Phytochemistry, 1980, Vol. 19, pp. 1913–1918. © Pergamon Press Ltd. Printed in England. 0031–9422/80/0901–1913 $02.00/0

ADENYLATE CYCLASE FROM *PHYCOMYCES* SPORANGIOPHORE

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(*Received* 29 *October* 1979)

Key Word Index—*Phycomyces blakesleeanus*; fungus; adenylate cyclase; cyclic AMP; blue light response; catecholamines; sensory processes.

Abstract—Transient changes in cyclic AMP levels accompany the light-growth response of the sporangiophore of *Phycomyces blakesleeanus*. Furthermore growth is regulated by endogenous hormones. Since adenylate cyclase may perform a role in these events, some properties of the enzyme from the sporangiophores of *Phycomyces blakesleeanus* are reported here. The enzyme is mostly particulate and activity is dependent on a divalent cation possibly Mg^{2+}; Mn^{2+} and Ca^{2+} are inhibitory. Its K_m is 0.5 mM and the pH optimum is 7.8. Low levels of GTP markedly enhance activity. Nucleoside triphosphates, including ATP at high concentrations, are inhibitory while AMP and ADP and to a lesser extent IMP increase activity. Ouabain, NaF, and alloxan also inhibit *Phycomyces* cyclase. Pyruvate, imidazole, nucleoside monophosphates other than AMP and IMP, histamine, glucagon, octopamine, γ-aminobutyric acid and norepinephrine have little or no effect. However, high concentrations of epinephrine and dopamine tripled activity. The effect of dopamine was shown to be saturable. Adenylate cyclase extracted in the dark was significantly activated upon simultaneous exposure to light and substrate. An inference is made that sensory transduction in *Phycomyces* may involve adenylate cyclase, although the interaction may or may not be a direct one.

INTRODUCTION

The sporangiophore of the fungus *Phycomyces blakesleeanus* is a giant, single cell which responds with a transient growth increase in minutes to light, gravity, stretch and the proximity of objects [1]. The light response has been particularly well characterized [2] and behavioral mutants are available [3]. The complete dissection of the molecular basis of this response system may be possible. It has been demonstrated that the level of adenosine 3′,5′-monophosphate (cyclic AMP) decreases *ca* 60% within 1 min of light stimulation and recovers within the next minute. This pulse precedes the initiation of the growth response by about 2 min. The sporangiophore grows at the same rate in air as when immersed in the fluorocarbon perfluorotributylamine. The addition of theophylline or dibutyryl cyclic AMP into the fluorocarbon results in rapid and transient growth decreases consistent with the hypothesis that cyclic AMP may mediate the growth response system [4]. Mutants with aberrant growth responses exhibit abnormal cyclic AMP responses ([5] and unpublished). More recently we have shown that cyclic nucleotide phosphodiesterase from *Phycomyces* sporangiophore is blue light-activated *in vitro* [6]. These results plus the report that *Phycomyces* sporangiophore exhibits a primitive hormone control system [7] prompted this study of the adenylate cyclase (EC 4.6.1.1) from *Phycomyces* sporangiophore. We found adenylate cyclase is also sensitive to blue light and is activated by certain catecholamines.

RESULTS

Subcellular fractionation

Table 1 indicates the results of incubations with subcellular fractions. Some adenylate cyclase activity was found in the 'nuclear' pellet. Cyclase has occasionally been found associated with the nucleus [8]; however, in the case of *Phycomyces* some of the activity found in the low-speed pellet may also be associated with sporangiospores and with material aggregated to cell-wall debris. In this experiment the supernatant solution from the 23 500 *g* spin was centrifuged at 100 000 *g* for 60 min to obtain the microsomal fraction. This fraction which contained membrane fragments consistently had low activity. The activity of the supernatant after this centrifugation was relatively high, composing about a sixth of the total activity. However, the specific activity was low. Our interest was focused on the 23 500 *g* pellet because of its higher specific activity. Furthermore, there exists now strong evidence from behavioral studies that the light receptor in the stalk of the sporangiophore of *Phycomyces* is oriented relative to its longitudinal axis [9]. This implies that the elusive receptor protein is associated with particulate material. If adenylate cyclase is involved with the initial light reception, it also should be found in a particulate fraction. Therefore, all the experiments related in this paper refer to the 23 500 *g* fraction.

