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Association of Formation and Release of Cyclic AMP with Glucose Depletion and Onset of Chlorophyll Synthesis in *Poterioochromonas malhamensis*[1]

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ABSTRACT

Depletion of glucose from the culture medium by *Poterioochromonas malhamensis* results in cessation of growth and accumulation of cyclic adenosine 3':5'-monophosphate (cAMP), followed by formation of chlorophyll and an increase in extracellular cAMP. Readdition of glucose to the culture medium causes *P. malhamensis* to release its intracellular cAMP into the medium. These results suggest that formation of the photosynthetic apparatus in *P. malhamensis* may be repressed by glucose, and that high cAMP conveys the regulatory information that the glucose supply is inadequate. This pattern is reminiscent of cAMP-mediated escape from catabolite repression in bacteria.

In both animals and bacteria, high cAMP concentrations are associated with a depletion of glucose (26). In vertebrate tissues cAMP activates a protein kinase which causes inactivation of glycogen synthetase and, through activation of phosphorylase kinase, causes activation of phosphorylase (22). Thus, the glucose supply of the organism can be replenished at the expense of glycogen. In most bacteria, high cAMP causes escape from catabolite repression of enzymes which make possible the utilization of carbon sources other than glucose (17) when glucose is not available. Enzymes needed for meeting nitrogen requirements of bacteria are also subject to catabolite repression which is mediated by cAMP (19, 20).

The role of cAMP in carbohydrate metabolism has not been investigated in photoautotrophic organisms which synthesize their own glucose from CO_2 rather than absorbing and utilizing it from their environments. In circumstances where chloroplast development is completed, formation of cAMP in response to glucose deprivation may not occur. However, in systems in which chloroplast development is nutritionally, environmentally, or hormonally controlled, it may be possible to find an involvement of cAMP in such regulation.

Poterioochromonas malhamensis, a chrysophycean alga, provides an attractive model system to investigate the role of cAMP in regulation of photosynthetic carbon metabolism. Whereas most algae have functional chloroplasts even when growing in the presence of reduced carbon, *P. malhamensis* does not develop its photosynthetic apparatus until starved for fixed carbon. It grows heterotrophically very well on glucose and develops its chloroplasts, but grows slowly when CO_2 is its only carbon source (18).

We have reported previously that *P. malhamensis* synthesizes and secretes cAMP (5). The level of cAMP found in this organism varied greatly between 3 and 3000 pmol/g fresh weight (5). In an effort to determine the reason for the variation, a study of cAMP in cells and medium during growth of *P. malhamensis* cultures was performed. We report here that cAMP begins to accumulate in *P. malhamensis* as the cells exhaust the medium of glucose and initiate Chl formation.

MATERIALS AND METHODS

Cells. *P. malhamensis* Peterfi. (formerly *Ochromonas malhamensis*) UTEX strain 1297, was grown as described in Bressan *et al.* (5), except that the glucose concentration was varied where indicated. The usual procedure was to start 50 replicate cultures per glucose treatment, each containing 500 ml of medium, and to harvest two replicate flasks of each treatment for each day's analyses.

Growth Assay. Cultures were transferred to tared polycarbonate centrifuge bottles and sedimented in a Sorvall GSA rotor at 2000 rpm (650*g*) for 10 min at 22 C. The medium was removed by aspiration. The bottle plus pellet of cells was weighed to determine the fresh weight which was taken as the growth parameter. Routine microscopic examination and periodic plating of the cultures onto nutrient agar revealed no bacterial contamination.

cAMP Assay. cAMP was extracted from cells or medium and partially purified by procedures described in Bressan *et al.* (5), and Handa and Bressan (8), through the steps of absorption to and elution from Dowex 50 (Sigma) and quantitated by the competitive binding assay, and/or the protein kinase stimulation assay (5, 8).

Chlorophyll Determination. Cells in 2-ml culture aliquots were sedimented at 2800*g* for 10 min in a clinical centrifuge. The supernatant medium was removed by aspiration, and the Chl in the cell pellet was extracted with 0.7 ml 80% (v/v) acetone. The extraction was facilitated by repeated passage of the suspension through a Pasteur pipet. After centrifugation the supernatant was transferred to another tube and brought to constant volume with 80% acetone (v/v). The Chl content was determined by spectrophotometric measurements at 663 and 645 nm as described by Arnon (2).

