

Formation of Cyclic Adenosine-3'-5'-monophosphate (cAMP) from Adenosine-5'-phosphosulfate (APS) in *Chlamydomonas reinhardtii* CW 15?

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Levels of cyclic adenosine monophosphate (cAMP) have been determined in the green alga *Chlamydomonas reinhardtii* strain CW 15. In the early exponential growth no cAMP was detected, however, in the middle of the exponential growth phase 2.4 pmol cAMP/mg protein were found and the cAMP level increased to 18.8 pmol/mg protein in the stationary phase.

An enzyme fraction has been isolated from *Chlamydomonas*, which produced cAMP *in vitro* using adenosine-5'-phosphosulfate (APS) as a substrate. This protein fraction was isolated by ammoniumsulfate precipitation, DEAE-cellulose chromatography and Sephadex-G-100 gel filtration. A solvent system was developed for thin layer chromatography to separate cAMP from APS, adenosine-5'-phosphoramidat (APN), 5'-AMP and adenosine. The product was identified as cAMP by cochromatography, coelectrophoresis, treatment with phosphodiesterase, chromatography on Biorad-columns and by the protein-binding assay. The so far purified protein fraction produced 151 pmol cAMP/mg protein and hour *in vitro*, using APS as a substrate.

Introduction

Cyclic nucleotides have major regulatory functions in microorganisms and animals, however, it is not clear, whether these principles can be applied to algae and plants. Even the existence of cAMP in plants and speculative role for this nucleotide is a matter of dispute, since "cAMP-like" substances showed similar but not the same behaviour on the separation methods used when compared with authentic cAMP [1, 2]; furthermore no proof of an ATP-cyclase has been published for plant material.

A new pathway for cAMP formation was suggested by the work of Tsang and Schiff [3], who reported that adenosine-5'-phosphosulfate should be used as a substrate for cAMP formation; however, a careful analysis of this "cAMP" showed recently, that this "cAMP" was a nitrogen-analog of ADP, identified as adenosine-5'-phosphoramidate (APN)

[4, 5]. However, the idea fascinated us, that the high hydrolysis energy of APS (about 18 kcal) should possibly be used for a cyclisation of APS to cAMP. Since we knew, that APS could react chemically with ammonia to form APN, which has chromatographic and electrophoretic properties closely to cAMP [5] we developed a method to separate cAMP from APS and APN by thin layer chromatography. The approach to find such an cAMP-forming enzyme was to isolate APS-sulphydrolases, which hydrolase the sulfur bond of APS and to analyze, if one of these purified enzyme fractions would form cAMP during this reaction. We will report here on the isolation of such a protein fraction and on the identification of one nucleotide formed as cAMP. A short note of our results has been published [6].

Materials and Methods

Organism

Chlamydomonas reinhardtii strain CW 15 was obtained from Dr. Theimer of this Institute. Cells were grown in the "high salt" medium of Sueoka [7] with modifications to tenfold higher magnesiumsulfate [8, 9]; each batch was controlled for contamination by light microscopy and plating. Growth parameters were determined by counting cell numbers.

Abbreviations: cADP, adenosine-3'-5'-pyrophosphate; cAMP, adenosine-3'-5'-monophosphate; APN, adenosine-5'-phosphoramidate; APS, adenosine-5'-phosphosulfate; cGMP, guanosine-3'-5'-monophosphate; IBMX, 1-methyl-3-isobutyl-xanthin; PAPN, 3'-phosphoadenosine-5'-phosphoramidate; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; PBA, protein-binding assay; PDE, phosphodiesterase; DTE, dithioerythritol.

