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ADENOSINE 3′,5′-CYCLIC MONOPHOSPHATE PHOSPHODIESTERASE FROM *PHYCOMYCES BLAKESLEEANUS*

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Key Word Index—*Phycomyces*; fungus; phosphodiesterase; cyclic AMP; sensory system.

Abstract—Cyclic AMP phosphodiesterase has been extracted from *Phycomyces* sporangiophore. The material is stable at −20° for several months. Activity depends on the presence of a divalent metal ion (e.g. magnesium). The enzyme may be multiple; at pH 8, two K_ms are observed, 3 and 12.5 μM, and an Arrhenius plot has a 'break' at *ca* 21°. No cooperativity is seen. Inhibition by dibutyryl cyclic AMP is marked, but cyclic GMP is not inhibitory (except at lower pH and high concentration) and cyclic GMP is not hydrolysed. The enzyme is thermolabile above 30°. Calcium is not stimulatory. Millimolar concentrations of pyrophosphate and nucleoside triphosphates are required for significant inhibition. Reductants, ascorbic acid, cysteine, dithiothreitol, glutathione, β-mercaptoethanol, NADH, sodium dithionite, sodium sulfite and the mild oxidant, ferricyanide, have little or no effect. Gallic acid, an abundant endogenous reducing agent, is inhibitory. Histamine and imidazole are slightly inhibitory. Methylxanthines are inhibitory but at high concentrations. Inhibition to 50% required 15, 15, 30 and >4 mM for aminophylline, theophylline, caffeine and 3-isobutyl-1-methylxanthine, respectively. The enzyme may be involved in the behavioral responses of the organism.

INTRODUCTION

Cyclic nucleotides may be involved in the growth and development of *Phycomyces blakesleeanus*. The mature sporangiophore undergoes a transient spurt of growth when stimulated by blue light. An early event upon stimulation is a sharp dip and recovery in cyclic AMP level before growth is observed. Added cyclic AMP elicits a negative growth reponse [1]. Small concentrations of cyclic AMP in the media causes gross morphological and physical changes in the mycelia [2] and interferes with the early sexual interaction in related Mucorales [3]. In order to understand the role of cyclic nucleotides in these processes, adenosine 3′,5′-cyclic phosphodiesterase (EC 3.1.4.17) from *Phycomyces* must be sufficiently characterized and compared to other systems. Presented here are a few properties including subcellular location, K_m, activation energy, stability, and the effects of nucleotides, reducing agents and xanthine inhibitors. Elsewhere we reported evidence that phosphodiesterase is blue light-sensitive [4] and that certain mutants may contain aberrant phosphodiesterase [5].

RESULTS

Cyclic 3′,5′-AMP phosphodiesterase activity has been isolated in soluble form from *Phycomyces* stage IVb sporangiophore by differential centrifugation (Table 1). Most of the activity is precipitated by 40% saturated ammonium sulfate. It should be noted, however, that moderate changes in the procedure, such as lower salt or the presence of Mg^{2+}, may allow up to 10–15% of the activity to remain in the particulate fractions. All experiments, other than the subcellular localization reported here, used phosphodiesterase precipitated with $(NH_4)_2SO_4$ immediately after the 31000 *g* centrifugation, omitting the high speed run. The disappearance of cyclic AMP was linear with time up to 60 min incubation with enzyme, and the rate was proportional to enzyme concentration up to 350 μg protein per assay in the standard 30 min incubation.

The enzyme requires a divalent or transition metal ion (Table 2). EDTA completely inhibits activity; magnesium at least partially fulfils this requirement. An apparent magnesium K_m of 0.6 mM is obtained from a double reciprocal plot of values in the table. In the presence of adequate magnesium, calcium ions and EGTA have no further effect (Table 3).

Phosphodiesterase is fairly stable. At −20°, activity remains constant after 2–3 months storage. Repeated freezing and thawing has little effect. At 0°, about 1% activity is lost per hr and 24° *ca* 4%/hr.

The material exhibits two K_ms on a Lineweaver–Burk double reciprocal plot, 3 and 12.5 μM, with cyclic AMP as substrate at pH 8 (Fig. 1). The high affinity Michaelis constant is comparable to the enzyme from animals. A Hill plot indicates no cooperativity for either activity, unlike the enzyme isolated from *Neurospora* [6]. An Arrhenius plot reveals two straight lines intersecting at *ca* 21° giving activation energies of 10.2 and 13.4 kcal/mol (Fig. 2). Possibly two separate molecular entities exist.

