION

Shapiro et al. (36) demonstrated the presence of cAMP in acid extracts of nongrowing cells of *P. aeruginosa* that had been harvested by centrifugation and assayed by a modification of the Gilman method (9). Resting cells incubated in phosphate buffer were reported to contain a ninefold higher intracellular concentration of cAMP than did cells incubated in the same buffer containing 1% glucose (36). Makman and Sutherland (20) had reported similar results from experiments with resting cells of *E. coli*, for which glucose is a strongly preferred carbon source (25). In the present experiments, with actively growing cells of *P. aeruginosa*, the

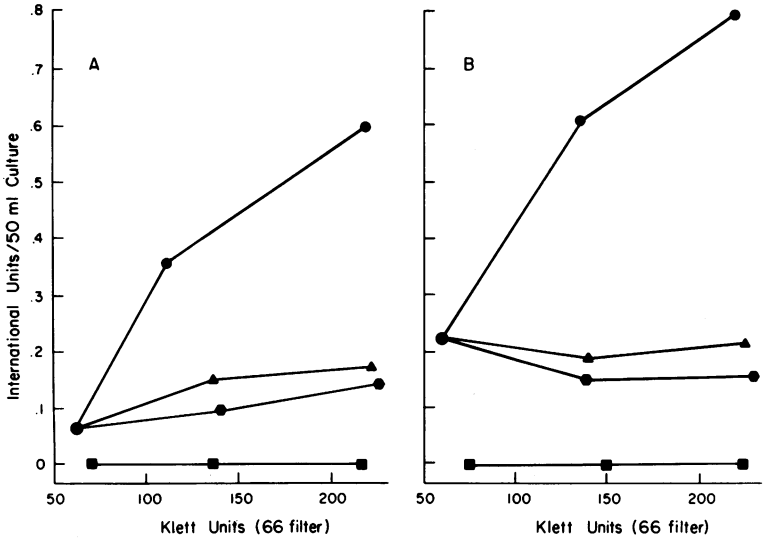

FIG. 5. *Differential rates of synthesis of mannitol dehydrogenase in P. aeruginosa PAO. (A) Enzyme activities (IU per 50 ml of culture) plotted as a function of culture density (Klett units with a number 66 filter) are shown for cells grown on 30 mM mannitol (●), 20 mM glucose (■), 30 mM mannitol plus the addition of 20 mM glucose immediately after removal of the first sample (▲), and 30 mM mannitol plus addition of 20 mM glucose and 5 mM sodium cAMP immediately after removal of the first sample (⬢). (B) Enzyme activities (IU per 50 ml of culture) plotted as a function of culture density (Klett units with a number 66 filter) are shown for cells grown on 30 mM mannitol (●), 40 mM succinate (■), 30 mM mannitol plus the addition of 20 mM succinate immediately after removal of the first sample (▲), and 30 mM mannitol plus the addition of 20 mM succinate and 5 mM sodium cAMP immediately after removal of the first sample (⬢).*

TABLE 3. *cAMP concentrations and activities of adenylate cyclase and cAMP phosphodiesterase in Pseudomonas aeruginosa, Escherichia coli K-12, and Bacteroides fragilis ATCC 25285*

Organism (gram-negative rod)	Physiological type	Intracellular cAMP concn (10^{-5} M)[a]	Extracellular cAMP concn (10^{-9} M)[b]	cAMP phosphodiesterase[c]	Adenylate cyclase[d]
P. aeruginosa PAO	Obligate aerobe	0.8–3.2	3.5–19.8	21	+
E. coli K-12	Facultative anaerobe	1.0–4.0	110–230	203	+
B. fragilis ATCC 25285	Obligate anaerobe	ND[e]	ND	<1	−

[a] Ranges of intracellular cAMP concentrations determined irrespective of the carbon source or the growth stage of the culture.

[b] Range of extracellular cAMP concentrations determined from early log to early stationary phase of growth irrespective of carbon source.

[c] Cells of *P. aeruginosa*, *E. coli*, and *B. fragilis* were grown in chemically defined media containing 30 mM glucose. Enzyme activity is expressed as nanomoles of cAMP hydrolyzed per 5 min per mg of protein in the soluble fraction of cell extracts (105,000 × *g*, 2 h).

[d] Enzyme activity is expressed only as detectable (+) or not detectable (−) in crude cell extracts or in the particulate fraction of cell extracts (105,000 × *g*, 2 h) as described in Materials and Methods.

[e] ND, No detectable cAMP.

intracellular concentrations of cAMP remained relatively unchanged during the growth cycle even when the culture entered stationary phase due to the depletion of the carbon source (Table 1; Fig. 2A). Moreover, varying the nature of the sole carbon source had little effect on intracellular cAMP levels in the organism (Table 1). The extracellular cAMP concentration increased with the culture absorbance (Fig. 2A; Table 2) but was unaffected by the nature of the carbon source (Table 2). The range of extracellular concentrations of cAMP was markedly lower (up to 60-fold) in cultures of *P. aeruginosa* than in cultures of *E. coli*, although both organisms contained very similar intracellular concentrations of cAMP (Table 3). It would be highly speculative to infer that this difference in extracellular pools reflects a significant difference in the physiological role(s) of cAMP in these bacteria. However, it is known that *E. coli* rapidly excretes large quantities of cAMP under nutritional conditions that cause catabo-

lite repression of inducible enzymes (3, 20, 22), and there is evidence that excretion is an important mechanism for regulation of the intracellular cAMP pool in *E. coli* (34).

