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Intracellular and Extracellular Cyclic Nucleotides in Wild-Type and White Collar Mutant Strains of *Neurospora crassa*

TEMPERATURE DEPENDENT EFFLUX OF CYCLIC AMP FROM MYCELIA

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ABSTRACT

Cyclic AMP and cyclic GMP were released into the growth medium of mycelia of *Neurospora crassa* wild-type strains St.L.74A and Em5297a and by *white collar-1* and *white collar-2* mutant strains. After growth for 6 days at 18°C, there were 2.19 (St.L.74A), 5.83 (Em5297a), 1.38 (*white collar-1*), and 1.10 (*white collar-2*) nanomoles of cyclic AMP per gram dry weight of mycelia in the growth medium. These values corresponded to concentrations of cyclic AMP of between approximately 10 and 50 nanomolar. The corresponding values for extracellular cyclic GMP were typically less than 6% of the values for cyclic AMP. Following transfer to fresh medium, cyclic AMP efflux was demonstrated for each of the strains, and the amount of cyclic AMP exported into the fresh medium was greater at 25°C than 6°C. Intracellular cyclic AMP and cyclic GMP were also measured in each of the strains. The values for cyclic AMP were in the same range as those in the literature (approximately 0.5 to 1.5 nanomoles per gram dry weight of mycelia). However, the corresponding intracellular cyclic GMP values were less than 1% of the cyclic AMP values, *i.e.* more than 50 times lower than the value previously reported for the St.L.74A wild-type. Transfer of mycelia after 6 days at 18°C to fresh media and incubation for 2 hours at 25°C or 6°C did not consistently affect the intracellular level of cyclic AMP or cyclic GMP in the strains examined. We could detect no change in intracellular cyclic AMP when mycelia of the St.L.74A wild-type strain were irradiated with blue light for periods of up to 3.0 hours at 18° C, or in cyclic AMP and cyclic GMP for irradiation times of up to 1 minute at 6°C. We propose that the plasma membrane of *Neurospora crassa* is permeable to cyclic nucleotides, and the export of cyclic nucleotides into the growth medium may be a means of regulating intracellular levels. We conclude that three factors that affect carotenogenesis in *Neurospora crassa* (blue light, temperature, and the white collar mutations) have no appreciable effect on the total measurable intracellular cyclic nucleotides in this organism. There was no extracellular or intracellular cyclic AMP or cyclic GMP in the *crisp-1* mutant strain, which suggested either that adenylate cyclase (which is absent in *crisp-1*) catalyzes the synthesis of both cyclic AMP and cyclic GMP or that the *crisp-1* mutation somehow results in a deficiency of two enzymes (adenylate and guanylate cyclase).

Cyclic AMP and cyclic GMP have important regulatory roles in the metabolism of animals, fungi, bacteria, lower plants, and possibly higher plants. In animals these two nucleotides act as 'second messengers.' Their intracellular levels, which control enzyme activity and gene expression, change in response to extracellular signals such as hormones, neurotransmitters, odor molecules, and light (11, 12, 22, 29). In fungi, cyclic AMP has a number of proposed functions (for a review see Pall [24]). A unifying hypothesis suggesting that cyclic AMP stimulates glycolysis in fungi has been proposed (24), whereas in bacteria cyclic AMP is involved in the control of gene expression (11). Although the presence of cyclic AMP (2) and cyclic GMP (21) in higher plants has been rigorously proven, their functions remain unknown.

Cyclic AMP is also exported from many types of cells including metazoan cells (for a review see Barber and Butcher [1]). Many algae and vascular aquatic plants secrete cyclic AMP into the water in which they live, and an ecological role for cyclic AMP as a regulatory molecule in natural ecosystems has been postulated (for a review see Francko [10]). In addition, in the slime mold *Dictyostelium discoideum*, extracellular cyclic AMP is involved in cellular interaction (for a review see Devreotes [9]).

In *Neurospora crassa* the presence of cyclic GMP has been reported (25), and cyclic AMP may be involved in morphological development (24). The *crisp-1* morphological mutants can be restored to wild-type morphology by adding cyclic AMP to their growth medium (25, 28). Work in this laboratory is concerned with photoinduced carotenogenesis in *N. crassa* (16), with the aim of identifying the blue light photoreceptor and characterizing the pathway of phototransduction. Progress towards these goals may be made by identifying blue light-induced changes in the levels of metabolic signal molecules, and by characterizing the metabolic defect in the white-collar mutants, which are blocked in the light induction of enzymes of the carotenoid biosynthetic pathway (16) and in other blue light-mediated responses (8, 15). The possible importance of extracellular cyclic nucleotides in metabolic control processes, the observation that cyclic AMP added to the growth medium of *N. crassa* inhibits blue light-induced carotenogenesis and conidiation (13), and reports of photoinduced changes in cyclic AMP levels in *Phycomyces blakesleeanus* (4) and *N. crassa* (18) prompted us to look for naturally occurring cyclic nucleotides in the growth medium of *N. crassa* and to attempt to determine whether cyclic nucleotides are directly involved in the physiological control of carotenoid biosynthesis in this organism. Therefore, we have examined whether three factors that affect carotenoid biosynthesis, *i.e.* blue light, temperature (14), and the white collar mutations (16), also affected the levels of intracellular cyclic nucleotides. Following our initial observation of cyclic AMP in the growth medium of a wild-type strain, we have also examined the effect of two of these factors, *i.e.* temperature and the white collar mutations, on the levels of extracellular cyclic nucleotides. The rationale for making the latter observation was that even if the intracellular levels of cyclic nucleotides in the white collar mutants were normal, a possible defect in transport could result in aberrant