However, under these assay conditions, the enzyme activity appears to be specific for cyclic AMP. At 4 μM, cyclic AMP as substrate, and at pH 8, up to 100 μM cyclic GMP inhibited hydrolysis very little (<10%). In contrast, at pH 7.2, 50 μM cyclic GMP inhibited activity *ca* 60% (Fig. 3). Labeled cyclic GMP (4 μM) was not degraded at either pH.

Table 1. Subcellular distribution of phosphodiesterase

Step	Amount of protein (mg)	Specific activity (pmol/min/mg protein)	Total activity pmol/min (%)
Homogenate	960	15.1	14500
1000 *g*			
pellet	180	1.0	180 (1.5)
supernatant	780	14.6	11400 (100)
31000 *g*			
pellet	20	0.3	6.2 (0.05)
supernatant	710	16.0	11400
100000 *g*			
pellet	24	2.8	66 (0.5)
supernatant	700	15.9	11200 (98)
$(NH_4)_2SO_4$			
40%	150	48.0	7200 (63)
75%	70	3.9	270 (2.4)
100%	30	1.8	54 (0.5)
supernatant	450	~1.0	—

The standard cyclic AMP phosphodiesterase assay is done with 4 μM cyclic AMP in 5 mM $MgCl_2$, 40 mM Tris–HCl, pH 8 at 24° ± 1°. 50–300 μg of extracted protein is incubated 30 min in assay solution. Proteins were determined by the Lowry procedure [26] after dialysing to remove interfering phenolics. % of total activity uses the supernatant after the 1000 *g* centrifugation as reference.

Phosphodiesterase from *Phycomyces* is unstable (Fig. 4). Pre-incubation for 2 min at slightly elevated temperature greatly decreased activity. Only half the usual activity remained after 2 min at 45°. Such instability is characteristic of an allosteric enzyme or an enzyme complex. Because so many processes of growth and development are light sensitive in *Phycomyces*, we compared the stability of phosphodiesterase extracted in the light and in the dark. The slight difference found is probably not significant.

Phosphodiesterase activity is surprisingly insensitive to added nucleoside triphosphates (Table 4). Millimolar concentrations must be encountered before an appreciable inhibition in degradation of cyclic AMP at 4 μM occurs. This is most likely an artifact due to chelation of Mg^{2+}.

Several reducing agents have been implicated as modulating phosphodiesterase activity in other systems. However, in *Phycomyces*, β-mercaptoethanol, dithiothreitol, sodium sulfite, sodium dithionite, ascorbic acid, reduced glutathione, NADH and NADPH, and cysteine all had little or no effect (Table 5). Curiously 5 mM gallic acid has a slight (20%) inhibitory effect. This substance is found in a great abundance in *Phycomyces* sporangiophore. Mild oxidizing agents such as ferricyanide have little effect. Histamine and imidazole are slightly inhibitory in contrast to their stimulation of mammalian phosphodiesterase. Dibutyryl cyclic AMP strongly inhibits the *Phycomyces* enzyme.

To be effective, higher concentrations of the classical methylxanthine inhibitors were required (Fig. 5) than for mammalian phosphodiesterase. Inhibition to 50% required *ca* 15 mM aminophylline or theophylline, and 30 mM caffeine. The solubility limit of 3-isobutyl-1-methylxanthine was reached before attaining 50% attenuation (>4 mM).

Table 2. Effect of magnesium and EDTA on phosphodiesterase activity

Compound	Concentration (mM)	% Activity (5 mM $MgCl_2$ = 100%)
$MgCl_2$	0	60
	0.5	63
	1.0	71
	2.5	78
	5	100
	10	96
EDTA	2.5	28
	5	0

The assay was conducted in 40 mM Tris–HCl, pH 8 for 30 min at 24° ± 1° in the presence of the indicated amounts of magnesium or EDTA.