Glucose Determination. Glucose in culture media was determined by the method of Nelson (15) and Somogyi (23).

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RESULTS

Changes in Intracellular Level of cAMP. Measurements of cAMP in *P. malhamensis* with cAMP binding protein or with protein kinase gave virtually identical results (Fig. 1B). The intracellular level of cAMP rose, then fell during the growth cycle of *P. malhamensis* on media containing various levels of glucose as the carbon source (Figs. 1 and 2). The rise in cAMP began as depletion of the glucose in the medium became appreciable and passed through a peak soon after the stationary growth phase was reached (Fig. 1). To determine if the apparent correspondence of the rise in cAMP with depletion of glucose in the medium was causally related, comparisons were made between otherwise identical cultures initially containing two different levels of glucose, *e.g.* 2 or 10 g liter^{-1} (Fig. 2, A and B). In this experiment, the glucose concentration reached its minimum 4 days after inoculation when initially it had been at 2 g liter^{-1}, but 10 days after inoculation when initially it had been 10 g liter^{-1}. In both cases the concentration of cAMP began to rise as that of glucose in the medium began to decline. Under both culture conditions, maximum intracellular concentrations of cAMP were reached approximately 6 days after glucose minima were reached (Fig. 2, A and B), indicating a correlation between depletion of glucose and accumulation of cAMP. A shift in time of the concomitant onsets of rise in cAMP and the depletion of glucose related to change in initial glucose content of the medium was evident with initial glucose levels differing by as little as a factor of 2 (data not shown).

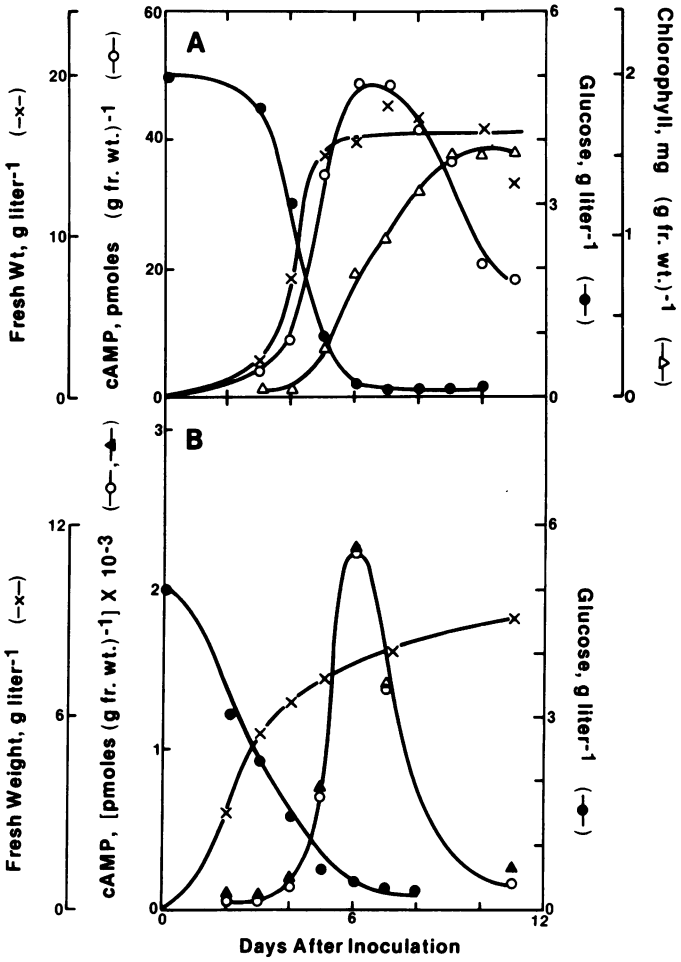

FIG. 1. Changes in cAMP and Chl concentrations in *Poterioochromonas* cells during the growth cycle. Also shown is the fresh weight gain of the cells and the decline of glucose concentration in the medium with culture age. Differences in intracellular cAMP levels between experiments is indicated by A and B. The cells represented in A were grown under lower light and temperature than those in B. Intracellular cAMP in B: ○, by binding assay; ▲, by protein kinase stimulation assay.