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(high) extracellular levels, which could result in inhibition of carotenogenesis.

We report that *N. crassa* exported both cyclic AMP and cyclic GMP into its growth medium. More cyclic AMP was released during a 2 h period after transfer to fresh medium at 25°C than 6°C. The white collar mutants had levels of extracellular cyclic nucleotides in the same order of magnitude as the wild-type strains. The values for the level of intracellular cyclic AMP in the wild-type strains were in the same range as those in the literature (23, 25), but the intracellular values of cyclic GMP were considerably lower than that previously reported for the St.L.74A wild-type strain (25). Temperature, blue light, and the white collar mutations did not have appreciable and consistent effects on the total measurable intracellular levels of cyclic AMP or cyclic GMP. The *crisp-1* mutant strain had zero intracellular and extracellular cyclic AMP and cyclic GMP.

MATERIALS AND METHODS

Strains. The wild-type strains of *Neurospora crassa* (Em5297a, FGSC[2] 352 and St.L.74A, FGSC 987) and the mutant strains *wc-1* (allele P829, FGSC 128) and *cr-1* (allele B123, FGSC 488) were obtained from FGSC, University of Kansas Medical School, Department of Microbiology, Kansas City, KS, 66103. The *wc-2* mutant used was obtained from D. D. Perkins, Stanford University (allele 234[w], Perkins stock 9896-5, identical in *wc* genotype to FGSC 3818, which is a sibling).

Chemicals. Cyclic AMP and cyclic GMP assay kits and the [8-^{3}H]cyclic AMP and [8-^{3}H]cyclic GMP used as tracers were from the Amersham Corporation.[3] Scintillation fluid (Ultrafluor) was from National Diagnostics. All other chemicals were of the highest quality commercially available.

Growth and Harvesting of Mycelia. The strains were grown in 125 ml Erlenmeyer flasks in 20 ml aliquots of Vogel's minimal medium N (30) supplemented with 2% (w/v) sucrose and 0.8% (v/v) Tween 80, unless otherwise indicated.

For comparison of intracellular and extracellular levels of cyclic AMP and cyclic GMP in various strains of *Neurospora*, the following treatments were used: 6 d growth in standing culture in the dark at 18°C; or 6 d growth in standing culture in the dark at 18°C followed by suction filtration on filter paper, transfer to 9 cm plastic Petri dishes, addition of 2 ml fresh medium and incubation for 2 h in the dark at 25, 18, or 6°C, unless otherwise indicated. All manipulations of mycelial pads were carried out under red safe light (60-W, natural dark ruby incandescent lamps). For intracellular cyclic nucleotide determinations, the mycelial pads following these treatments were either rapidly filtered by suction on filter paper and then frozen by immersion in liquid N_2, or filtration was omitted, and the pads were freeze-clamped. For freeze-clamping, the Petri dish containing the mycelial pad was placed on a block of expanded polystyrene and the pad frozen by compression beneath an aluminum plate cooled in liquid N_2. For determination of extracellular cyclic nucleotides, the growth medium was collected from the cultures by suction filtration through filter paper and stored frozen at −20°C.

For measurement of the effect of blue light on intracellular cyclic AMP levels in the St.L.74A wild-type strain, three different treatments were used. Mycelial pads were grown for 6 d in the dark at 18°C then: (a) excess growth medium was decanted, the pads placed on filter paper on a Büchner funnel at 18°C, irradiated immediately at 18°C with blue light similar to that in Harding (14), rapidly filtered by suction, and frozen in liquid N_2; (b) the 6 d old mycelial pads were filtered by suction, placed in 9 cm plastic Petri dishes, 2 ml of fresh medium added, the pads incubated in the dark at 18°C for 2 h, irradiated with blue light in the dishes with the lids removed, rapidly filtered by suction, and frozen in liquid N_2; (c) as for (b) above, except that the pads were incubated at 6°C (instead of 18°C) for 2 h and at the end of the irradiation, filtration by suction was omitted and the pads were freeze-clamped. In all cases, the dark control pads were treated in an identical fashion to those irradiated, except that irradiation was omitted.