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Determination of cAMP

In vivo measurements

Cells were harvested at different growth stages and heated for 5 min in a boiling water bath to inactivate phosphodiesterases. Before cells were broken in a french press at 10 000 PSI, 10 000 cpm of carrierfree [³H]cAMP were added for recovery calculations. After determination of the protein content of the crude extract it was heated again for 10 min as described above. Particulate matter was removed by centrifugation for 30 min at 40 000 × *g*. The supernatant was fractionated on Biorad-columns. In small Pasteur pipettes (Ø = 0.5 cm) the ion exchange resin Biorad 1 × 8 (100 to 200 mesh) was added to 0.5 cm followed by 4.5 cm of Biorad 1 × 2 (200–400 mesh), both in the chloride form. Samples were applied to these columns. Materials not bound to the resin were washed out with 10 ml of water and cAMP was eluted afterwards with 5 ml of 0.05 M HCl into scintillation vials. These vials were lyophilized over night to remove the HCl. After addition of 1.0 ml of water the cAMP content was determined according to Gilman [10, 11] using the protein binding assay in the calibration range of 2 to 40 pmol. All results are corrected for the recovery of the tracer cAMP.

In vitro measurements

Cyclic AMP formation *in vitro* was measured by incubation of the purified protein fraction in a system containing in a total volume of 0.1 ml: 10 mM Tris-HCl pH 9.0; 2 mM APS and if indicated 10 mM MgCl₂. After incubation for 1 h at 37 °C the protein was denaturated by boiling. After addition of tracer-cAMP (4000 cpm) aliquots were separated on thin layer plates (Silica-gel GF₂₅₄ activated for 30 min at 110 °C) using the solvent system isopropanol/conc. ammonia/ethanol (6/3/1) which separated APS, APN, and cAMP (Fig. 5a). The *R_f*-values for APS was 0.47, for APN 0.54 and for cAMP 0.65. The cAMP band, visible in the UV-light, was eluted and treated with the cAMP phosphodiesterase of bovine, which seems to be specific for cyclic nucleotides [12]. Treated and untreated samples were separated on Biorad-columns as described above and analyzed with the protein binding assay for cAMP.

Determination of APS-degrading activities

APS-degrading activities were measured by the liberation of labelled sulfate from labelled APS. The test system contained in a total volume of 0.1 ml: Tris-HCl pH 9.0: 10 mM; [³⁵S]APS 20 µM (1000 cpm pro nmol); MgCl₂: 10 mM if indicated; and protein; incubation for 1 h at 37 °C. After 1 h the reaction was terminated by boiling and the inactivated protein was removed by centrifugation for 3 min at 10 000 × *g*. A sample was diluted with water and 200 µmol of nonlabelled sodiumsulfate to 1 ml, and an aliquot was measured before and after adsorption of APS to 500 mg of charcoal according to Austin *et al.* [13].

Analysis of nucleotides produced during APS-degradation

For the analysis of different nucleotides [8-¹⁴C]-APS was used and the products were separated in the usual manner, on TLC-plates; with these technique we could separate APS, cAMP, 5'-AMP, APN, adenosine, and adenine.

Preparation of APS

APS was prepared according to Cooper and Trüper [14] using the APS-reductase system of *Thiobacillus denitrificans*. Because the APS prepared in this way was contaminated with cAMP (about 0.001%) it had to be purified further using thin layer chromatography and separation on Sephadex-G-25 fine columns, when APS was needed for cAMP formation.

Protein purification

Cells were harvested by centrifugation and washed once with the extraction buffer (0.02 M Tris-HCl pH 8.0 containing 10 mM ME). For 1 g of algae 3 ml of extraction buffer was added; the cells were broken in a french press at 10 000 PSI. After centrifugation for 10 min at 10 000 × *g* the supernatant was used as crude extract. It was fractionated by ammoniumsulfate precipitation, DEAE-cellulose chromatography and Sephadex-G-100 gel filtration as described in the text. The purity of the final enzyme preparation was assayed by disc-gel electrophoresis [15] with 7.5% acrylamid gel (5½ h, 3 to 4 mA per gel). After staining with coomassie blue the gels were scanned at 550 nm for protein bands.

Determination of protein

Protein was determined as described previously [16].

Radioactivity determination

Radioactivity was determined according to Patterson and Green [17] using a Beckmann LS-100 scintillation counter.

Scanning of thin layer plates

A Turner fluorimeter 111 equipped with a Camag-T-scanner was used with filter no. 110–810 for the primary light source and filter no. 110–823 10% for the photomultiplier side; the obtained signal was recorded with W + W recorder 1100.