DISCUSSION

P. blakesleeanus is eukaryotic, carrying a genome only 6.7 times larger than *E. coli* [7], yet having many refined developmental and growth controls. Signals include blue light, hormones and other chemicals, and temperature. Since many analogous processes in other systems typically involved cyclic nucleotides, it is imperative to examine the metabolism of these substances in *Phycomyces*. Hundreds of well-characterized mutants in responsiveness and development are available for biochemical inquiries. This work on phosphodiesterase was undertaken to initiate our attempt to understand the underlying biochemical principles behind the much better understood behavioral and genetic studies.

One significant observation gleaned from this work and related investigations is that few generalities can be made concerning phosphodiesterase activity. In particular, no useful phylogenic conclusions are apparent. Phosphodiesterase from most sources possess K_ms ranging from 3 to 200 μM, just greater than the endogenous levels of cyclic AMP. Multiple forms of the enzyme are commonly reported—perhaps 7 isozymes

Table 3. Effect of calcium and EGTA on phosphodiesterase activity

Compound	Concentration	Activity (pmol/min/mg protein)	% Control
$CaCl_2$	0 μM	10.9	100
	1	11.2	103
	20	9.9	91
	50	11.0	101
	100	11.3	104
	250	10.9	100
	500	11.8	108
EGTA	0.1 mM	12.1	111
	1	12.2	112

Phosphodiesterase was incubated in 5 mM $MgCl_2$, 40 mM Tris–HCl, pH 8 at 24° ± 1° in the presence of 4 μM cyclic AMP and the indicated concentrations of calcium and EGTA. ([ethylene bis(oxyethylene-nitrilo)]tetraacetic acid).

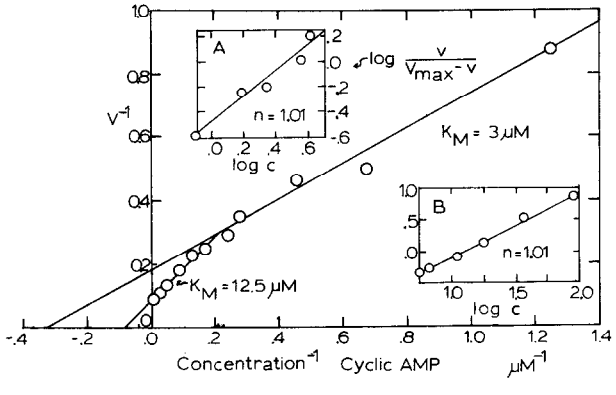

Fig. 1. Double reciprocal plot for the hydrolysis of cyclic AMP by cyclic AMP phosphodiesterase. The rate of hydrolysis, v, is in pmol/min/mg protein. The two apparent Michaelis constants are 12.5 and 3 μM. (A) A Hill plot of $\log v/V_{max} - v$ vs log [cyclic AMP] [μM]. For the right side of the Lineweaver–Burk plot (< 5 μM), the slope $n = 1.01$. (B) Similar to (A) but for [cyclic AMP] between 5 and 100 μM, $n = 1.01$. Note different scales. The system exhibits no cooperativity or deviations from linearity. pH 8; temperature 24° ± 0.5°.

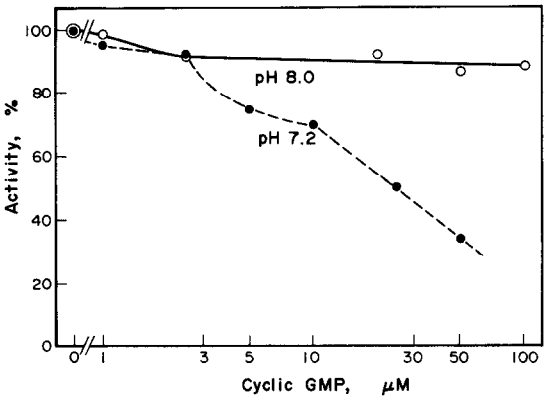

Fig. 3. Activity of cyclic AMP phosphodiesterase in the presence of the indicated concentrations of cyclic GMP. Enzyme incubated with 4 μM cyclic AMP at pH 8 and 7.2 (titrated with HCl). At low pH inhibition is observed.