These experiments on cAMP pools were conducted with two wild-type strains of *P. aeruginosa* (PAO and OSU-64) and the results were essentially the same. It should be noted that the range of intracellular cAMP concentrations in these strains was 10-fold or more higher than that reported by Shapiro et al. (36). The difference most likely was due to our use of a rapid filtration method for cell harvesting, since our values also were more than 10-fold lower when cell samples were harvested by centrifugation (12,000 × *g*, 10 min) (Hylemon and Phibbs, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P198, p. 174). The importance of rapid cell sampling and extraction to quantitative assay of cellular cAMP has been emphasized by Rickenberg (32).

It has been firmly established that cAMP is a key regulatory effector in the control of synthesis of inducible catabolic enzymes in *E. coli* and many other bacteria (32). Gilboa-Garber et al. (8) have reported what appears to be the only experimental evidence for a similar regulatory role for cAMP in *Pseudomonas*. These workers reported that formation of an inducible cholinesterase, in a clinical isolate identified as *P. aeruginosa*, was sensitive to catabolite repression when cells were grown in the presence of glucose and that repression was substantially released when the growth medium contained 5 mM cAMP (8). The effect of a more highly preferred carbon source such as succinate on the induced formation of cholinesterase was not reported.

P. aeruginosa utilizes certain organic acids (acetate, Krebs cycle intermediates) preferentially to glucose or other carbohydrate compounds and these organic acids exert strong catabolite repression control over induction of the transport systems and catabolic enzymes required for carbohydrate utilization (14, 17, 23, 38; Fig. 3). However, the addition of up to 5 mM sodium cAMP failed to relieve the catabolite repression by succinate of inducible glucose-6-phosphate dehydrogenase synthesis (Fig. 4). Similarly, exogenously added cAMP was ineffective in overcoming the strong catabolite repression by glucose or succinate of inducible mannitol dehydrogenase synthesis (Fig. 5). In replicate experiments not shown here, nearly identical negative results were obtained when 5 mM dibutyryl cAMP was used in place of sodium cAMP. Moreover, the repression of other inducible hexose catabolic enzymes (i.e., glucokinase and KDPG aldolase) also was unaffected by the exogenous addition of cAMP. These results are not necessarily in conflict with those of Gilboa-Garber et al. (8) since it is possible that certain enzymes in this organism may require cAMP for induction and others may not. It is also possible that strains may vary in permeability to exogenous cAMP. In this regard, we have made repeated attempts to measure the uptake and accumulation of [^{3}H]- and [^{14}C]cAMP by *P. aeruginosa* over wide ranges of cAMP concentration and specific radioactivity. Under no condition could cAMP uptake be detected. Therefore, the failure of added cAMP to affect the catabolite repression of induced enzyme formation may reflect impermeability of these strains of *P. aeruginosa* to exogenous cAMP.

It may be possible that the observed twofold change in the intracellular concentration of cAMP in *P. aeruginosa* (Table 1) may be sufficient to allow function as a regulatory effector. In this regard, Buettner et al. (3) have proposed that catabolite repression is sensitive to threshold concentrations of cAMP. Since the transcription of catabolite-sensitive operons in *E. coli* is not regulated by cAMP alone but by a complex between cAMP and its binding protein, perhaps the level of this complex is altered without a change in the intracellular concentration of cAMP, as suggested by Haggerty and Schleif (11). Effects on the synthesis of cAMP-binding proteins has also been suggested to be involved in catabolite repression control (3). At this time, there appears to be no information on whether *Pseudomonas* contains such a cAMP-binding protein.

We have demonstrated the presence of a membrane-associated adenylate cyclase in *P. aeruginosa* but have been unable thus far to study any catalytic regulatory properties of the enzyme because of the presence of high activities of competing enzymes (e.g., adenosine triphosphatase). cAMP phosphodiesterase activity was detected in the soluble fraction of cell extracts of *Pseudomonas* and the specific activity remained relatively constant under all growth conditions that were used. In preliminary studies (not shown) the enzyme from *P. aeruginosa* OSU-64 was partially purified by ammonium sulfate fractionation; pyruvate and certain other organic acids were found to have no effect on the substrate (cAMP) saturation kinetics of the enzyme preparation. Additional studies on the catalytic properties of these enzymes from *Pseudomonas* may lead to some better understanding of the physiological role of cAMP in this bacterium.

Present information on the physiological role(s) of cAMP in bacteria has derived primarily from studies of *E. coli* and other facultative anaerobic bacteria (32). There is limited information on the distribution and function of cAMP over a range of physiologically and taxonomically dissimilar bacteria. cAMP has not been detected in the typical gram-positive aerobic sporeformers, *Bacillus subtilis* (20), *Bacillus megaterium* (35), and *Bacillus licheniformis* (2), although numerous inducible catabolic enzymes are regulated by catabolite repression control in these bacteria. In a preliminary report from this laboratory, evidence was presented against the presence of cAMP, adenylate cyclase and cAMP phosphodiesterase in *Bacteroides fragilis*, a gram-negative, obligately anaerobic bacterium (15). The present results from comparative experiments provide further insight into the significant variation that occurs in the distribution of cAMP and its associated enzymes among widely divergent physiological types of gram-negative bacteria.

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