Chemicals

[³⁵S]sulfite, [³⁵S]sulfate, and [8-¹⁴C]-5'-AMP were obtained from Buchler (Braunschweig, W.-Germany). Adenosine, ADP, ATP, AMP, cAMP, cGMP and cAMP phosphodiesterase were purchased from Boehringer (Mannheim, W.-Germany). Theophyllin, IBMX, and APN were obtained from Sigma (München, W.-Germany). Sephadex-G-25 fine, G-50, G-100 was ordered from Pharmacia (Freiburg, W.-Germany) and DEAE-52 cellulose, acrylamid, and bis-acrylamid were obtained from Serva (Heidelberg, W.-Germany). All other chemicals not mentioned were purchased from Merck (Darmstadt, W.-Germany).

Results

cAMP levels in vivo

Cells of *Chlamydomonas reinhardtii* CW 15 were inoculated into fresh medium with 5×10^6 cells/ml. Cyclic AMP levels were determined after 14, 32, 53 and 72 h, as is shown in Table I. No cAMP was detected in the early exponential growth curve, however, the level of cAMP increased during the exponential growth to reach a very high level during the stationary phase (Fig. 1). In the same time, were the cyclic AMP pool increased drastically (53 to 72 h) the cells lost their flagellae and became immotile, which could be observed with light microscopy.

The effects of phosphodiesterase inhibitors on the cyclic AMP levels are somewhat confusing, as can be seen from the data of Table I. Theophyllin seems to have only an effect when incubated for a long time, whereas IBMX affected the cyclic AMP level also in short time experiments. However, these two methylxanthines seem to act differently, since only treatment with IBMX inhibited the mobility of the cells, whereas theophylline showed no influence on cell motility, even under long term experiments.

Purification of APS-sulphydrolases

It is difficult to determine cAMP formation in crude extracts of *Chlamydomonas*, since this organism contains phosphodiesterases, which split cAMP to 5'-AMP [12]. Therefore we isolated different APS-sulphydrolases and purified these activities partly, to

Table I. cAMP levels *in vivo*.

Growing time [h]	Treatment	Cell number	pmol cAMP/g fresh weight	pmol cAMP/mg protein	%
14	–	5.5×10^6	n.d.	n.d.	
32	–	2.0×10^7	71.4	2.4	
53	–	1.2×10^8	508	4.3	100
53	Theophyllin				
	a) long time	1.2×10^8	788	8.3	193
	b) short time	1.2×10^8	188	1.6	37
53	IBMX				
	a) long time	0.6×10^8	406	5.3	123
	b) short time	1.1×10^8	931	6.3	155
72	–	2.6×10^8	2166	18.8	

cAMP was determined as described in materials and methods; Theophyllin: 10 mM; IBMX: 0.4 mM; short incubation = 90 min; long time = during whole growing time; n.d. = not detected.

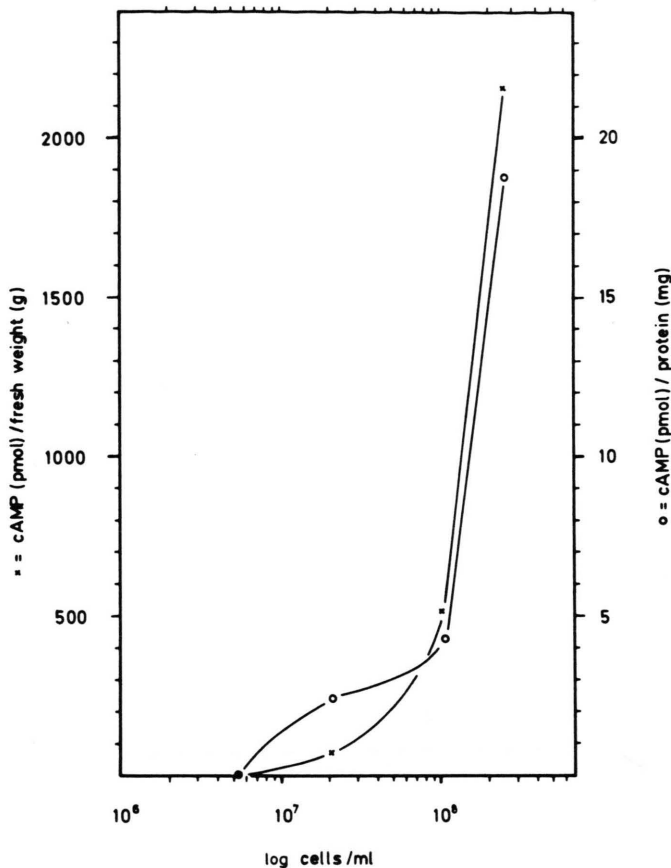


Fig. 1. cAMP levels *in vivo*. The results have been corrected for the recovery of tracer cAMP.