Table 1. Subcellular fractionation

Subcellular fraction	Total protein (mg)	Sp. act. ± s.d. (pmol/min mg protein)	Total activity (pmol min)
1. 1100 g, 10 min pellet	125	3.9 ± 0.1	490 (0.29)
2. 23 500 g, 10 min pellet	40	24.0 ± 0.4	960 (0.56)
3. 100 000 g, 60 min, pellet	32	0.5 ± 0.1	16 (0.009)
4. 100 000 g, 60 min. supernatant solution	480	0.5 ± 0.1	240 (0.14)
	677		1706 (1.00)

Mature sporangiophores, 5–6 cm tall, grown in the light, were plucked, cut and ground in a mortar in 2–3 vol of 25 mM Tris–HCl, 5 mM $MgCl_2$, 0.25 mM Na_2EDTA and 1 mM dithiothreitol, pH 7.6. After filtration through cheesecloth, the filtrate was subjected to differential centrifugation as indicated. Adenylate cyclase was incubated 20 min in a 55 μl mixture containing 0.34 mM ATP-[8-^{14}C] (4.1 mCi nmol), 1 mM cyclic AMP, 10 mM aminophylline, an ATP-regenerating system (phosphocreatine and creatine phosphokinase), 1 mg/ml bovine serum albumin, 100–300 μg sporangiophore extract (protein) and the above additives in 25 mM Tris–HCl, 0.25 mM Na_2EDTA, and 0.5 mM DTT, pH 7.6, at 24°. Cyclic AMP was separated by PEI-cellulose TLC.

Time course

The rate of synthesis of cyclic AMP was constant for 40 min and then diminished thus suggesting the choice of 20 min incubation periods.

Kinetic constants

A double reciprocal plot of the rate of cyclic AMP production reveals an apparent K_m of 0.5 mM for ATP and in this preparation a V_{max} of 0.13 nmol/min per mg protein (Fig. 1). *Phycomyces* cyclase seems to be substrate inhibited at high concentrations of ATP. This inhibition is not merely an effect of magnesium chelation, but appears to be true substrate inhibition because the magnesium concentration remained in substantial excess relative to the nucleotide.

Cation dependence, pH effect.

Adenylate cyclase from sporangiophores was Mg^{2+} dependent. Table 2 shows that the addition of Na_2 EDTA-inhibited the enzyme 60% relative to standard conditions. Activity was not affected by doubling the $MgCl_2$ concentration to 10 mM. On the other hand, Ca^{2+} is inhibitory. In mammalian cyclase, Mn^{2+} is about as effective as Mg^{2+} [10]; however, adenylate cyclase from *Phycomyces* was strongly inhibited by Mn^{2+}. The pH optimum in Tris–HCl lies between 7.5 and 8.2.

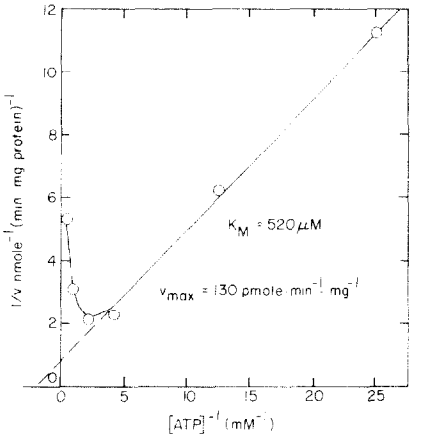

Fig. 1. A Lineweaver–Burk plot of the reciprocal rate of cyclic AMP production vs the inverse of the ATP concentration. 24°. Standard assay conditions.