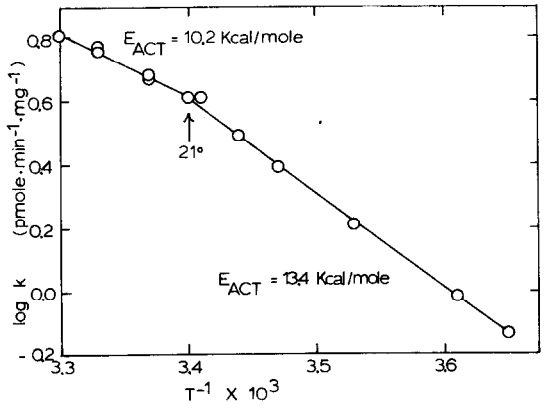

Fig. 2. An Arrhenius plot for phosphodiesterase activity: the logarithm of the apparent rate constant for cyclic AMP hydrolysis vs T^{-1}. The apparent rate constant k is measured between 0 and 30°. A 'break' in the plot appears at *ca* 21°. The two activation energies are 10.2 (high temperature) and 13.4 kcal/mol. 4 μm cyclic AMP; pH adjusted to 8 at each temperature.

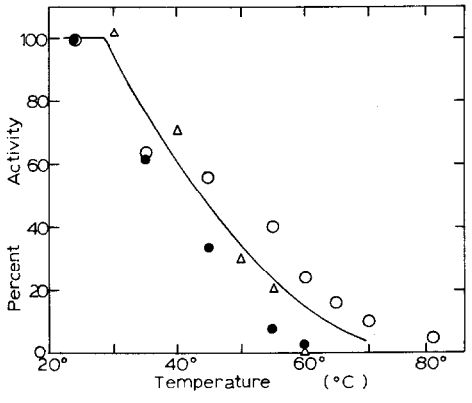

Fig. 4. Activity of phosphodiesterase after 2 min exposure to indicated elevated temperatures. *Phycomyces* phosphodiesterase was kept at the indicated temperatures 2 min and plunged into an ice bath before assaying for activity at 24° ± 0.5°. Triangles and open cicles are two separate extractions and assays. Closed circles refer to a preparation made in the dark and under dim red light, but assayed under normal room light conditions.

Table 4. Effect of nucleoside triphosphate and pyrophosphate on phosphodiesterase activity

Compound	Activity in presence of 0.5 mM NTP or PP_i pmol/min/mg protein	% Control	Concentration required for 50% inhibition of activity (mM)
None	11.2	100	—
ATP	11.2	100	2.8
GTP	11.8	105	1.6
TTP	10.1	90	3.0
UTP	11.9	106	2.9
PP_i	11.8	105	9.0

Activity of cyclic AMP phosphodiesterase was measured at 4 μM cyclic AMP in 5 mM $MgCl_2$, 40 mM Tris–HCl, pH 8 at 24° ± 1°; 30 min incubation. Various concentrations of the nucleotide triphosphates and pyrophosphates were added. The right column was obtained by interpolating data. At the highest concentrations required, the pH was back-titrated to 8.

in a rat [8], some of which appear to be interconvertible in rat liver [9]. Some organisms have a very insensitive activity, with a K_m in the millimolar range; however, in the case of *Dictyostelium discoideum*, the higher K_m moiety is due to interaction with an inhibitor [10]. Two high affinity activities, $K_m = 3$ and 12.5 μM, are suggested in *Phycomyces* sporangiophore (Fig. 1), but no high K_m species is implied by the data. The Arrhenius plot (Fig. 2) and cyclic GMP inhibition (Fig. 3) also imply two or more activities. Proof of multiple forms must await further purification.

Phycomyces phosphodiesterase has an absolute requirement for divalent metal ions. Most other phosphodiesterases also require metal ions; usually, as in *Phycomyces*, magnesium suffices. Ferrous ion is, however, required by the *E. coli* enzyme [11]. In contrast, yeast activity is inhibited by added metals [12]. In brain and heart preparations, micromolar calcium concentrations enhance the hydrolysis of cyclic AMP by phosphodiesterase via a calcium-binding protein activator [13]. No such calcium effect has been reported from nonmetazoan sources in agreement with our results.