analyze for the possibility of cAMP formation with the purified enzyme fractions, were activities of phosphodiesterases would have been removed. Cells were harvested in the late exponential phase (80 h) and broken in a french press as described in materials and methods. The crude extract was fractionated by ammoniumsulfate between 35% and 55% saturation, dialyzed and separated on a DEAE-cellulose column. Fig. 2 shows an elutionsprofile of this column. When APS-sulphydrolase activity was analyzed without addition of magnesiumchloride, two peaks could be found; however, when the assays were done in the presence of $MgCl_2$ a third peak between 300 and 400 ml effluent was seen. Only the first peak of this DEAE-cellulose column between 10 and 60 ml effluent showed a distinct formation of cAMP, therefore this fraction was purified further on a Sephadex-G-100 column. The elution profile of this column is shown in Fig. 3. Only one peak of APS-sulphydrolase activity was found, which did not

need magnesium ions for activity. The active fractions were pooled and analyzed for APS-degradation, cAMP formation and further metabolism of adenine nucleotides.

Evidence for cAMP formation

With the so far purified enzyme system the nucleotides produced during APS-degradation were analyzed. The separation of the different nucleotides formed was achieved using thin layer chromatography; some data are shown in Fig. 4. It is evident that 5 different nucleotides can be found: 5'-AMP, an unknown nucleotide, APS, cAMP and adenosine. This suggested, that the so far purified enzyme fraction contains different enzymatic activities; and at least 4 protein bands could be detected in this fraction by disc-gel electrophoresis (Fig. 5). Analysis with ^{14}C labelled APS, APN, 5'-AMP and cAMP showed that small, but detectable amounts of the following reactions are present: a) a phosphatase is

Fig. 2. Chromatography of APS-sulphydrolases on DEAE-cellulose. The dialyzed extract was separated on a DEAE-cellulose column (12×2.1 cm) with a linear gradient of 0 to 0.2 M NaCl (400 ml) in 0.02 M Tris-HCl pH 8.0 containing 10 mM ME. APS-sulphydrolase activity was determined as described in materials and methods.

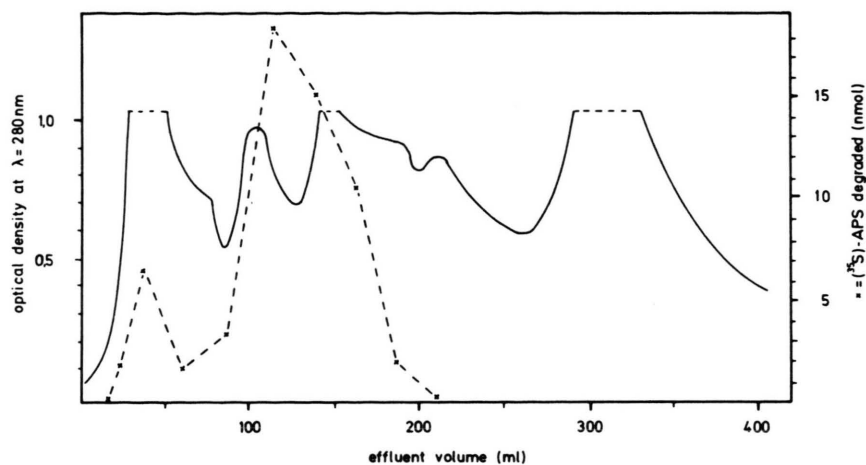


Fig. 3. Gel filtration of APS-sulphydrolase activity on Sephadex-G-100. The Sephadex-column (2.1×50 cm) was equilibrated and eluted with 0.02 M Tris-HCl pH 8.0 containing 10 mM ME and 100 mM KCl. The active fractions (66–104 ml) were pooled and concentrated in an Aminco-diaflow cell with a 10000 D filter.