Nucleotides

Submillimolar levels of GTP greatly enhanced cyclase activity (Table 3). This activation is reminiscent of hormone-sensitive cyclase from vertebrate sources [11]. Five millimolar IMP and AMP enhanced activity by 34 and 194%, respectively, while 5 mM UMP, TMP, GMP

Table 2. The effect of divalent cations on the activity of *Phycomyces* adenylate cyclase

Additive	Activity (pmol/min/mg protein ± s.d.)	(% ± s.d.)
5 mM $MgCl_2$	17.9 ± 1.4	100 ± 8
10 mM $MgCl_2$	17.2 ± 1.8	96 ± 10
5 mM $MgCl_2$ + 1 mM $CaCl_2$	1.1 ± 0.5	6 ± 3
5 mM $MnSO_4$	2.0 ± 0.2	11 ± 1
10 mM $MnSO_4$	2.5 ± 0.7	14 ± 4
5 mM Na_2EDTA	7.2 ± 1.6	40 ± 9

Standard assay conditions as in Table 1 and the above additives. Cyclic AMP was separated by PEI-cellulose TLC. An experiment without additives is not included since it is a poor control because of our lack of knowledge concerning endogenous cations.

Table 3. The effects of various nucleoside phosphates on adenylate cyclase

Nucleoside phosphate	Activity (pmol/min/mg protein ± s.d.)	(%)
5 mM CTP	5.4 ± 4.3	10 ± 8
5 mM GTP	1.1 ± 1.1	2 ± 2
5 mM UTP	32.9 ± 6.4	61 ± 12
0.5 mM GTP	55.1 ± 5.4	102 ± 10
0.25 mM GTP	80.5 ± 7.6	149 ± 14
0.025 mM GTP	96.7 ± 6.5	179 ± 12
5 mM ADP	150 ± 16	277 ± 29
5 mM AMP	159 ± 23	294 ± 43
5 mM CMP	54.5 ± 7.0	101 ± 13
5 mM GMP	56.7 ± 6.5	105 ± 12
5 mM IMP	72.4 ± 4.3	138 ± 8
5 mM TMP	54.0 ± 7.0	100 ± 13
5 mM UMP	58.9 ± 4.9	109 ± 9
Control	54.0 ± 4.3	100 ± 8

Standard assay conditions as in Table 1.

Table 4. The effects of various agents on adenylate cyclase

Additive	Activity (pmol/min/(mg protein) ± s.d.)	(% ± s.d.)
Series A		
Control	41.7 ± 3.3	100 ± 8
2 μM Ouabain	37.5 ± 2.2	90 ± 6
10 μM Ouabain	24.6 ± 1.7	59 ± 4
20 μM Ouabain	20.0 ± 2.9	48 ± 7
50 μM Ouabain	17.9 ± 3.7	43 ± 9
200 μM Ouabain	17.1 ± 2.5	41 ± 6
1 mM NaF	33.4 ± 1.7	80 ± 4
4 mM NaF	25.4 ± 2.5	61 ± 6
8 mM NaF	17.5 ± 0.8	42 ± 2
20 mM NaF	20.8 ± 0.8	50 ± 2
40 mM NaF	9.6 ± 2.9	23 ± 7
Series B		
Control	24.3 ± 2.2	100 ± 9
10 mM Alloxan	13.4 ± 2.9	55 ± 12
5 mM Pyruvate	26.0 ± 1.7	107 ± 7
10 mM Imidazole	23.3 ± 0.7	96 ± 3
2 mM *N*-Ethylmaleimide	42.3 ± 3.6	174 ± 15
25 μM Glucagon	20.6 ± 3.4	85 ± 14
50 μM Histamine	24.8 ± 1.2	102 ± 5
50 μM γ-Aminobutyric acid	22.8 ± 1.9	94 ± 8
50 μM Acetylcholine	31.6 ± 3.9	130 ± 16
50 μM Octopamine	22.1 ± 3.1	91 ± 13
50 μM 3-Methyl-5-heptanone	37.9 ± 2.9	156 ± 12

