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Cyclic Adenosine 3',5'-Monophosphate Levels in *Pseudomonas putida* and *Pseudomonas aeruginosa* During Induction and Carbon Catabolite Repression of Histidase Synthesis†

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Inducibility of histidase (histidine ammonia-lyase, EC 4.3.1.3) in *Pseudomonas putida* and *Pseudomonas aeruginosa* was observed to be strongly affected by succinate-provoked catabolite repression, but this did not occur as a consequence of reduced intracellular cyclic adenosine 3',5'-monophosphate levels, and repression could not be alleviated by exogenously added cyclic adenosine 3',5'-monophosphate. Milder repression of histidase by lactate was also not reversed by the addition of cyclic adenosine 3',5'-monophosphate. These results, along with data showing intracellular cyclic adenosine 3',5'-monophosphate levels remained essentially constant during growth on such diverse carbon sources as histidine, acetamide, glucose, and succinate, indicated that catabolite repression of histidase synthesis by efficient carbon sources was not mediated through variations in internal cyclic adenosine 3',5'-monophosphate.

It is generally recognized that transcription of the genes for histidine utilization (*hut*) in *Klebsiella aerogenes* is subject to catabolite repression by glucose and that cyclic AMP (cAMP) and its binding protein (CAP) activate transcription of this and other catabolite repression-sensitive operons in enteric organisms (15, 25). There is, however, no clear indication of a similar role for cAMP in controlling *hut* gene expression in *Pseudomonas* species. Potts and Clarke (17) suggested that *hut* genes of *Pseudomonas aeruginosa* are regulated by a cAMP-dependent CAP protein, as occurs in *K. aerogenes*, but this was not directly shown. What has been established for *Pseudomonas* spp. *hut* genes is induction of enzyme synthesis by urocanate and repression by growth on efficient carbon sources such as succinate and glutamate (7, 10, 11), but much less severe repression by the poorer carbon sources glucose and pyruvate (17); however, succinate is able to repress only when cells are grown with excess nitrogen (7, 17). If this succinate-provoked catabolite repression in *Pseudomonas* spp. is analogous to glucose-related catabolite repression in *Escherichia coli* and *K. aerogenes*, one would anticipate that cAMP levels would be lower in cells growing on succinate compared with those in cells grown on glucose, as was observed in *E. coli* for cells grown on glucose (a good carbon source) compared with those grown on glycerol (8). Repression of the histidine utilization enzymes in *Pseudomonas* spp. by succinate could therefore be ascribed to lowered intracellular cAMP.

Two reports have presented estimates for intracellular cAMP levels in *P. aeruginosa*. The first, by Shapiro et al. (21), gave values only for mid-logarithmic phase cells grown on glucose and then transferred to phosphate buffer, with and without glucose. These values, 0.3 mM and 2.9 mM, respectively, suggested that cAMP accumulates in nongrowing cells when glucose is absent, analogous to what was observed for *E. coli* by Makman and Sutherland (12). In a later, more extensive study conducted by Siegel et al. (22), intracellular cAMP was measured at various growth stages and with a variety of carbon sources, including glucose and succinate. This investigation revealed that intracellular cAMP levels did not change appreciably during growth or with different carbon sources. The reported values ranged, without trending, from 8 to 32 μM and averaged around 16 μM for six carbon sources at four growth stages.

Because we were interested in possible positive control by cAMP of *hut* gene expression in *Pseudomonas putida*, we considered it important to demonstrate whether cAMP levels and histidase (histidine ammonia-lyase, EC 4.3.1.3)

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synthesis were concomitantly affected by catabolite repression in *P. putida* and in *P. aeruginosa* PAO, where the bulk of data on cAMP levels has been obtained. The present report examines cAMP levels in several species of *Pseudomonas* and describes experiments which show that intracellular cAMP concentrations do not reflect the degree of catabolite repression of histidase synthesis in *P. putida* or *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* PAO was furnished by R. W. Bernlohr; this is the strain used by Siegel et al. (22). *P. putida* ATCC 12633, *Pseudomonas oxalaticus* ATCC 11883, and *Pseudomonas testosteroni* ATCC 17511 were purchased from the American Type Culture Collection. *E. coli* Crooks strain and strain 5336 (*cya*) have been described previously (16).