Methylxanthines at low levels inhibit mammalian phosphodiesterase. Higher concentrations are required by *Phycomyces* phosphodiesterase (Fig. 5). On the other hand, enzyme from *Dictyostelium* [14], *Candida* [15], pea seedlings [16], and *E. coli* [11] are insensitive, while the *Neurospora* [6], *Coprinus macrorhizus* [17], *Saccharomyces* [12], and *Chlamydomonas* [18] enzymes are inhibited. Imidazole has a slight inhibitory effect in *Phycomyces* but is a well known activator of mammalian phosphodiesterase. It has no effect in *Dictyostelium*,

Table 5. Effect of reducing agents and other compounds on phosphodiesterase activity

	Concentration (mM)	Compound	% Control (± 1 standard deviation)
Reductants	2	Ascorbic acid	100 ± 9
	5	Cysteine	99 ± 4
	5	Dithiothreitol	96 ± 5
	5	Gallic acid	80 ± 4
	2	Glutathione (reduced)	99 ± 10
	5	Glutathione (reduced)	95 ± 8
	50	β-Mercaptoethanol	96 ± 4
	1	NADH	105 ± 3
	1	NADPH	100 ± 2
	2	Sodium dithionite	96 ± 5
	2	Sodium sulfite	100 ± 7
	10	Sodium sulfite	97 ± 6
Others	0.5	Histamine	89 ± 10
	20	Imidazole	94 ± 2
	0.5	NaN_3	109 ± 5
	0.9	NaN_3	107 ± 8
	0.5	$K_3Fe(CN)_6$	87 ± 1
	1	$K_3Fe(CN)_6$	94 ± 10
	0.2	Dibutyryl cyclic AMP	16 ± 0
	1	Dibutyryl cyclic AMP	7 ± 1

Phosphodiesterase assayed at 4 μM cyclic AMP in 5 mM $MgCl_2$, 40 mM Tris–HCl, pH 8 for 30 min at 24° ± 1°. The pH was readjusted with HCl before incubation when required.

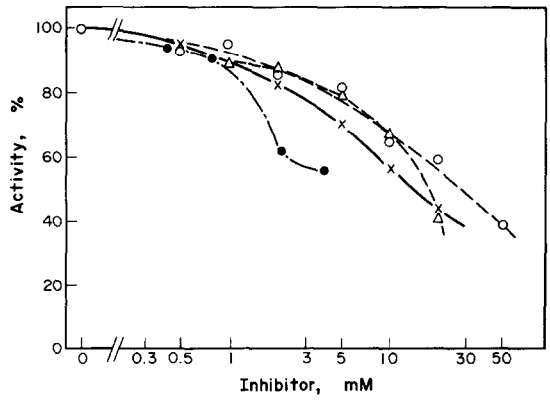

Fig. 5. Cyclic AMP phosphodiesterase activity in the presence of the indicated concentrations of methylxanthine inhibitors. Cyclic AMP is at 4 μM and assays are run at pH 8 and 24° ± 0.5°. ——○——, Caffeine; ——△—— aminophylline; ——×——, theophylline and ——●——, 3-isobutyl-1-methylxanthine.

Serratia marcenscens, pea seedlings [16] and *Candida* [15]; imidazole slightly activates *Physarum* enzyme and enhances the cyclic nucleotide hydrolysis by *Neurospora* [6] and by *Tetrahymena pyriformis* extracts [19].

Nearly all reducing agents, including thiol reagents, and mild oxidizing agents, such as ferricyanide, had no effect on *Phycomyces* enzyme. *E. coli* requires a reducing agent presumably to maintain the iron in a Fe^{2+} state [11]. Thiol reagents inhibit phosphodiesterase activity from *Dictyostelium* [20] but not from yeast [12]. In mammalian systems, ascorbate and dehydroascorbate inhibit activity from Walker carcinoma [21] and the former inhibits corneal epithelial phosphodiesterase [22]. Gallic acid inhibits phosphodiesterase in *Phycomyces* sporangiophore. Gallic acid tends to form free radicals via a free electron mechanism. Since gallic acid is abundant in sporangiophore [23], the possibility exists that a free radical modulation may be physiologically significant in *Phycomyces*. However, in preliminary *in vitro* experiments (unpublished) a variety of free radical scavengers do not suppress the inhibition.

Teshima *et al.* [24] conclude as we did, but for brain phosphodiesterase, that ATP inhibits activity merely by chelating magnesium. Also in brain phosphodiesterase, Cheung, in an early experiment [25], found an abrupt drop in activity at 45° as in our Fig. 4. Along the same lines, it may be significant that *Phycomyces* does not grow above 30°, where we observed the enzyme beginning to be inactivated.