Adenylate cyclase assay run in triplicate contained in 55 μl 0.34 mM ATP-[8-^{14}C] (4.1 mCi/mmol), 1 mM cyclic AMP, 10 mM aminophylline, an ATP regenerating system (creatine phosphate and creatine phosphokinase), 1 mg/ml bovine serum albumin, 100–300 μg of protein and additives in 25 mM Tris–HCl, 5 mM $MgCl_2$, 0.25 mM Na_2 EDTA and 0.5 mM DTT, pH 7.6. Incubation time 20 min. The temperature was 24°. Cyclic AMP was separated by PEI-cellulose TLC.

and CMP did not affect cyclase activity. The nucleotide triphosphates CTP, UTP, GTP and ATP inhibit *Phycomyces* adenylate cyclase at the same concentrations. To test the effect of 5 mM ADP required a modification of the assay to exclude the ATP regenerating system and consequently shortening the incubation period to 5 min. Under these conditions ADP served as a potent activator.

Other agents

Adenylate cyclase from preparations of metazoan sources is activated several-fold by fluoride [12–14]; on the other hand, adenylate cyclase from various microorganisms is reportedly unaffected or inhibited by the halide [15, 16]. The enzyme from *Phycomyces* sporangiophore was inhibited about 50% by 10 mM NaF (Table 4). Ouabain also inhibited the cyclase activity of *Phycomyces* extracts (Table 4). The cardiac glycoside activates brain enzyme [17] but inhibits cyclase from lipocytes [18]. The intermediary metabolite pyruvate markedly stimulates the enzyme from *Brevibacterium* [19] but has no significant effect on *Phycomyces* enzyme (Table 4).

Table 4 indicates that adenylate-cyclase activity was considerably reduced in the presence of alloxan [20]. Imidazole, a potent inhibitor of cyclic phosphodiesterase, has no effect on *Phycomyces* cyclase. *N*-Ethylamaleimide (2 mM) enhanced the activity of *Phycomyces* cyclase by 74%. Small concentrations of 3-methyl-5-heptanone in the vapor phase greatly reduce the growth rate of *Phycomyces* sporangiophore [21]. We found that 50 μM of the ketone enhanced adenylate cyclase 56%.

Hormones and transmitters

The possibility that *Phycomyces* cyclase activity may be affected by mammalian hormones and putative neurotransmitters was next examined (Table 4). A slight positive modulation was found for acetylcholine while octopamine, histamine, glucagon and γ-aminobutyric acid had little effect.

Because we had some evidence that the growth regulatory substance emitted by the spores and detected in the growing zone is a catecholamine, possible dopamine (unpublished), we decided to examine further the effects of higher concentrations of epinephrine, norepinephrine and dopamine. Norepinephrine never effected a higher than 20% increase activity above the base level, while dopamine and epinephrine caused 2–4-fold increases at 500 μM (Fig. 2). The effect of dopamine saturated at 3–5 mM.

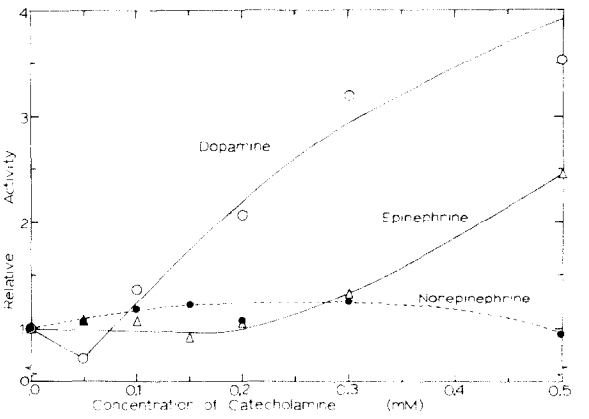

Fig. 2. The activation of adenylate cyclase from *Phycomyces* sporangiophore by the catecholamines, dopamine (○—○), epinephrine (△—△) and norepinephrine (●—●). Standard assay conditions, 0.34 mM ATP, 24°. The basal level of adenylate cyclase was 17.7 pmol/min/mg protein.