Growth conditions. Growth of *P. aeruginosa* was conducted at 37°C in the salts mixture of Hylemon and Phibbs (9) with 20 mM glucose, 33 mM sodium succinate, 20 mM sodium pyruvate, 15 mM L-histidine, or 60 mM acetamide as the carbon source. In some experiments, salts A (13) without citrate and with Na_2SO_4 replacing $(NH_4)_2SO_4$ was used in place of the Hylemon and Phibbs salts mixture. *P. putida* was grown on the same media, but at 30°C. *P. oxalaticus* was grown on oxalate medium (18) at 30°C, and *P. testosteroni* was grown at 30°C on 0.1% *p*-hydroxybenzoate as the carbon source in the medium of Stanier et al. (24). The two *E. coli* strains were grown at 37°C on a glycerol-fumarate-salts A medium supplemented with 1% Difco Casamino Acids (16). All cultures received vigorous reciprocal shaking; growth was usually conducted in 400 ml of medium contained in a 2-liter Erlenmeyer flask, with samples taken as required for enzyme or cAMP analysis.

Enzyme and protein assays. Samples (10 ml) of cultures were taken at prescribed times and immediately chilled in ice. The samples were then centrifuged at 10,000 × *g* for 10 min at 4°C, and the cells were stored overnight at −20°C. Cell extracts were prepared in 50 mM potassium phosphate (pH 7.5) by sonic treatment. Histidase was assayed spectrophotometrically at 277 nm by the procedure of Rechler (19), except the assays were scaled down to a 0.2-ml final volume. Activities are expressed as micromoles of urocanate formed per minute. Protein was determined by the method of Bradford (2), with bovine serum albumin as the standard.

Amidase (EC 3.5.1.4) assays were performed by the method of Brammar and Clarke (3).

Measurements of intracellular and extracellular cAMP. Cultures were inoculated to 2×10^7 cells per ml from an overnight sample in the same medium. At various times, usually corresponding to absorbance values at 660 nm (A_{660}) of 0.35 and 0.55, 25-ml samples were rapidly filtered (<30 s) on 90-mm membrane filters (0.45-μm pore size; Millipore Corp.) and immediately washed with 5 ml of cold 0.2 M Na_2SO_4. Each filter was submerged in 15 ml of 5% trichloroacetic acid at 0°C. Except when the cAMP radioimmunoassay was to be conducted, this solution contained 5,000 cpm of [^{3}H]cAMP (0.2 pmol) to permit recovery estimation. After 1 h, the extract was transferred to a conical centrifuge tube along with an additional 2 ml of acid solution used for washing. This solution was centrifuged at 4,000 × *g* for 10 min, and the supernatant was transferred to a neutral alumina column prepared as described by Cook et al. (6). The sample was rinsed into the column with 3 ml of 5% trichloroacetic acid, washed twice with 4 ml of 50% aqueous acetone, and eluted from the alumina column directly onto a column of Bio-Rad AG-1X8-formate (6) with three 10-ml washes of 50 mM 1,2-diaminopropane-formate (pH 6.5). The latter column was washed with 10 ml of water, 10 ml of 0.1 M formic acid, and finally 10 ml of 2 M formic acid. The 2 M formic acid eluate was dried overnight at 37°C with a gentle dry air stream; the wall of each conical sample tube was washed down with 1 ml of 95% ethanol, and this solvent was evaporated with air. Samples were then dissolved in 0.2 ml of 5 mM potassium phosphate (pH 7.5), 50 μl was counted to determine recovery, and portions of 25 and 50 μl were assayed for cAMP by using the human erythrocyte ghost binding method of Epstein et al. (8). Alternatively, samples were redissolved in 0.25 ml of 5 mM 2-(*N*-morpholino)ethane sulfonate (pH 7.0), and 100-μl portions were assayed for cAMP by a commercial kit radioimmunoassay procedure (New England Nuclear Corp.).

Samples for estimation of extracellular cAMP were obtained from the filtrate of the membrane filtration. A 15-ml portion of the filtrate (total volume, 30 ml) was made 5% in trichloroacetic acid and applied directly to the alumina column as described above.