While the rise in cAMP and the peaking of cAMP after glucose depletion was observed invariably, the absolute intracellular levels of cAMP before and after glucose depletion differed widely from experiment to experiment. The values before glucose depletion ranged from as few as 4 to as many as 76 pmol/g fresh weight (Fig. 1, A and B), and those after glucose depletion ranged from 47 to 2230 pmol g fresh weight^{-1} (Fig. 1, A and B). The ratios of the peak values of cAMP after depletion to the minimum value before depletion ranged between 4.7 and 30 (Figs. 1 and 2). The high degree of variability in absolute levels, and the somewhat smaller variability in ratios of cAMP before and after glucose depletion, may mean that whatever cAMP does in this system involves an all or none mechanism in which the response is triggered by an above threshold level of cAMP above a background of cAMP, rather than a mechanism in which the response is incrementally dependent on cAMP concentration or on ratios of final to initial concentrations of cAMP.

Changes in Chl Content. The Chl content of the cells began to increase soon after the onset of the rise in cAMP (Figs. 1A and 2A), and reached its maximum after glucose had been exhausted from the medium. The subsequent decline in intracellular cAMP began sometimes before, and sometimes after, the rise in Chl content was complete.

Changes in Extracellular cAMP. Shortly after intracellular cAMP began to rise, about the time that growth of the culture ceased as a result of exhaustion of glucose, cAMP began to appear in the culture medium (Figs. 2 and 3). When glucose was added back to cells which had exhausted their initial glucose supply, and were actively synthesizing and releasing cAMP into the culture medium (Fig. 2D), the intracellular cAMP level declined rapidly (Fig. 2B), with a concomitant rise in extracellular cAMP (Fig. 2D).

In an experiment in which the response of the cells on glucose at 2.5 and 5 g liter^{-1} was compared, the cells exhausted the lower initial amount of glucose about 1.5 days sooner than the higher initial amount (Fig. 3B), with final fresh weight yield being proportional to the initial glucose concentration (Fig. 3A). This clearly indicates that cell growth was limited because of unavailability of glucose in the medium. About a day after exhaustion of glucose, a peak of cAMP occurred in the culture medium followed by a decline during the next two days. Then, on the 4th day after exhaustion of the glucose supply, a second peak of cAMP appeared in the culture medium (Fig. 3B). Such peaks were not as dramatic, and were not observed consistently in other experiments (see Fig. 2, C and D) in which, because of limitations of culturing equipment, physical conditions (*e.g.* light and temperature) differed from those in the experiment shown in Figure 3B. Nevertheless, the remarkable similarity in the patterns, and the fact that the peaks of cAMP were displaced by about the same time interval as the exhaustion of the glucose supply in the two sets of cultures, indicates that production of cAMP waves is a reproducible phenomenon related to glucose exhaustion.

DISCUSSION

There have been many attempts to find a cAMP-dependent protein kinase in higher plants, but all have been unsuccessful (1). These negative results have raised doubts about the occurrence of cAMP-dependent protein kinase systems in photosynthetic eucaryotes. Such kinases were considered likely on the basis of the animal model. However, if the procaryotic (bacterial) model is the appropriate one for plants, then cAMP-dependent protein kinases would not be expected; rather cAMP-dependent derepression of enzymes that make possible the substitution of poor carbon

