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SYNTHESIS AND RELEASE OF ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE BY *CHLAMYDOMONAS REINHARDTII*

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Key Word Index—*Chlamydomonas reinhardtii*; Chlorophyta; biosynthesis; adenosine 3′:5′-cyclic monophosphate; release; radiolabeling; purification.

Abstract—One of the labeled compounds synthesized by *Chlamydomonas reinhardtii* when ^{32}Pi was supplied was isolated from both the cells and the medium in which the cells had grown. This compound copurified with authentic [8-^{3}H]cAMP by TLC to a constant ratio of ^{32}P/^{3}H. The compound was degraded by beef heart cyclic nucleotide phosphodiesterase to a product which cochromatographed with authentic 5′AMP, at the same rate as the hydrolysis of authentic cAMP-[^{3}H] to 5′AMP-[^{3}H]. In both cases, 1-Me-3-*iso*Bu-xanthine, a specific inhibitor of the phosphodiesterase, totally blocked the reaction. It is concluded that the compound synthesized by *C. reinhardtii* was cAMP, 85% of which was released into the medium.

INTRODUCTION

Amrhein and Filner [1] reported the presence of a substance in *Chlamydomonas reinhardtii* which co-chromatographed with authentic cAMP, stimulated cAMP-dependent protein kinase from rabbit muscle, and was destroyed by beef heart cyclic nucleotide phosphodiesterase (PDE). However, the kinetics of the degradation differed from those for authentic cAMP apparently because of the presence of an endogenous inhibitor of PDE. These authors concluded that in all likelihood the substance was cAMP [1].

There are severe limitations on the characterization of any putative cAMP if one uses only an activity assay: the loss of the compound can be followed but not the appearance of the product derived from it.

Also, the chemical purity of the cAMP cannot be adequately assayed because only compounds which affect the assay can be detected. The rather low levels of cAMP found in plant species [1, 8, 9] make it difficult to obtain enough of it for characterization by chemical methods. These limitations can be largely overcome if the substance is radiolabeled and we report here results obtained by feeding Pi-[^{32}P] to *Chlamydomonas reinhardtii* to this end.

RESULTS

In order to achieve the highest possible specific radioactivity in cAMP using ^{32}P, we first determined the lowest concentration of Pi at which *C. reinhardtii* could grow normally and found it to be 0.04 mM. We used 0.1 mM, to provide a margin of safety and were surprised to find that *C. reinhardtii* was sensitive to radioactive Pi as evidenced by reduced growth rate at specific radioactivities above 500 μCi/μmol. The specific radioactivity used was therefore 100 μCi/μmol, which was sufficient to permit detection of cAMP-[^{32}P] at 1 pmol/g fr. wt.

The acid extract of *Chlamydomonas* cells grown in the presence of Pi-[^{32}P] contained labelled compounds which were eluted from neutral Al_2O_3 near but not exactly coincident with the cAMP-[8-^{3}H] standard (Fig. 1A). Most of the ^{32}P-labelled compounds was separated from standard cAMP by TLC on Si gel (Fig. 1B) but part migrated with standard cAMP-[^{3}H]. This peak of ^{32}P matched that of ^{3}H in two subsequent purification steps by TLC (on cellulose, and Si gel (Fig. 1C and D). The ratio of ^{32}P/^{3}H remained constant over the last two steps (Table 1).

The fraction of ^{32}P from the used radiolabelled low Pi TAP medium which was eluted from Al_2O_3 by water behaved identically with the cAMP-[^{3}H] standard (Fig. 2A). Subsequent TLC on Si gel (Fig. 2B), however, showed that some of this ^{32}P was not cAMP but further purification of the cAMP-like fraction on cellulose (Fig. 2C) and Si gel (Fig. 2D) gave a peak which migrated with cAMP.