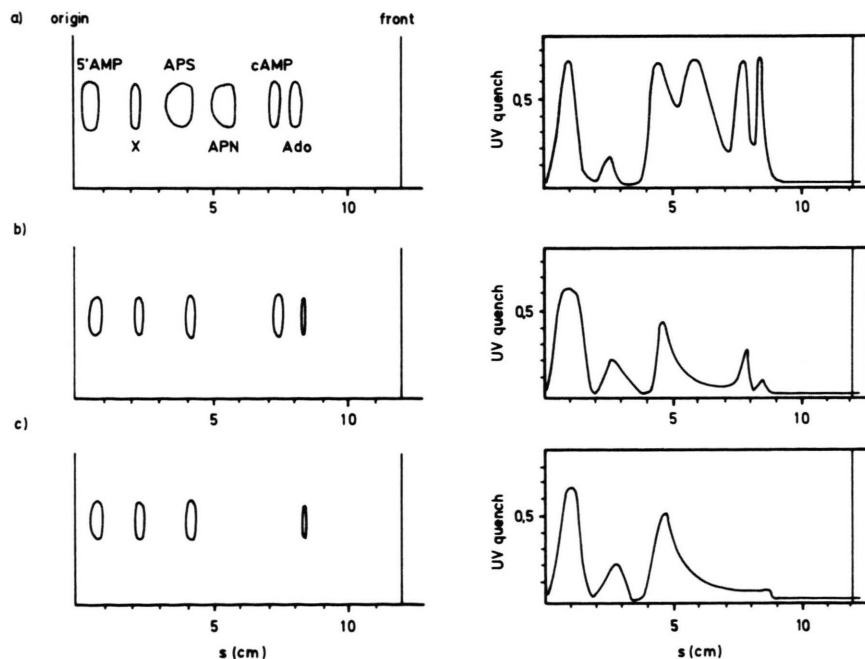
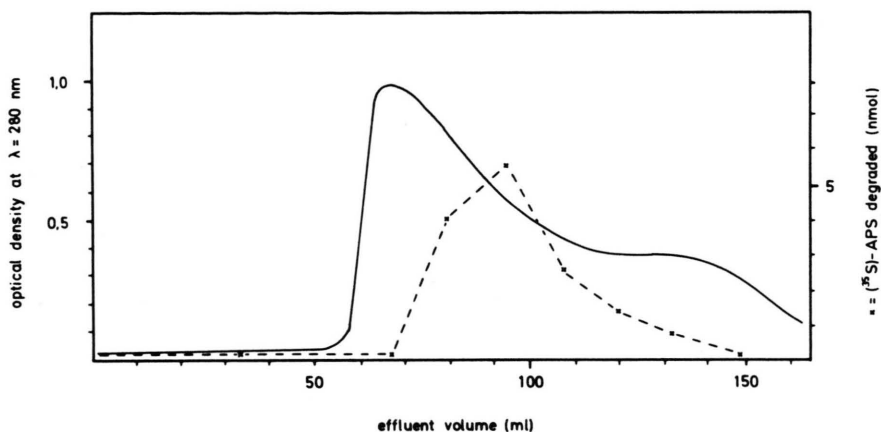


Fig. 4. Nucleotides formed from non-labelled APS a) reference nucleotides; b) metabolism of APS with the purified enzyme fraction; c) conditions as b with 50 mM NH_4Cl ; λ = unknown nucleotide.

present, which splits 5'-AMP to adenosine b) that a phosphodiesterase is present, which splits cAMP to 5'-AMP c) that an enzymatic activity is present, which splits APN to 5'-AMP; d) that an enzyme is present which hydrolyzes APS. It should be noticed that the data of Fig. 5 show, that a substance with properties of cAMP could be detected, when APS was incubated with the so far purified enzyme fraction, however, no nucleotide with properties of APN could be detected, which was tentatively identified as cAMP [3]. Addition of NH_4Cl , which should