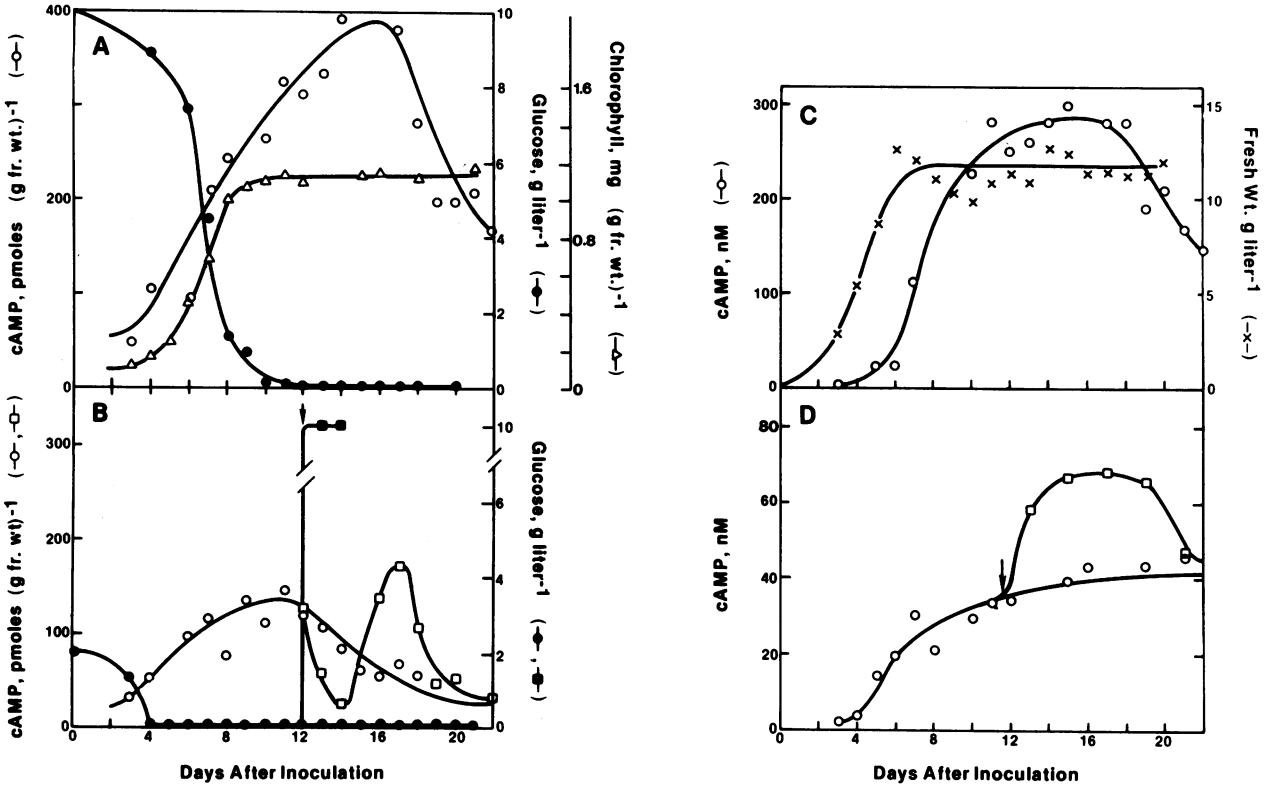

FIG. 2. Effect of initial level of glucose in the medium on intracellular and extracellular cAMP concentrations. A, C: medium initially containing 10 g/l glucose. Intracellular cAMP and Chl levels are given in A. cAMP concentration in the medium and gain in fresh weight are shown in C. B, D: medium initially containing 2 g/l glucose. Intracellular cAMP levels and the effect of addition of 10 g/l glucose to the medium on day 12 (arrow) on intracellular cAMP (□) are shown in B. Extracellular cAMP and the effect of addition of 10 g/l glucose (arrow) on release of cAMP into the medium (□) are shown in D.

sources for glucose would be expected. CO_2 plus light can be considered a poor substitute for glucose, and the chloroplast in *P. malhamensis* can be considered to be an elaborate derepressible enzyme system for using CO_2 as a substitute for glucose. The rises in cAMP and then in chlorophyll as *P. malhamensis* exhausts the medium of glucose are reminiscent of escape from catabolite repression in bacteria. This is, as far as we are aware, the first demonstration that high cAMP and low glucose availability are clearly correlated in a photosynthetic member of the Plant Kingdom.

Berchtold and Bachofen (3) attempted to detect such a relationship in *Chlorella fusca*, but saw, at best, less than a doubling of intracellular cAMP in cells deprived of glucose. In these experiments, the Chl content was directly, rather than inversely, related to the glucose concentration, and the change in Chl concentration occurred throughout a wide range of glucose concentrations which had no effect on cAMP. Correlations of low glucose and high cAMP, however, have been found in fungi (14, 27).