It is evident from the results that some of the ^{32}P-labelled material isolated from either the cells or the medium in which they had grown copurified with authentic cAMP-[^{3}H] to a constant ratio of ^{32}P/^{3}H, strongly suggesting that it is indeed cAMP. To verify this, regions of the chromatograms (Figs. 1D and 2D) containing authentic cAMP-[^{3}H] and the ^{32}P compound were treated with PDE which converted the compounds to 5′AMP-[^{3}H] and a comigrating ^{32}P compound (Fig. 3). The kinetics of this change for ^{32}P were identical to those for ^{3}H in cAMP. Furthermore, MIX, a specific inhibitor of PDE, blocked degradation of both authentic cAMP-[^{3}H] and the ^{32}P compound

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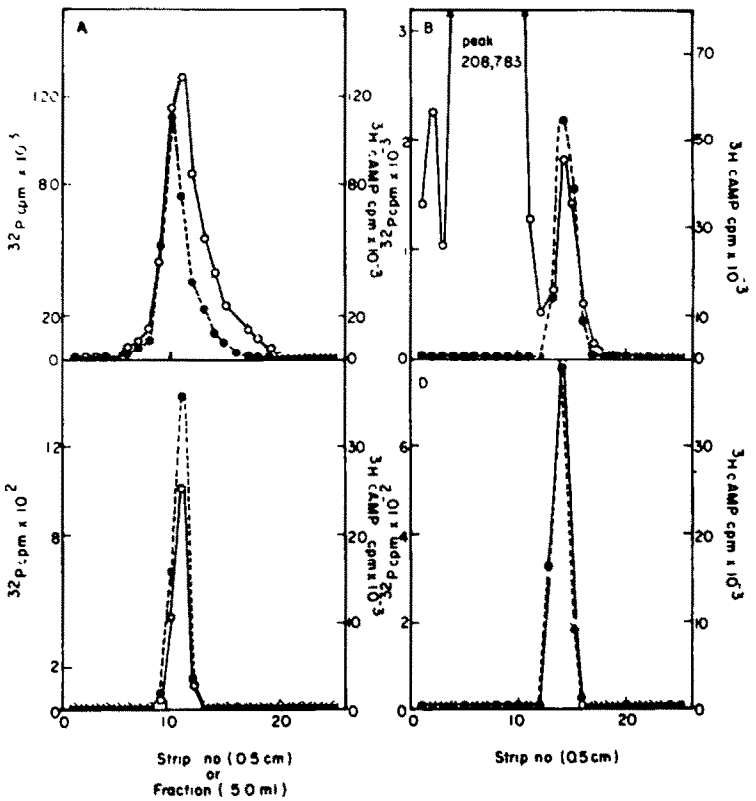

Fig. 1. Chromatographic profiles of ^{32}P from *Chlamydomonas* cell extracts compared to cAMP-[^3H]. The cAMP-[^3H] was added to the acid before extraction. A: Elution profile of ^{32}P and [^3P]-cAMP from alumina column, B: migration of ^{32}P and ^3H from fractions 9–12 of A on Si gel TLC, C: migration of ^{32}P and cAMP-[^3H] from fractions 13–16 of B on cellulose TLC, D: migration of ^{32}P and cAMP-[^3H] from fractions 10–12 of C on Si gel TLC. ^3H:●; ^{32}P:○.

Table 1. Purification of putative cAMP-[^{32}P] from *Chlamydomonas*

Purification step	^3H cpm ($\times 10^{-3}$)	% Recovery	^{32}P cpm ($\times 10^{-3}$)	$^{32}P/^3H$
		Cells		
Alumina column	317	45.3	444	1.75
Silica gel TLC solvent A	296	42.3	4.62	0.015
Cellulose TLC solvent B	242	34.5	2.42	0.01
Silica gel TLC solvent C	230	32.7	2.3*	0.0101
		Medium		
Alumina column	131	18.8	33.5	0.255
Silica gel TLC solvent A	125	17.8	12.8	0.108
Cellulose TLC solvent B	99	14.1	4.74	0.048
Silica gel TLC solvent C	82	11.7	4.69†	0.057

The neutralized $HClO_4$ cell extract and the charcoal eluate of cell free medium, from a 500 ml culture containing Pi-[^{32}P] at 100 μCi/mol, which yielded 2 g fr. wt, were purified by the indicated steps. The ^{32}P cpm are corrected for decay. The ^3H cpm are corrected for difference in counting efficiency at each purification step.