Extracts of cells or portions of extracellular fluids, when purified through the double column procedure, contained 80 ± 5% of the ^{3}H recovery standard in the 2 M formic acid eluate. Incorporation of a known quantity of unlabeled cAMP in random samples confirmed the ability of the erythrocyte binding assay to give reliable results on samples which had been carried through the entire procedure. When the radioimmunoassay procedure was conducted, recovery estimates were determined from a standard sample of cAMP which was carried through the column procedure and assayed at the same time as the unknowns.

Calculations of intracellular cAMP concentrations were based on values of internal cell volume, determined from cell numbers and the relationship 7.5×10^{-13} ml of internal volume per bacterium (4). Cell numbers were estimated by A_{660} measurements with a Gilford Model 240 spectrophotometer (1-cm path length); an A_{660} of 0.18 equalled 10^8 cells per ml.

Repression of histidase formation in *P. aeruginisa* and *P. putida*. The usual procedure for repression of histidase formation was to prepare an overnight culture in minimal medium containing 0.2% glucose or 0.2% sodium pyruvate as the carbon source. The next day a 300-ml portion of the same medium was inoculated with the overnight culture to an A_{660} of 0.05, and the new culture was incubated with shaking at the appropriate temperature. Periodically, samples were taken for histidase assay as described above. At the times indicated in each figure, a solution of filter-

sterilized L-histidine (0.5 M, neutralized) was added to yield a final concentration of 5 mM. At a later time, the culture was split into three portions; to one was added nothing; to the second was added either 1 M sodium succinate or 1 M sodium lactate to a final concentration of 10 mM; and to the third was added lactate or succinate plus cAMP, the latter at either 10 or 30 mM final concentration.

In some cases, repression by succinate was initiated in cultures which had been pregrown on histidine medium to provide a high and constant specific activity for histidase. These cultures were grown in 15 mM L-histidine as the sole carbon and nitrogen source. Repression was achieved by the addition of succinate to 10 mM concentration and $(NH_4)_2SO_4$ to 0.1%.

RESULTS

cAMP concentrations under different growth conditions. Table 1 illustrates the results of cAMP analyses for *P. aeruginosa* grown under a variety of conditions. Three findings were apparent. First, the levels of cAMP were essentially invariant on the different media. Growth on acetamide or histidine did not result in consistently higher internal cAMP concentrations than those seen with succinate or glucose as the carbon source, despite reports that cAMP is required for the induction of amidase (23) and possibly histidase (17). The induction of these enzymes is necessary for utilization of acetamide and histidine, respectively, in *P. aeruginosa* (5); in fact, enzyme assays for amidase and histidase showed at least a 40-fold elevation of these activities in extracts of cells grown on acetamide or histidine, respectively, as the carbon source compared with those of cells grown on succinate or glucose and ammonium nitrogen.

A second point noted in our results was that starvation for carbon in a manner similar to that used for *E. coli* by Makman and Sutherland (12) did not result in a large increase in intracellular cAMP. Whereas this observation did not confirm the finding of Shapiro et al. (21), our estimates for cAMP levels (0.65 μM with glucose and 0.94 μM after starvation) were intermediate between their values and thus do not completely eliminate slightly larger differences being possible under other experimental conditions.

TABLE 1. *cAMP levels in P. aeruginosa grown with various carbon sources*

Carbon source	Culture density (cells/ml)	Assay[a]	Intracellular cAMP (μM)[b]
Glucose	3.2×10^8	EBP	0.65 ± 0.20
	3.1×10^8	RIA	0.59 ± 0.07
Succinate	3.0×10^8	RIA	0.72 ± 0.18
Acetamide	3.0×10^8	EBP	0.45 ± 0.08
	2.8×10^8	RIA	0.67 ± 0.24
Histidine	3.2×10^8	EBP	0.68 ± 0.13
	2.5×10^8	RIA	0.56 ± 0.19
Glucose, then starved	3.2×10^8	EBP	0.94 ± 0.21

[a] EBP, Erythrocyte ghost binding protein; RIA, radioimmunoassay.

[b] Results shown are averages ± standard deviations for four to six independent analyses.