Within 1 min after stimulating *Phycomyces* sporangiophore with blue light, the first known biochemical change occurs: a 60% drop in the cyclic AMP level [1]. The growth response, starting 2 min later, may be mediated by, or in some indirect manner, be dependent on, the change in the cyclic nucleotide pool. For the attenuation of this metabolite in *Phycomyces*, an obvious mechanism would be the activation of a phosphodiesterase [4]. Since phosphodiesterase from various sources appears to have different characteristics, relevant parameters in *Phycomyces* had to be ascertained before attempting to relate activity with the stimulus–response system.

EXPERIMENTAL

Enzyme extraction. P. blakesleeanus (−)NRRL1555 was grown in 30 × 50 cm trays under fluorescent lights on commercial instant potato media supplemented with 0.1% yeast extract (Difco) and 0.25 ppm thiamine HCl. Spores were germinated by heat shocking at 48–50°, and 10^4 spores were inoculated per tray. Sporangiophores initiated *ca* 50 hr after germination. Synchrony of development was obtained by chilling at 4° in the dark overnight and then exposing the trays to room light and temp. (24°). Mature stage IVb sporangiophores were returned to the cold room in the light *ca* 30 min before harvesting. Sporangiophores were plucked and cut into 3 mm pieces, which fell directly into a mortar containing 150 mM NaCl and 10 mM Tris–HCl, pH 7.4. The material was ground with a pestle and squeezed through 3 layers of cheese-cloth. The cutting, grinding and squeezing were repeated. The filtrate was centrifuged at 4° at 1100 *g* for 10 min in a Sorvall SS-34 rotor to remove cellular debris. The supernatant solution was recentrifuged at 31000 *g* for 30 min. If the pellets were to be assayed, they were taken up in 10 mM Tris–HCl, pH 7.4. The remaining soln was centrifuged again at 100000 *g* for 60 min in a Beckman Type 30 rotor for the expts involving subcellular distribution of phosphodiesterase. The supernatant soln was made up to 40% satn with $(NH_4)_2SO_4$, equilibrated 10 min and centrifuged at 7700 *g* for 5 min. The pellet was resuspended in 10 mM Tris–HCl, pH 7.4.

Protein was precipitated from the remaining soln with 75% and then 100% of saturation with $(NH_4)_2SO_4$. For most of the expts, it sufficed to obtain only a 40% $(NH_4)_2SO_4$ ppt. of the 31000 *g* supernatant soln. All of the protein extracts were dialysed 4 hr against 10 mM Tris–HCl, pH 7.4 at 4°, diluted and frozen in 1 ml aliquots at −20°. Protein was determined by the method of ref. [26]. No loss of activity was seen after 2–3 months storage.

Standard phosphodiesterase assay. The assay is a modification of ref. [27]. All determinations were done in quadruplicate in plastic scintillation vials. In a total vol. of 110 μl were 4 μM cyclic AMP-[^{3}H] (10^5 cpm), 50–300 μg phosphodiesterase extract, 3 μg snake venom (*Crotalus atrox*, Sigma), 50 μg BSA and additives in 5 mM $MgCl_2$, 40 mM Tris–HCl, pH 8. The reaction was started after thermal equilibration (at 24°) by the addition of substrate and the mixture allowed to incubate 30 min. The reaction was terminated by adding 0.8 ml of a slurry (60% settled vol.) of AG1-X2, 200–400 mesh, anionic exchange resin. After equilibrating 10 min, 10 ml of scintillation fluid (1 l. toluene, 585 ml Triton X-100, 5 g PPO and 0.16 g POPOP) was added to the sample and radioactivity was counted in a scintillation counter. Blanks were as assay, including additives, but were stopped immediately by resin; such blanks were included for every set of experimental points. Internal standards were utilized for quench corrections. Standard deviations are less than 10% unless indicated.

PC was used routinely to check results, especially in critical expts. Instead of quenching the reaction with resin, sample tubes were boiled 1 min and centrifuged. The clear supernatant (50 μl) was removed and spotted on Whatman 3MM paper. Chromatograms were developed for 18 hr in the descending mode in 95% EtOH–1 M NH_4OAc, pH 7.5(7:3). Unlabeled cyclic AMP, adenine, adenosine and AMP were added to visualize the spots by UV. Appropriate areas were then cut out and counted.