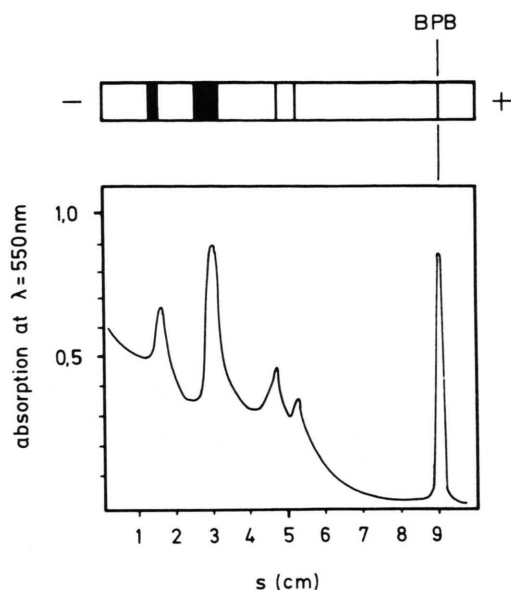


Fig. 5. Disc-gel electrophoresis of the purified protein fraction on 7.5% polyacrylamid-gel at pH 8.8. The bands visualized by staining for protein are shown above and the scan at 550 nm is shown below. s: distance from origin; BPB: marker bromophenole-blue.

Table II. Formation of cAMP from APS *in vitro*.

Treatment	I_0/I_x		pmol cAMP in the testtube		pmol cAMP/mg protein \times h
	- PDE	+ PDE	- PDE	+ PDE	
a) APS 1.0 μmol	10.1	n.t.	10.0	—	—
b) APN 1.0 μmol	1.8	n.t.	<2.0	—	—
c) TLC-region after chromatography of APS	1.4	n.t.	<2.0	—	—
d) enzyme fraction after incubation with APS	16.0	1.1	77.5	<2.0	151

For cAMP formation 2.5 mg of purified protein were incubated with 5 μmol Tris-HCl pH 9.0; 1 μmol APS in a total volume of 0.5 ml; incubation for 1 h at 37°C.

The reaction mixture was separated in the TLC-system, the cAMP band was eluted and separated on a Biorad column before analysis of cAMP with the protein-binding assay. I_0/I_x was determined according to Gilman [10]. n.t. = not tested; PDE = phosphodiesterase.

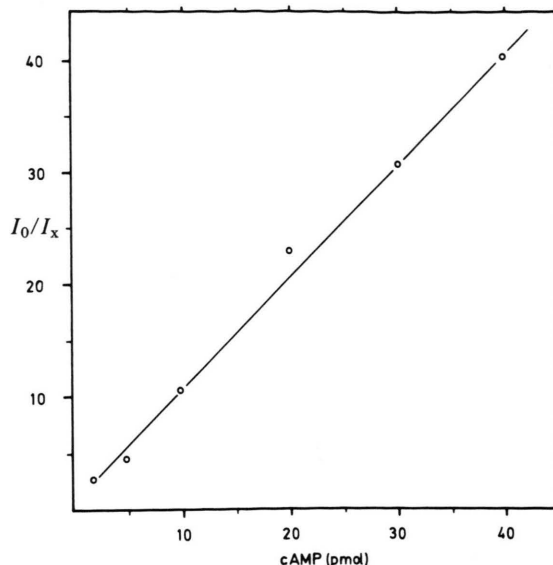


Fig. 6. Calibration curve of the protein-binding assay.

favour the formation of APN, inhibited cAMP formation and again no APN was found.

To identify the substance with R_f of cAMP, [^{14}C]-labelled APS was used and the cAMP like substance was eluted from the plate and half of it was treated with the phosphodiesterase from bovine heart and the products were analyzed again on thin layer plates. After treatment with phosphodiesterase, this nucleotide moved as 5'-AMP. Since other substances like APS, APN or APPA would show the formation of 5'-AMP after hydrolysis with suitable enzymes, it was necessary to analyze this nucleotide in the protein binding assay, which was used for cAMP

determinations *in vivo*. These data together with the calibration curve for cAMP-determinations are shown in Fig. 6 and Table II. APS in high concentrations seems to interfere with the protein binding assay, whereas APN and silica gel did not inhibit when analyzed for cAMP after separation on Biorad columns. Therefore samples were separated first on thin layer plates and afterwards on Biorad columns before analyzing the cAMP content. The data of Table II d show that indeed a substance can be found which binds in the cyclic AMP assay and which is inactive in this assay system after treatment with phosphodiesterases (Table II).