* Corresponds to 16 pmol/g fr. wt in cells.

† Corresponds to 90 pmol/g fr. wt in medium.

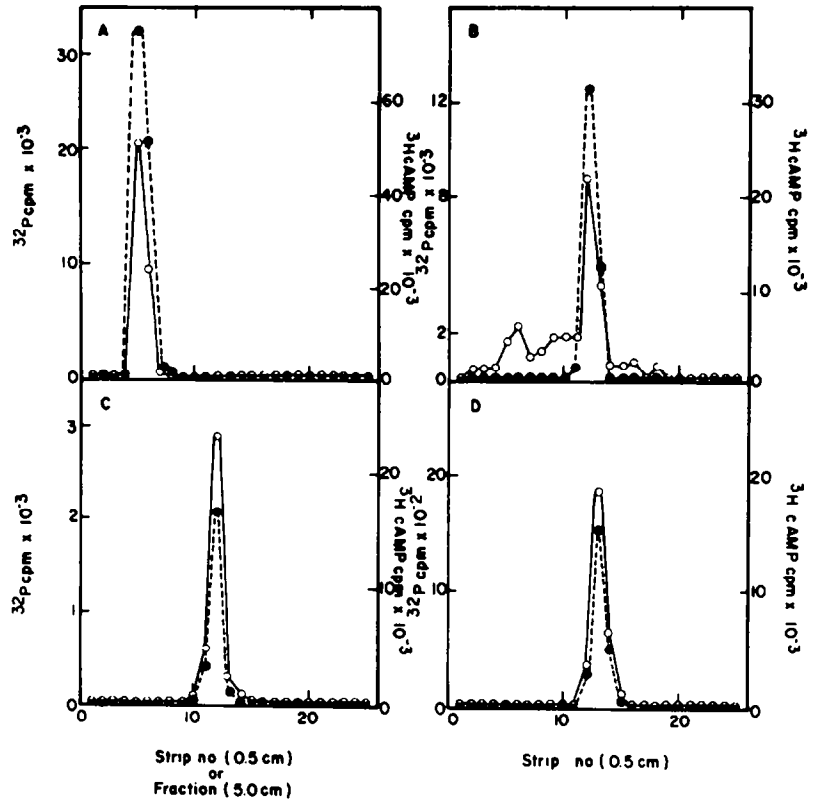

Fig. 2. Chromatographic profiles of ^{32}P from the medium of the *Chlamydomonas* culture. Purification steps as in Fig. 1. A: Elution of ^{32}P and ^{3}H from neutral alumina, B: migration of fractions 5 and 6 from A, C: migration of fractions 12 and 13 from B, D: migration of fractions 11 and 12 from C.

regardless of source. When the ^{32}P compounds from cells or medium were mixed together and with authentic cAMP-[^{3}H], only one ^{32}P peak was observed, and it migrated on PEI cellulose plates exactly with cAMP-[^{3}H] (Fig. 4). These results indicate that the ^{32}P compound from either cells or medium is therefore cAMP.

Thus during 5 days of growth on medium containing Pi-[^{32}P], *C. reinhardtii* synthesizes cAMP-[^{32}P] and releases much of it to the medium. Since the cells

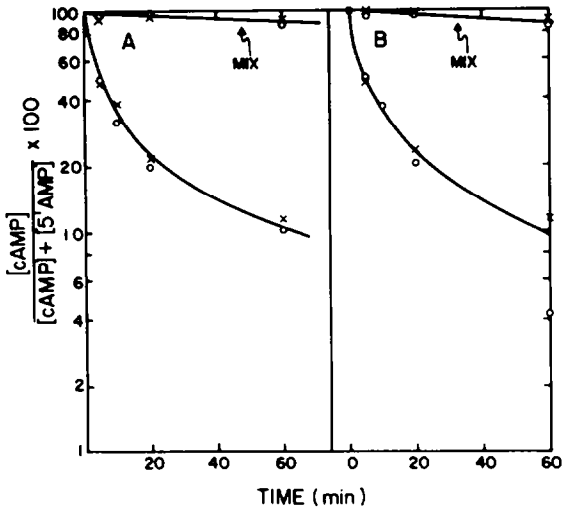

Fig. 3. Time course of degradation by PDE of ^{32}P compound from cell extract (A) or medium (B), purified with cAMP-[^{3}H]. The reactions were performed in the presence or absence of MIX, as indicated. ^{32}P:O; ^{3}H:X.