Light

The adenylate cyclase was from sporangiophores grown in light but extracted in the dark after 30 min dark adaptation, and stored at −20° in the dark. The assay procedures are detailed in the Experimental. Enzymatic activity measured during moderate intensities of continuous incandescent blue light (1 μW/cm^2) was 60–70% higher than that measured in the dark (Table 5).

DISCUSSION

Among the many metabolic processes controlled by cyclic AMP are some of the neuronal synaptic, the hormonal and quite possibly the sensory systems. One purpose of this study is to present some properties of an adenylate cyclase from a lower eukaryote which has much in common with that from metazoan sources. Full advantage may therefore be taken of the behavioral and

Table 5. *In vitro* effect of light on adenylate cyclase from *Phycomyces* sporangiophore

Condition	Specific activity (pmol/min/mg protein ± s.d.)	(% Dark control ± s.d.)
Dark	19.4 ± 4.1	100 ± 21
Continuous blue light (1 μWatt/cm^2)	31.0 ± 6.7	160 ± 35
Stroboscopic flash	34.3 ± 9.3	177 ± 48

Adenyate cyclase isolated in the dark or under dim red light was incubated in the standard assay mixture in quartz microcells and simultaneously exposed to labelled ATP substrate and to darkness, to continuous blue light, or to a stroboscopic flash of white light for 30 min. The assay mixture run in triplicate contained in 55 μl 0.34 mM ATP-[8-^{14}C] (4.1 mCi/mmol), 1 mM cyclic AMP, 10 mM aminophylline and an ATP-regenerating system in 25 mM Tris–HCl, 5 mM $MgCl_2$, 0.25 mM Na_2 EDTA and 0.5 mM DTT, pH 7.6. The temperature was 24°. Cyclic AMP was separated by PEI-cellulose TLC.

morphological mutants of the organism to elucidate the possible role of adenylate cyclase in a responsive system. The results recorded here on *Phycomyces* cyclase can be summarized by first listing the mammalian-like properties, e.g. Mg dependence, Ca inhibition, pH optimum, activation by low GTP labels, inhibition by higher concentrations of nucleoside triphosphates, effect of ouabain, lack of activation by pyruvate, and activation by the catecholamines (however, at much higher concentrations); and second by listing some properties unlike that from higher organisms: subcellular distribution, inhibition by manganese and sodium fluoride and the large enhancement of activity by AMP and possible by ADP. To be more specific, for example, GTP has a marked effect of hormonally sensitive adenylate cyclase from mammalian cells [11, 22] while AMP enhances activity of *Dictyostelium* cyclase [23, 24].

The results most worth noting are the activation of adenylate cyclase activity by catecholamines and by moderate light levels. In *Phycomyces* dopamine and epinephrine greatly increase activity while norepinephrine seems to have little effect. The enhancement by the first two catecholamines is similar to that in adenylate cyclase from retinal homogenates [25] although at an order of magnitude higher concentration. In tissue slices of mammalian superior cervical ganglion, dopamine and norepinephrine enhance adenylate cyclase activity [26]. In most systems, however, epinephrine and norepinephrine have similar properties while dopamine is without effect. Thus our results are not reconcilable with the current identification of receptor sites with the classical α and β adrenergic receptor scheme of Ahlquist [27].