Third, the data were in accord with the general conclusion drawn by Siegel et al. (22) that internal cAMP concentrations were not lower in cells grown on succinate than they were in glucose-grown cells, contrary to the expectation that conditions of strong catabolite repression (i.e., growth on succinate) would decrease internal cAMP levels. However, the values we observed were much lower, at least 10-fold, than those reported by Siegel et al. (22), notwithstanding the facts that several media were identical, the organism was the same, and a similar cAMP binding protein assay was employed. Moreover, when a radioimmunoassay procedure (^{125}I type) was used in place of the erythrocyte binding protein method, essentially similar results were obtained (Table 1).

Further investigation of the source of the difference between our results and those of Siegel et al. (22) centered on three apparent differences in technique: conditions for extraction, washing versus not washing of cells before extraction, and purification versus no purification of cAMP before analysis. We obtained values similar to those reported by Siegel et al. (22) when extracts were not purified through the alumina and AG-1-formate columns (Table 2). Contrary to their claim, we found that the bulk (>80%) of this unpurified material which responded as cAMP in the binding protein assay was not destroyed by cyclic nucleotide phosphodiesterase (data not shown), but we have not pursued its identification further. We conclude that our higher values observed for unpurified samples, and perhaps the values reported by Siegel et al. (22), were the result of a contaminating material which responds in the cAMP binding protein assay. We also believe that this same situation may have existed for the analysis of extracellular cAMP by Siegel et al. (22), because we were unable to detect cAMP in column-purified samples of culture filtrates above the level of 0.2 pmol per 25-ml portion of filtrate obtained from mid-log phase growth (3×10^8 cells per ml); Siegel et al. (22) reported an external concentration of 400 pmol/ml for comparable growth in the same medium.

The levels of cAMP in several other species of

Pseudomonas were examined briefly, and these are reported in Table 3. Significantly less cAMP was found in each of the other pseudomonads than had been detected in *P. aeruginosa*. In fact, the very low levels were satisfactorily measured only by the radioimmunoassay procedure. The level in *P. testosteroni* grown on *p*-hydroxybenzoate was just slightly above that for a *cya* mutant of *E. coli*. A more extensive set of growth conditions and media is required before it can be concluded that *P. testosteroni* makes cAMP in a meaningful amount. Also, the cAMP level for glycerol-grown *E. coli* Crooks strain, analyzed by radioimmunoassay or by the erythrocyte protein binding assay, was 8 μM, a value consistent with those reported for other *E. coli* strains grown on glycerol (15); this would suggest that our assay procedures do, in fact, estimate intracellular cAMP with an efficiency similar to that of other investigators.

TABLE 2. *Apparent cAMP levels in P. aeruginosa as affected by sample preparation*[a]

Treatment[b]	Purification[c]	Apparent cAMP (μM)[d]
Wash, cold trichloroacetic acid extract	Yes	0.59 ± 0.18 (4)
Wash, hot HCl extract	Yes	0.63 ± 0.31 (6)
No wash, hot HCl extract	Yes	0.95 ± 0.50 (4)
Wash, cold trichloroacetic acid extract	No	7.5 ± 3.9 (4)
Wash, hot HCl extract	No	12 ± 6.5 (4)
No wash, hot HCl extract	No	23 ± 12 (4)

[a] Cells were grown to a density of 2.5×10^8 per ml with glucose as the carbon source.

[b] Wash treatment was with 5 ml of cold 0.2 M Na_2SO_4. Extraction was either by 15 ml of 5% trichloroacetic acid at 0°C for 1 h, or by 15 ml of 0.1 N HCl at 95°C for 10 min.

[c] Purification refers to absorption and elution from columns of neutral alumina and AG-1-formate, as described in the text. When no purification was attempted, samples were simply lyophilized after extraction with HCl; when trichloroacetic acid was used, it was removed by ether extraction before lyophilization.

[d] Average ± standard deviation, with the number of determinations indicated within parentheses. Assay was by the erythrocyte binding protein method.

TABLE 3. *cAMP levels in several species of Pseudomonas and in E. coli*[a]

Organism	Carbon source	Culture density (cells/ml)	Intracellular cAMP (μM)[b]
P. putida	Glucose	2.9×10^8	0.09 ± 0.02
	Histidine	2.9×10^8	0.12 ± 0.05
	Succinate	3.2×10^8	0.07 ± 0.02
P. testosteroni	*p*-Hydroxybenzoate	3.1×10^8	0.04 ± 0.02
P. oxalaticus	Oxalate	2.5×10^8	0.21 ± 0.10
E. coli Crooks	Glycerol	3.0×10^8	8.0 ± 2.2[c]
E. coli cya	Glycerol	2.0×10^8	0.02 ± 0.01

[a] Radioimmunoassay was employed for cAMP determinations.