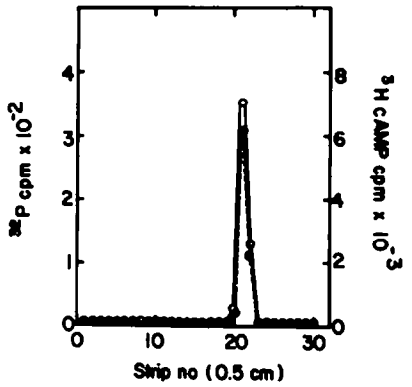

Fig. 4. Chromatographic profile of a mixture of ^{32}P purified from cell extract and medium with cAMP-[^{3}H] through the steps indicated in Table 1 and Figs. 1 and 2. The mixture was chromatographed on PEI cellulose in solvent D.

underwent more than a 100-fold multiplication in the presence of Pi-[^{32}P], the specific activity of the cAMP-[^{32}P] can be taken to be equal to that of the Pi-[^{32}P] added to the medium; the concentration of cAMP in the cells is thus calculated as 16 pmol/g fr. wt, or *ca* 16 nM. The medium contained *ca* 90 pmol/g fr. wt, or 85% of the total in the culture, at a concentration of 0.73 nM.

DISCUSSION

There have been numerous attempts to detect cAMP in flowering plants and other photosynthetic members of the plant kingdom [2, 3]. Prior to this study, attempts to incorporate radioactive precursors

into cAMP in such organisms have not yielded unequivocally positive results. Plants synthesize compounds which mimic cAMP in numerous chromatography systems, but which were ultimately separated from the cyclic nucleotide [4–7]. These organisms also contain compounds which mimic cAMP or interfere in an assay considered by those who study cAMP in animals and bacteria to be highly specific [1, 7, 9]: this is the competitive inhibition of binding of authentic cAMP-[^{3}H] to the cAMP-binding protein [10]. These interfering compounds appear to be ubiquitous in plants, but none has been identified. They merit more attention than they have so far received, both as natural products and as compounds with possible physiological or ecological significance or pharmacological utility.

In our experience fractions which interfere with the cAMP-binding assay usually do not activate protein kinase. Nevertheless, the protein kinase assay alone should not be considered an adequate criterion for the initial identification of cAMP in any extract, because of the possible existence of other compounds which can activate the enzyme. At the very least, copurification with authentic cAMP and destruction of the protein kinase stimulating activity of the putative cAMP by the PDE reaction should be demonstrated and the kinetics of the destruction should be shown to match those for authentic cAMP. Any discrepancy needs to be explained. We have shown here that the endogenous inhibitor of PDE which Amrhein and Filner [1] encountered can be eliminated by a somewhat different purification procedure. Inhibitors of PDE which copurify with cAMP to a certain extent also appear to be ubiquitous in photosynthetic members of the plant kingdom [1, 8, 9] and require more study.

The strongest evidence for occurrence of cAMP in photosynthetic members of the plant kingdom has been obtained by application of the protein kinase assay and the PDE reaction to activities purified from sterile cultures of a green single-celled alga, *C. reinhardtii* [1], a moss, *Funaria hygrometrica* [8] and cells from a flowering plant, *Lolium multiflorum* [9]. In these studies cAMP was found in quite low concentrations: often much less than 100 pmol/g fr. wt. Because bacteria contain much higher concentrations of cAMP and excrete it, no assay of cAMP can be used with confidence on *unsterile* plant material, unless the contribution of the microbial contaminents has been assessed.

A major shortcoming of assays based on the interaction of pmol amounts of putative cAMP, especially when applied to impure fractions, is that when the activity is destroyed by PDE or some other well-defined reaction, it is difficult or impossible to assay the resultant pmol increments of product to prove that the assumed reaction actually took place. Labeling with ^{32}P, as we have described here, greatly facilitates this determination.