This failure to reflect the adrenergic receptor theory is not wholly detrimental to the utility of *Phycomyces* as a model system. Only one study of cyclase from other lower eucaryotic organisms presents an enzyme which seems appreciably sensitive to catecholamine. In *Tetrahymena pyriformis*, epinephrine enhances activity 4-fold while again norepinephrine is without effect [28]. *T. pyriformis* has been reported to contain catecholamines and serotonin [29] and to contain moderate levels of cyclic AMP [30]. Some evidence suggest that *Euglena gracilis* possesses a cyclase sensitive to catecholamines although the response is quite small—*ca* 20% [31]. No prokaryotic system has been shown to respond to catecholamines, serotonin, histamine, glucagon or prostaglandins. The sensitivity of the *Neurospora crassa* enzyme to glucagon and insulin reported by Flawia and Torres [32, 33] serves as the most developed precedent for the effect of mammalian hormones on the adenylate cyclase from other nonmetazoan systems.

Phycomyces adenylate cyclase seems to be involved in the reception of light. Our experiments did not prove that cyclase activity is directly effected by light. For example, light could activate a protein kinase which phosphorylates and activates adenylate cyclase. A popular but largely untested theory is that the interconversion of phytochrome by light absorption leads to a conformational change which activates adenylate cyclase [34]. This in turn may lead to an alteration of the ion permeability of the membrane [36]. Analogously, in *Phycomyces* changes in a nearby receptor protein in a membraneous milieu may induce a conformational change in the cyclase molecule. The observation of either direct or indirect activation of a subcellular preparation of cyclase by moderate light intensity is remarkable and so far unique.

Our previous work showing that the *in vivo* cyclic AMP levels decreased and recovered within 2 min of light stimulation does not contradict the results reported here. The metabolic regulation of cyclic nucleotide concentration is quite complex. The rapidity of the change in metabolite concentration may imply the occurrence of an initial increased phosphodiesterase activity shortly followed by an enhanced adenylate cyclase activity. The regulation may be subtle involving mediation by cations such as Ca^{2+} [37–39].

EXPERIMENTAL

Enzyme extraction. Phycomyces blakesleeanus (NRRL-1555(−)) was grown under fluorescent light on commercial instant potatoes supplemented with 0.2% yeast extract for 3 days, until the first appearance of immature stage I sporangiophores. They were then transferred to a cold room overnight at 4° in the dark and subsequently removed after about 12 hr and simultaneously exposed to light. This procedure tends to synchronize the maturation of sporangiophores. Mature stage IVb sporangiophores, 4–6 cm tall, were harvested about 28 hr later. Colonies were transferred back to the 4° cold room under fluorescent light 30 min before harvesting. The sporangiophores were plucked, weighed, cut into 3 mm pieces and ground in a mortar and pestle in 2–3 vols of 25 mM Tris, 5 mM $MgCl_2$, 0.25 mM Na_2 EDTA and 1 mM dithiothreitol, pH 7.6. Cell wall and debris were excluded by squeezing through 3 layers of cheesecloth. The cutting, grinding and squeezing were repeated. The combined filtrate was subjected to centrifugation at 1100 *g* for 10 min and the pellet discarded. The supernatant solution was recentrifuged at 23 500 *g* for 15 min. These pellets were taken up in the grinding soln and frozen immediately at −20°. The enzyme could be stored for up to 3 weeks with little loss of activity. (About 20% of the activity was lost on freezing and thawing.) Protein concns were assayed according to ref. [40]. Most expts were done with the resuspended 23 000 *g* pellet. The adenylate cyclase preparation from *Phycomyces* was not stable at 4° or at −75°.