Discussion

Keates [18], Amrhein [1] and Niles and Mount [19] failed to detect cAMP in plants, and Lin [2] suggested that higher plants do not require cyclic nucleotides. However, Ashton and Polya [20, 21] and Truelsen and Wyndaele [22] demonstrated recently the existence of cAMP in plants by different methods. The demonstration of cAMP in *Chlamydomonas reinhardtii* CW 15 as shown in this paper confirms earlier results of Amrhein and Filner [12]. Cyclic AMP can be found during the exponential growth phase and especially during the stationary phase. When the cells lost their flagellae, the cAMP level rose drastically, a phenomenon which might indicate a physiological role for cAMP in this organism (Handa *et al.* [23]). This was indicated by results of Rubin *et al.* [24, 25] which showed a reaction between cAMP and the microtubulin of the flagellae in *Chlamydomonas*. The motion of flagellae can be inhibited by methylxanthines (Theophylline and IBMX) which seem to inhibit phosphodiesterases, which are located on the plasmamembrane of the flagellae. Our results with these substances are inconclusive, because inhibition of phosphodiesterases with IBMX increased the cAMP level after short-time and long-time incubations, whereas theophyllin influenced the cAMP level only in long-time incubations; however, different uptake mechanisms could be responsible for this difference. It should be mentioned that cultures grown with IBMX lost their flagellae and had longer generation times when compared with controls.

Although a role of cAMP for plant metabolism has not been demonstrated up to now, it seems to be

necessary for the motion of flagellae [26], for the potential of membranes [27] and perhaps for the circadian rhythm of leaf movements [28]. If cAMP occurs in plants is further questioned by results concerning the distribution of the ATP-cyclase in photosynthetic organisms. Hintermann and Parish [29] found the ATP-cyclase only in blue-green algae, green algae, and fungi, but not in higher plants. The purification of an "APS-cyclase" by Tsang and Schiff [3] seemed to indicate a new pathway for cAMP formation, however, recent data have shown that the nucleotide found was in fact adenosine-5'-phosphoramidate (APN) and not cAMP [4]. The surprising finding of Rosenberg and Pall [30] that the *cr-1* mutant of *Neurospora crassa* contained only small amounts of ATP-cyclase in comparison with the wild type, however, that it had the same high levels of cAMP as the wild type suggested the existence of an unknown pathway for cAMP formation.

The idea that the high energy of the sulfate-phosphate anhydride bond of APS could be used for the cyclisation to cAMP was attractive to us, therefore we decided to isolate enzymes hydrolyzing APS. With this assay system we separated 6 different APS-sulphhydrolases, by ammoniumsulfate precipitation, DEAE-cellulose chromatography and Sephadex-gel filtration; the isolation of that fraction which gave rise to cAMP is described in this paper. The first indication for cAMP formation resulted from separation on thin layer plates, where cAMP (R_f 0.7) was separated from APN (R_f 0.56). Incubation with ^{14}C labelled APS yielded a labelled "cAMP" like substance which comigrated with cAMP in electrophoresis and chromatographic systems and which was split by beef heart phosphodiesterase (which is known to be specific for cyclic nucleotides [32]) to 5'-AMP. cGMP can be excluded, since it is eluted from the Biorad columns with 0.5 M HCl. We feel that the combination of thin layer chromatography and separation on Biorad columns in combination with the protein binding assay is very good evidence that the substance found is indeed cAMP, although we have not been able to analyze this substance in the protein kinase assay, which had not been available to us. A reaction similar to APS-cyclisation had been suggested for the formation of cADP from PAPS by Su and Hassid [33]. One might speculate that substances like APN and PAPN should possibly be also good substrates for similar cyclisations. However,

our data do not indicate, that APN is an intermediate in cAMP formation from APS, although this possibility has not been ruled out completely. APS is an important sulfonucleotide in the reduction of sulfate to cysteine in plants and algae [34]; therefore the balance of APS-degradation, cAMP-formation and sulfate reduction may be important for the situation *in vivo*.