[b] Results are expressed ± standard deviation for four to six analyses.

[c] An essentially similar result was obtained when the erythrocyte ghost binding protein assay was used.

One question remains concerning the effect of washing cells with cold 0.2 M Na_2SO_4 compared with not washing or washing with a prewarmed solution. Our results, with the exception of those for unwashed samples shown in Table 2, were all obtained with cells subjected to the cold wash procedure of Epstein et al. (8). It might be concluded from examination of Table 2 that washing cells with cold solutions was inferior to not washing, in that nearly 50% less cAMP was found in washed compared with unwashed cells. We believe that this conclusion may not be justified because of the possibility that unwashed cells could contain interfering substances which would lead to erroneously high cAMP values, even if purification were conducted. We have not investigated the possibility that a warm, rather than cold, wash would increase our values by preventing loss due to cold shock. This might be a useful improvement in our methodology, although Epstein et al. (8) apparently examined this point and chose to use a cold wash.

Effect of succinate on histidase synthesis. Succinate is known to be a potent promotor of catabolite repression in *P. aeruginosa* (14, 23). To ascertain that histidase synthesis was subject to catabolite repression and to see whether exogenous cAMP could overcome this repression, the induction of histidase was achieved, and then succinate with or without cAMP was added to the culture. Figure 1 illustrates the results for *P. aeruginosa*. Although succinate drastically affected the differential rate of synthesis of histidase, 10 mM cAMP was not able to counter the repression.

A related experiment was conducted with *P. putida*, differing mainly in the fact that histidase was induced by growth on histidine medium until a constant specific activity was reached (Fig. 2). Upon addition of succinate to the medium, histidase specific activity fell roughly as predicted if enzyme synthesis had nearly ceased and growth continued at the observed generation time of 55 min. Again, cAMP was unable to block the decline in rate of synthesis of histidase. This experiment, along with that shown in Fig. 1, points to the conclusion that catabolite repression promoted by succinate is not a consequence of depleted internal cAMP levels in either *P.*

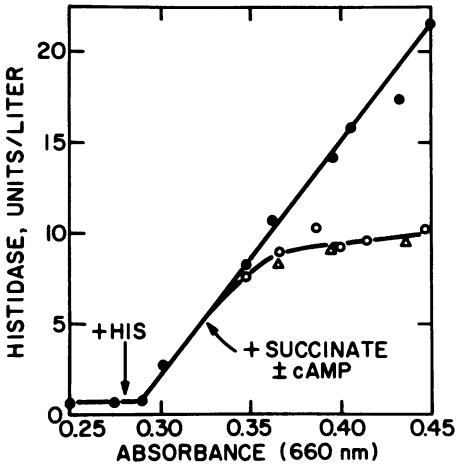

FIG. 1. *Induction of histidase in P. aeruginosa and repression by added succinate. Cells were grown at 37°C in glucose minimal medium, and L-histidine was added to 5 mM at the point indicated. Approximately 15 min later, the culture was divided and treated as follows: ●, no addition; ○, 10 mM sodium succinate; △, 10 mM succinate and 10 mM cAMP.*

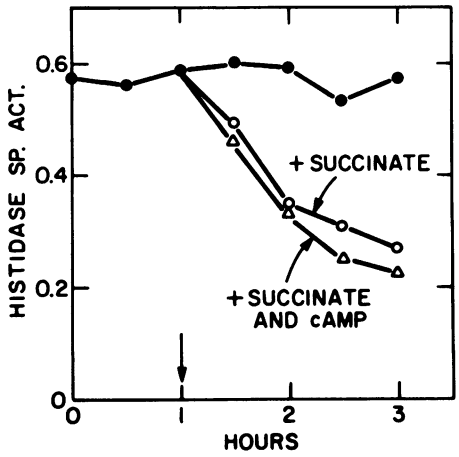

FIG. 2. *Repression of histidase synthesis in P. putida by succinate. A culture which had been growing in histidine minimal medium containing nitrogen-free salts A for over 10 generations was diluted to an A_{660} of 0.12 in fresh medium. After 1 h, when the A_{660} was 0.20, the culture was divided and treated as follows: ●, no addition; ○, 10 mM sodium succinate plus 7.5 mM $(NH_4)_2SO_4$; △, 10 mM succinate and 7.5 mM $(NH_4)_2SO_4$ plus 10 mM cAMP.*

putida or *P. aeruginosa*.