Adenylate cyclase assay. The standard assay was done at 24° in a centrifuge tube containing 0.34 mM ATP-[8-^{14}C] (4.1 mCi/mmol), mM cyclic AMP, 10 mM aminophylline, 15 mM phosphocreatine, 60 μg/ml creatine phosphokinase, 1 mg/ml BSA, 100–300 μg of the protein and various additives in 55 μl of 25 mM Tris–HCl, 5 mM $MgCl_2$, 0.25 mM Na_2 EDTA and 0.5 mM dithiothreitol pH 7.6. Enzyme was generally added to start the reaction which was allowed to incubate for 20 min, stopped by the addition of 20 μl 3 mM ATP and 10 mM cyclic AMP and boiled for 1 min. The tube was centrifuged and two 10 μl aliquots of the clear supernatant soln spotted on a polyethyleneimine–cellulose thin layer plate (EM Laboratories) which was eluted with 0.05 M $LiCl_2$ for 3.0 hr [41]. The cyclic AMP spots were visualized by the quenching of fluorescence and scraped. The material was allowed to elute 24 hr in 10 ml Aquasol (New England Nuclear) and counted. ATP spots were counted and found to be constant. Expts were always done 3× giving 6 determinations for each. Each set of expts was accompanied by an incubation excluding the enzyme and the amount of cyclic AMP found (*ca* 0.05% of the added ATP) subtracted from the experimental quantities (generally, 0.5–1.0%).

A few expts were conducted using descending PC to separate the nucleotides. Solution (40 μl) was spotted on Whatman 3 MM paper and eluted with EtOH–1 M ammonium acetate, pH 7.5 (15:6) for 18 hr. Spots of cyclic AMP and ATP were cut out and counted in 20 ml of a scintillation fluid containing 4 g 2,5-diphenyloxazole and 50 mg 2,2′-*p*-phenylene-bis(5-phenyloxazole) per 1. toluene using the ratio of counts to determine

synthesis of cyclic AMP. This technique as well as eluting the TLC with higher concns of $LiCl_2$ was used to check the consistency of the results. An occasional expt was conducted with no added cyclic AMP and checked by assaying the amount of cyclic AMP synthesized using the protein binding assay for cyclic AMP of ref. [42]. Simultaneous comparisons indicated the same rate of cyclic AMP synthesis. In addition, samples incubated with radiolabelled ATP in the absence of unlabelled cyclic AMP were treated with 0.1 mg/ml bovine heart cyclic nucleotide phosphodiesterase (Sigma) for 20 min at 30°. Aliquots were subjected to TLC as above. Spots corresponding to cyclic AMP contained no label. The isolatable product before phosphodiesterase treatment is therefore authentic cyclic AMP.

Unlabelled chemicals were purchased from Sigma, Mallinckrodt, and Matheson, Coleman & Bell. The tetrasodium adenosine 5'-triphosphate-[8-^{14}C] (52 mCi/mM), from New England Nuclear, was tested by TLC occasionally and further purification was unnecessary.

Light experiment. Sporangiophore adenylate cyclase was isolated as indicated from cultures grown in the light except that all steps starting from the 30 min incubation were conducted in the dark or under red light. In one series of enzyme assays, portions of the enzyme extract were incubated either in the dark or in blue light under standard incubation conditions. The incubations, however, were done in quartz spectrophotometer cuvettes set in a specially designed chamber with a light diffuser on one side. The light source was an incandescent tungsten bulb 1.5 m distant collimated by a lens system and fitted with a Corning 5–61 broad blue filter. The width of the beam was adjusted to the width of the chamber, 30 cm. Infrared radiation was eliminated by an Eastman heat filter. The intensity of the light was 700 lx (1.0 μW/cm^2, well within the so-called normal range of the photoresponse of *Phycomyces* as defined in ref. [1]); it varied < 5 ftcd across the inside of the chamber as measured by a Gossen Lunasiz photocell. One set of experiments used a Xe tube stroboscopic flash (Graflex) every 40 sec for the duration of the incubation. Temps (24°) measured simultaneously with a black-coated thermometer varied less than 0.5° during the expt and between dark and light expts. Triplicate simultaneous expts were made under each of 3 conditions: (a) total darkness, (b) continuous blue illumination, and (c) stroboscopic flashing. In the latter two cases, enzyme was added and the light sources turned on at time zero. The incubation mixtures were quenched and assayed as above.

Acknowledgements—This study was supported by the National Institute of General Medicine, National Institutes of Health (GM19390). We would also like to thank Mr. Raymond Sheline for technical assistance during the preliminary aspects of this report.

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