We found in preliminary expts that extracts from *Phycomyces* sporangiophores have considerable 5′-nucleotidase activity in each subcellular fraction. PC confirmed that after incubation only 4% of the 5′-AMP product remained as such while 96%

was further degraded to adenosine. No deamination was detected. These findings are similar to the extracts of *Neurospora* [6]. In addition to the endogenous nucleotidase, 3 μg *Crotalus atrox* venom was also included in each assay [7]. Frequent checks by PC indicated that nucleotidase activity was always in excess. Cyclic AMP phosphodiesterase activity with venom present and in the absence of *Phycomyces* extract or with boiled extract was always less than 1–2% of the overall activity.

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REFERENCES

1. Cohen, R. J. (1974) *Nature* **251**, 144.
2. Tu, J. C. and Malhotra, S. K. (1977) *Can. J. Microbiol.* **23**, 378.
3. Jones, B. E. and Bu'Lock, J. D. (1977) *J. Gen. Microbiol.* **103**, 29.
4. Cohen, R. J. and Atkinson, M. M. (1978) *Biochem. Biophys. Res. Commun.* **83**, 616.
5. Cohen, R. J. (1978) *Plant Sci. Letters* **13**, 315.
6. Scott, W. A. and Solomon, B. (1973) *Biochem. Biophys. Res. Commun.* **53**, 1024.
7. Dusenberry, R. L. (1975) *Biochim. Biophys. Acta* **378**, 363.
8. Monn, E. and Christiansen, R. O. (1971) *Science* **173**, 540; Thompson, W. J. and Appleman, M. M. (1971) *Biochemistry* **10**, 311.
9. Pichard, A. L. and Cheung, W. Y. (1976) *J. Biol. Chem.* **251**, 5726.
10. Riedel, V., Gerisch, G., Muller, E. and Beug, H. (1973) *J. Mol. Biol.* **74**, 573.
11. Perlman, R. L. and Pastan, I. (1971) in *Current Topics in Cellular Regulation* (Horecker, B. and Stadtman, E. R., eds.) pp. 755–809. Academic Press, New York; Nielson, L. D., Monard, D. and Rickenberg, H. V. (1973) *J. Bacteriol.* **116**, 857.
12. Fujimoto, M., Ichikawa, A. and Tomita, K. (1974) *Arch. Biochem. Biophys.* **161**, 54.
13. Liu, Y. P. and Cheung, W. Y. (1976) *J. Biol. Chem.* **251**, 4193.
14. Chang, Y. Y. (1968) *Science* **161**, 57.
15. Gunasekaran, M., Hughes, W. T. and Maguire, W. J. (1976) *Microbios Letters* **3**, 47.
16. Lin, P. P.-C. and Varner, J. E. (1972) *Biochim. Biophys. Acta* **276**, 454.
17. Uno, I. and Ishikawa, T. (1973) *J. Bacteriol.* **113**, 1249.
18. Fischer, U. and Amrhein, N. (1974) *Biochim. Biophys. Acta* **341**, 412.
19. Ramanathan, S. and Chou, S. C. (1973) *Comp. Biochem. Physiol.* **46B**, 93.
20. Pannbacker, R. G. and Bravard, L. J. (1972) *Science* **175**, 1014.
21. Tisdale, M. J. (1975) *Biochem. Biophys. Res. Commun.* **62**, 877.
22. Buck, M. G. and Zadunaisky, J. A. (1975) *Biochim. Biophys. Acta* **389**, 252.
23. Dennison, D. S. (1959) *Nature (London)* **184**, 2036.
24. Teshima, Y., Yamazaki, R. and Kakiuchi, S. (1974) *J. Neurochem.* **22**, 789.
25. Cheung, W. Y. (1967) *Biochemistry* **6**, 1079.
26. Lowry, O. H., Roberts, N. R., Wu, M. L., Hixon, W. S. and Crawford, E. J. (1954) *J. Biol. Chem.* **207**, 19.
27. Chasin, M. and Harris, D. N. (1972) in *Methods in Cyclic Nucleotide Research* (Chasin, M., ed.) pp. 169–198. Dekker, New York.