Intracellular cAMP levels during onset of catabolite repression. Although the preceding experiments documented the effect of succinate addition on histidase synthesis and showed that this was not prevented by cAMP addition to the medium, we could not establish that externally added cAMP influenced the intracellular cAMP pool. This was because it has not been possible to determine accurately the intracellular concentration of cAMP when millimolar amounts were added to the medium. Assuming, however, that succinate might exert its repressing effect on histidase synthesis by transiently decreasing the intracellular cAMP level (Table 1 reveals that no major or permanent decrease in cAMP level exists during growth on succinate), we chose to monitor cAMP levels during the onset of catabolite repression. No significant change was noted in the internal level of cAMP in *P. putida* as a result of succinate-related catabolite repression of histidase synthesis (Fig. 3).

Lactate-dependent catabolite repression of histidase in *P. putida*. The most direct evidence that cAMP controls amidase synthesis in *P. aeruginosa* comes from the work of Smyth and Clarke (23) who observed a reversal of lactate-provoked catabolite repression of amidase by the addition of cAMP. We therefore performed a similar experiment, wherein *P. putida* was grown on a pyruvate-minimal medium and then was induced for histidase. Shortly thereafter, lactate was added with or without cAMP (Fig. 4). As expected, lactate-dependent catabolite repression was much less severe than succinate-related catabolite repression, but this weaker form of catabolite repression was also not reversed by cAMP under the conditions employed, nor when the higher level of 30 mM cAMP was used (data not shown).

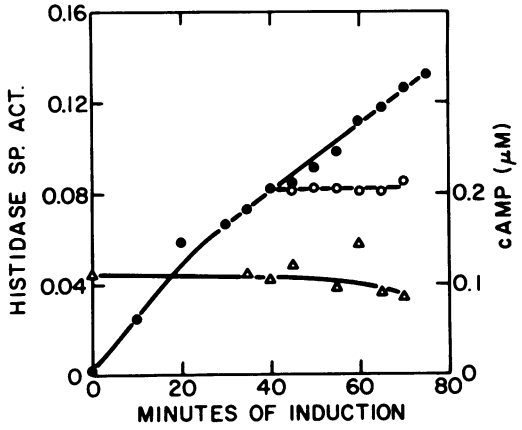

FIG. 3. *Intracellular cAMP levels in P. putida during induction of histidase and subsequent repression by succinate. A culture growing on glucose-minimal medium, upon reaching an A_{660} of 0.2, was made 5 mM in L-histidine. After 40 min, when the A_{660} was 0.34, the culture was split, and to one portion was added 10 mM sodium succinate. Symbols: ●, histidase specific activity in histidine-treated cells; ○, histidase specific activity after succinate addition; △, intracellular cAMP concentration.*

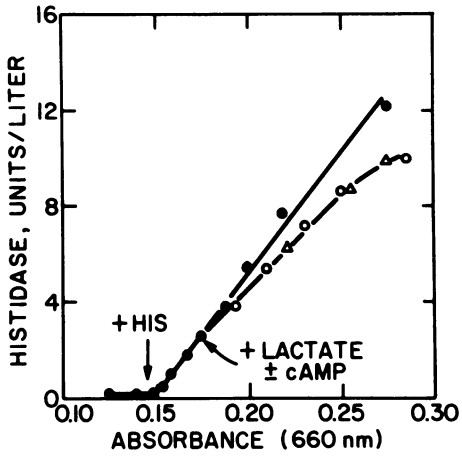

FIG. 4. *Induction of histidase in P. putida and repression by* DL-*lactate. A culture was grown in glucose-minimal medium and induced for histidase at the point indicated. Shortly thereafter, the culture was divided into thirds and treated as follows:* ●, *no addition;* ○, *10 mM sodium* DL-*lactate;* △, *10 mM lactate and 10 mM cAMP.*