We have shown an example of cAMP release into the medium by a photosynthetic organism. The initial observation on this was made by Amrhein and Filner (unpublished results). Handa and Johri [8] observed a loss of cAMP from *Funaria* during washing which may be also indicative of release and Ashton and Polya [9] found that *Lolium* cells release cAMP into the medium. We have also observed release of cAMP by the chrysophycean alga, *Ochromonas malhamensis* and by a cyanobacterium, *Anabaena variabilis*. Bacteria [11], fungi [12, 13] and animal cells [14, 15] are known to release or secrete cAMP. In bacteria, the role of cAMP release appears to be the control of intracellular concentrations of the nucleotide [11] while in the fungus *Dictyostelium discoideum*, secreted cAMP functions as an intercellular message [13]. The function of cAMP release in animal cells is not yet understood.

Increase of cAMP in *Chlamydomonas* is associated with inhibition of flagellar functioning and regeneration [16]. *Chlamydomonas* possesses a PDE which can be inhibited in extracts by methylxanthines. When exposed to the methylxanthine, aminophylline, the cAMP level in *Chlamydomonas* rises *ca* 10-fold [1] which indicates that the enzyme may play a major role in controlling intracellular cAMP in this organism. If the endogenous inhibitor of PDE in *C. reinhardtii* is also active against the alga's own PDE *in vivo*, then it would play an important role in regulation of cAMP level in this organism. Since it appears that *C. reinhardtii* may be well equipped to regulate its internal cAMP level without resorting to excretion of the compound, the intriguing question remains: why does the organism release the bulk of the cAMP that it synthesizes?

It is noteworthy that the levels of cAMP found in *Chlamydomonas* cells in this study were of the order of those reported by Amrhein and Filner [1] for cells treated with aminophylline. Toward the end of this study, we observed that a substantial proportion of the colony forming units in the cultures used were palmelloids [17], i.e. clusters of unflagellated cells. Similar clusters normally exist briefly immediately after mitosis. However, this was true at all times during the cell cycle in synchronized cultures so the clusters were not cells completing mitosis. The palmelloids were cloned and shown to be self-propagating. The relationship between palmelloid phenotype and elevated cAMP level is currently under investigation.

EXPERIMENTAL

Chromatography systems. All TLC plates were precoated and were obtained from Brinkmann Instruments Inc. Solvent systems for TLC were: A: MeOH–EtOAc–conc NH_4OH–*n*-BuOH (3:4:4:7); B: MeOH–NH_4OAc (7:3); C: *iso*-PrOH–conc NH_4OH–H_2O (7:1:2); D: M LiCl. The Al_2O_3 columns were made of Baker chromatographic grade Al_2O_3.

Measurement of radioactivity. cAMP-[8-^{3}H] (25–40 Ci/mmol, depending on the batch) was used as the int. standard for estimating recoveries, and locating the cAMP regions on chromatograms. Samples from Al_2O_3 columns were placed in scintillation fluid containing Triton X-100 (4 g PPO, 0.1 g POPOP, 330 ml Triton X-100, 670 ml toluene) to measure ^{3}H and ^{32}P cpm by scintillation spectrometry. Sections from TLCs were placed directly into scintillation vials containing scintillation fluid from which Triton X-100 had been omitted, with the vol. made up with toluene. The spectrometer was set so that spillover of ^{3}H into the ^{32}P channel was insignificant for the ratios of ^{32}P/^{3}H used. Spillover of ^{32}P into the ^{3}H channel was determined for each measurement procedure and subtracted from the ^{3}H cpm. All ^{3}H measurements were corrected for differences from the

efficiency of ^{3}H measurement in the scintillation fluid containing Triton X-100, which was 32%.

Cell cultures. Chlamydomonas reinhardtii (+)-Mating type was grown in Tris–acetate–Pi (TAP) medium as described previously [1]. For radiolabeling with ^{32}P, cells were grown in 1 l. flasks, each containing 500 ml of low Pi TAP (0.1 mM instead of the 1 mM in standard TAP) plus 5 mCi of carrier-free Pi-[^{32}P], to produce a sp. act. of 100 μCi/μmol. The cells grew in this medium for 5 days on a reciprocal shaker at 28° under continuous illumination from cool-white fluorescent lamps (0.15 mW/cm^2). After inoculation with 2 ml containing 10^7 cells, the cultures multiplied exponentially for *ca* 2.5 days, after which they were in stationary phase for the remaining 2.5 days. No microbial contamination was seen when culture samples were examined by phase contrast microscopy before harvesting.