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- [1] N. Amrhein, Ann. Rev. Plant Physiol. **28**, 123–132 (1977).
- [2] T. Lin, Advances in Cyclic Nucleotide Research Vol. **4**, 439–461, Raven Press, New York 1974.
- [3] L.-S. M. Tsang and J. A. Schiff, Eur. J. Biochem. **65**, 113–121 (1976).
- [4] H. Fankhauser, L. Garber, and J. A. Schiff, Plant Physiol. **63**, S-897 (1979).
- [5] B. P. Cooper, F. E. Baumgarten, and A. Schmidt, Z. Naturforsch. **35 c**, 159–162 (1980).
- [6] A. Schmidt and F. Kühllhorn, Botanikertagung Marburg 226 (1978).
- [7] N. Sueoka, Proc. Nat. Acad. Sci. USA **46**, 89–91 (1960).
- [8] T. Lien and G. Knutsen, Arch. Microbiol. **108**, 189–194 (1976).
- [9] R. Kudielka, Thesis, München 1980.
- [10] A. G. Gilman, Proc. Nat. Acad. Sci. USA **67**, 305–312 (1970).
- [11] A. G. Gilman, Advances in Cyclic Nucleotide Research, Vol. **1**, pp. 389–398, Raven Press, New York 1972.
- [12] N. Amrhein and P. Filner, Proc. Nat. Acad. Sci. USA **70**, 1099–1109 (1973).
- [13] J. Austin, D. Armstrong, D. Stumpf, T. Luttenegger, and M. Drago, Biochimica Biophysica Acta **192**, 29–36 (1969).
- [14] B. P. Cooper and H. G. Trüper, Z. Naturforsch. **34 c**, 346–349 (1979).
- [15] B. J. Davis, Ann. N. Y. Acad. Sci. **121**, 404–427 (1964).
- [16] A. Schmidt, Plant Sci. Lett. **5**, 407–415 (1975).
- [17] M. S. Patterson and R. L. Green, Anal. Chem. **37**, 854–857 (1965).
- [18] R. A. Keates, Nature **244**, 355–357 (1973).
- [19] R. M. Niles and M. S. Mount, Plant Physiol. **54**, 372–373 (1974).
- [20] A. R. Ashton and G. M. Polya, Plant Physiol. **61**, 718–722 (1978).
- [21] A. R. Ashton and G. M. Polya, Biochem. J. **165**, 27–32 (1977).
- [22] T. A. Truelsen and R. Wyndaele, Physiol. Plant. **42**, 324–330 (1978).
- [23] A. K. Honda, J. Cherniak, R. A. Bressan, and P. Filner, Plant Research 78, MSU-DOE Plant Research Laboratory, 102 (1979).
- [24] R. W. Rubin, N. Amrhein, and P. Filner, 11. Ann. Meeting, Am. Soc. Cell Biol., 254 (1971).
- [25] R. W. Rubin and P. Filner, J. Cell. Biol. **56**, 628–635 (1973).
- [26] G. Hartfield and N. Amrhein, Biochem. Physiol. Pflanzen **169**, 531–536 (1976).
- [27] J. M. Trevillyan and M. L. Pall, J. Bacteriol. **138**, 397–403 (1979).
- [28] J. Bollig, K. Mayer, W. E. Mayer, and W. Engelmann, Planta **141**, 225–230 (1978).
- [29] R. Hintermann and R. W. Parish, Planta **146**, 459–461 (1979).
- [30] G. Rosenberg and M. L. Pall, Arch. Microbiol. **118**, 87–90 (1978).
- [31] F. Kühllhorn, Diplomarbeit München 1978.
- [32] R. W. Butcher and E. W. Sutherland, J. Biol. Chem. **237**, 1244–1250 (1962).
- [33] J. C. Su and W. Z. Hassid, Biochem. **1**, 474–480 (1962).
- [34] A. Schmidt, Encyclopedia of Plant Physiology, New Series, Vol. **6**, pp. 481–496, (M. Gibbs and E. Latzko, eds.), Springer Verlag 1979.