DISCUSSION

Our findings on the influence of carbon source on intracellular cAMP levels in *P. aeruginosa* and *P. putida* have led us to conclude that cAMP concentrations do not vary significantly with the carbon source, certainly not to the extent seen in *E. coli* where the levels in cells exposed to a potent repressing sugar (glucose) are 10 to 20% of those found in cells grown on a mildly repressing or nonrepressing carbon source (8). Siegel et al. (22) reached the same conclusion in their examination of catabolite repression of some inducible carbohydrate catabolic enzymes in *P. aeruginosa*. They observed that glucose 6-phosphate dehydrogenase and mannitol dehydrogenase were repressed by the addition of succinate during growth, but cAMP did not reverse this effect. They could not, however, exclude the possibility that added cAMP was unable to reverse succinate-related catabolite repression due to an impermeability of whole cells to cAMP.

In the specific case of histidase synthesis in *P. putida*, we have been able to show that catabolite repression due to succinate is not the consequence of a transient reduced internal level of cAMP (Fig. 3), nor is the level of cAMP in histidine-grown cells appreciably greater than that seen in cells grown on other carbon sources. Although Smyth and Clarke (23) found that catabolite repression of amidase by succinate could not be relieved by the addition of cAMP, their data on repression by lactate indicated that very high levels of cAMP (10 to 30 mM) in the medium could partially reverse this repression. Our findings with histidase did demonstrate mild repression (approximately 25%) by lactate, but 10 mM cAMP was not stimulatory to histidase synthesis in the presence of lactate. The observation that exogenous cAMP was unable to reverse either lactate- or succinate-provoked repression of histidase synthesis argues for the conclusion that catabolite repression of histidase is not mediated by a reduction in internal cAMP levels.

Our results illustrated in Fig. 1, 2, and 4, like those of Siegel et al. (22) and Smyth and Clarke (23), are subject to the assumption that millimolar levels of cAMP external to cells can result in micromolar changes in internal cAMP pools. Unfortunately, it is not possible to validate this assumption with current methodology. In our experience, the presence of extremely high external cAMP levels precludes accurate determination of intracellular cAMP concentration changes. Whereas it is quite likely that cells are largely impermeable to cAMP, experience with adenylate cyclase-negative *E. coli* has shown that external cAMP can be taken up to the extent that cAMP-dependent sugar utilization can occur (15). The question remains whether this is evidence for sufficient permeability to justify the conclusion that intracellular cAMP levels are increased when exogenous cAMP is supplied in large amounts. This uncertainty does not apply to the experiment shown in Fig. 3, wherein we found cAMP levels were unperturbed by the onset of succinate-related catabolite repression.

Insofar as we are aware, there have been no direct observations of an influence of cAMP on histidase production in either *P. aeruginosa* or *P. putida*, despite the suggestion by Potts and Clarke (17) that cAMP regulates *hut* expression in *P. aeruginosa*. Our data reveal that it is difficult to perturb the intracellular cAMP level by variations in the carbon source for growth; thus, proof of a requirement for cAMP in transcription of the *hut* genes of *Pseudomonas* spp. will not likely be obtained until such time as an adenylate cyclase-negative mutant is isolated. An extensive search for such a mutant based on three approaches, non-utilization of histidine in the absence of cAMP supplementation, resistance to the antibiotic fosfomycin (1), and non-utilization of fructose by a phosphotransferase system specific for fructose (20, 26), has not proven successful; the last of these assumes that a phosphotransferase system mutation in *P. putida* analogous to the *Salmonella typhimurium* enzyme I defect (*ptsI*) will reduce the rate of cAMP synthesis, as was observed in *S. typhimurium* (20).

The fact that histidine utilization in *P. putida* and *P. aeruginosa* can not be demonstrated to be cAMP regulated does not necessarily mean that there is no requirement for cAMP in expression of *hut* genes. It probably is correct, however, to conclude that cAMP does not function in *P. aeruginosa* and *P. putida* as a signal for insufficient fuel (carbon) to support full growth—a role ascribed to cAMP in several organisms (1), at least not when any of the carbon sources used in our study are considered. Thus, it seems unlikely that cAMP will be essential for *hut* gene expression, since variations in cAMP are uncommon, and no obvious control function would be served.

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