These results with *P. malhamensis* suggest the intriguing possibility that the biochemical logic underlying the regulation of chloroplast development may be escape from catabolite repression when the supply of fixed carbon becomes inadequate. The media commonly used to induce organization of cells into leaves (24), or to obtain photoautotrophic growth by cultured plant cells (16), appear to be "carbon-limited," judging from the typical yields (about 15 g dry weight of cells from 30 g of sucrose, some of which must be respired during growth of the cultures). In addition, the accumulation of starch by cultured cells appears to be correlated with the formation of meristemoids (25). Furthermore, Handa and Johri (9, 10) have shown that in the moss *Funaria hygrometrica*, endogenous cAMP is correlated with development of chloroplast-rich cells (chloronemata).

It has become clear that algae release cAMP into their aqueous environment, as we have shown for *P. malhamensis* (5), *Chlamydomonas reinhardtii* (4) and *Anabaena variabilis* (11). This appears to be generally true for fresh water algae (6). The finding that glucose causes *P. malhamensis* to release cAMP into the culture medium leads us to speculate that in fresh water systems in which nutrients are normally at low concentrations and then are suddenly raised, algae can be expected to release large amounts of cAMP into the water.

Waves of cAMP production or release have been encountered in other organisms (7, 21), the best known being the oscillations in aggregating populations of *Dictyostelium discoideum* (13). Our results suggest that waves of cAMP occur in *P. malhamensis* cultures, though at far lower frequency than in *Dictyostelium*. The carbon supply probably plays a role in generating the waves. There is currently no indication of a function for the released cAMP in contrast to the situation in *Dictyostelium* for which it is an aggregation attractant (13).

We showed that glucose added to a glucose-starved culture caused a drop in internal cAMP and a rise in external cAMP, implicitly suggesting that glucose merely caused release of internal cAMP. However, cAMP production did not stop upon addition of glucose. On the contrary, the amount of cAMP released in 48 h (Fig. 2D) was 4270 pmol/g fresh weight, while the net change in the intracellular cAMP concentration (Fig. 2B) during that time was only 110 pmol/g fresh weight. Therefore, glucose caused more than a 38-fold increase in the net rate of production of cAMP in the culture. It is difficult to imagine that *P. malhamensis* would engage in such activity, *i.e.* massive glucose-dependent production of extracellular cAMP, unless the nucleotide has a function.

If released cAMP has a function, in *P. malhamensis* (12) and other algae, the question arises: how does one organism in the

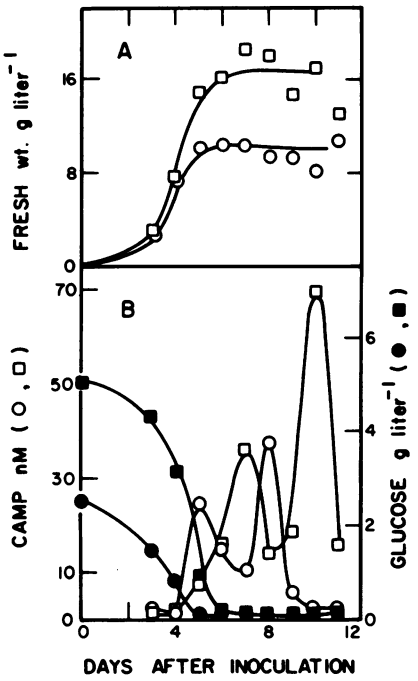

FIG. 3. "Carbon limited" growth of *Poterioochromonas* (A) and periodic release of cAMP into the medium after glucose depletion (B). In A the medium initially contained 2.5 g/l (○) or 5.0 g/l (□) of glucose. In B when the initial glucose concentration was 2.5 or 5.0 g/l the corresponding subsequent glucose concentrations in the medium are designated as ● and ■ and the released cAMP concentrations as ○ and □, respectively.

ecosystem discriminate the cAMP signal to which it is to respond from that produced by the other species in the vicinity? Both the absolute level of cAMP, and the ratio of intracellular cAMP before to that after glucose depletion showed a high degree of variability. The wide variation in amplitude and in peak to trough ratios of cAMP content suggest that it may be change *per se*, perhaps the frequency of change or the rate of change in cAMP concentration, which is important rather than the static concentration or ratios of cAMP. Perhaps in a mixed population, organisms can distinguish signals based on modulation of cAMP waves, each species sending and/or receiving on its species-specific frequency.

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