Processing of culture before chromatography. Cells were collected by centrifugation at 4000 g for 10 min at 25° in a Sorvall GSA rotor. The sedimented cells were resuspended in *ca* 3 vols of ice-cold 0.5N $HClO_4$. The suspension was placed in a −20° freezer for 2 hr or overnight, then thawed. We have found cAMP to be stable in 0.5 N $HClO_4$ at −20° for weeks. 50 pmol of cAMP-[8-^{3}H] (25 000 cpm/pmol) were added as an int. standard. The suspension of broken cells was then centrifuged at 12 000 g for 10 min at 40° in a Sorvall SS-34 rotor to separate the acid extract from the cell debris. The supernatant was neutralized with 5.0 N KOH and the precipitated $KClO_4$ was removed by sedimentation at 12 000 g for 10 min. 50 pmol of cAMP-[8-^{3}H] were also added to the 500 ml of cell-free medium. Then washed Norit A charcoal [8], 3 mg/ml, was added to the medium and it was stirred continuously for 2 hr or overnight at 22°, or at 4°. Neither the time of stirring nor the temp. in these ranges was critical. After collecting the charcoal by filtration on 2 layers of Whatman No. 4 filter paper, the charcoal was washed with H_2O, then eluted with EtOH–H_2O (1:1) containing 3% conc NH_4OH, v:v. This eluate and the neutralized $HClO_4$ extract from the cells were concd by flash evapn prior to column chromatography.

Chromatographic fraction of cell extract and medium. The neutralized cell extract and the medium fraction which was eluted from charcoal were partially purified separately by 4 consecutive chromatographic steps. The first step was column chromatography with neutral Al_2O_3. After loading the sample on a 1.5 × 12 cm column, it was eluted with H_2O and 5 ml fractions were collected. ^{3}H and ^{32}P in each fraction were determined, and the fractions containing the peak of ^{3}H were pooled and lyophilized. The redissolved residues were each applied as a band to a thin layer plate of Si gel GF 254 and developed in solvent A. One half cm strips were cut from the chromatograms and ^{3}H and ^{32}P were determined in each strip. The strips containing the ^{3}H peak were removed from the scintillation fluid, washed with toluene, then dried and eluted with 1.5–2.0 ml warm H_2O. This eluate was lyophilized and redissolved in *ca* 0.5 ml EtOH–H_2O (1:1), then chromatographed on cellulose TLC developed in solvent B. Again the plate was divided into strips, the ^{3}H and ^{32}P of each strip was determined, the strips with the peak of ^{3}H were eluted, and the eluate was rechromatographed, this time on Si gel plates developed in solvent C. Portions of the fractions purified from cell extract and from medium (through the step involving Si gel and solvent B) were mixed and chromatographed on polyethyleneimine (PEI) cellulose in solvent D.

Phosphodiesterase treatment of samples. Beef heart 3′:5′-cyclic nucleotide phosphodiesterase (PDE) purchased from Sigma, was used for all PDE treatments. Aliquots of both the cell extract fraction and the medium fraction which had been purified through the step involving Si gel and solvent C (Table 1) were treated with 5 μg of PDE. Reaction mixtures contained 50 μl of sample, 10 μl H_2O or 10 μl of 5 mM 1-Me-3-*iso*Bu-xanthine (MIX), and 5 μl of enzyme in 400 mM Tris–HCl (pH 7.4) and 200 mM $MgSO_4$. PDE reactions were stopped by boiling for 3 min. 10 μl each of 10 mM cAMP and 5′AMP were added to the reaction mixtures after boiling, and they were chromatographed on Si gel GF 254 in solvent A. The bands absorbing UV light and corresponding to cAMP and 5′AMP were removed from the plates and the ^{3}H and ^{32}P present were measured as described above.

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