Extraction and Measurement of Intracellular Cyclic Nucleotide Levels. The frozen pads were lyophilized, weighed, and ground to a fine powder with a mortar and pestle. The powder was extracted with 0.6 M $HClO_4$ (10 ml/g of powder) using a hand-held Teflon pestle tissue grinder. [8-^{3}H]cyclic AMP (0.081 pmol) and [8-^{3}H]cyclic GMP (0.11 pmol) were added as tracer to estimate recovery (5000 dpm each case). The extract was then centrifuged at 48,000*g* for 10 min. The supernatant was neutralized with 0.5 M tri-*n*-octylamine in Freon 11 (3), lyophilized, and resuspended in 1 ml of deionized H_2O. The extract was then loaded on to a column of Bio-Rad AG 50W-X4 (200–400 mesh, 4 cm × 1 cm), and cyclic AMP and cyclic GMP eluted with water. Those fractions containing cyclic AMP or cyclic GMP were pooled separately, lyophilized, and resuspended in 0.5 ml of Tris-HCl (50 mM, pH 7.5), then loaded on to columns of alumina (aluminum oxide 90, 70–230 mesh, E. Merck, 4 × 1 cm) equilibrated with Tris-HCl buffer. Cyclic AMP and cyclic GMP were eluted with the Tris-HCl buffer. Fractions containing cyclic AMP or cyclic GMP were pooled separately, lyophilized, and resuspended in water. An aliquot of each was subjected to liquid scintillation counting to determine recovery through the extraction procedure. Cyclic AMP and cyclic GMP were completely resolved on both the AG 50W-X4 and alumina columns, and most interfering substances which absorbed light at a wavelength of 254 nm were removed by the two column purification procedure, thus facilitating effective analysis by HPLC. Cyclic AMP was measured by competitive binding protein assay (Amersham) and selected results confirmed by HPLC, whereas cyclic GMP was measured by radioimmunoassay (Amersham).

Measurement of Extracellular Cyclic Nucleotide Levels. For the radioimmunoassay of cyclic GMP, aliquots of growth media were assayed without prior concentration or purification. For assay of cyclic AMP, [8-^{3}H]cyclic AMP tracer was added to the media samples before lyophilization. Samples for competitive binding protein assay were processed as described for the intracellular nucleotide extracts. To facilitate rapid sample preparation, Tween 80 was omitted from the growth media of samples destined for HPLC, so that the alumina chromatography step could also be omitted.

HPLC. A Beckman model 344 HPLC system equipped with a Beckman C-18 Ultrasphere-ODS reverse-phase column was used. Cyclic AMP prepared from extracellular extracts was eluted with a linear 30 min gradient, with 0.1% (v/v) acetic acid as the initial eluent, and 0.1% (v/v) acetic acid containing 20% (v/v) methanol as the final eluent. Cyclic AMP prepared from intracellular extracts was eluted isocratically with 0.1% (v/v) acetic acid containing 20% (v/v) methanol. Cyclic AMP was monitored at a wavelength of 254 nm and identified by comparison of its retention time with an authentic sample of cyclic AMP and also by co-elution with that authentic sample. The level of cyclic AMP was measured by comparison of its HPLC peak area with that of a known amount of authentic standard. Measurement of HPLC peak area was by electronic integration and triangulation.

RESULTS

Extracellular Cyclic Nucleotides. Two d after inoculation with conidia, there was an appreciable amount of cyclic AMP in the

[2] Abbreviations: FGSC, Fungal Genetics Stock Center; *wc*, *white collar*; *cr*, *crisp*.

[3] Reference to brand or firm name does not constitute endorsement by the Smithsonian Institution over others of a similar nature not mentioned.

growth medium of the St.L.74A wild-type strain (Fig. 1). During growth of the mycelial pads, the amount of cyclic AMP in the medium increased approximately in proportion to the dry weight of tissue (Fig. 1).

After 6 d incubation at 18°C, there was cyclic AMP and cyclic GMP in the growth medium of all the *Neurospora* strains examined, except the *cr-1* mutant (Table I). When the mycelial pads were then placed in fresh medium, more cyclic AMP was exported from the pads incubated for 2 h at 25°C than at 6°C. The St.L.74A wild-type strain was also incubated for 2 h at 18°C in fresh medium, and a value intermediate between those at 25 and 6°C was obtained (Table I). The levels of extracellular cyclic AMP and cyclic GMP in the two white collar mutants were of the same order of magnitude as those of the two wild-type strains.

The cyclic AMP values presented in Table I were obtained by competitive binding protein assay of media purified by the two column procedure described in "Materials and Methods." In a second experiment, extracellular cyclic AMP was measured by HPLC (Fig. 2). The resulting values were generally higher (between 1.7 and 6.4 times for the various experimental conditions) than those in Table I, but confirmed the presence of cyclic AMP in the growth media, and the temperature dependent nature of its efflux from the mycelia of the wild-type and white collar mutant strains. Control experiments, in which media samples with and without Tween 80 were purified and assayed by the competitive binding protein method, indicated that the differences between the results in Table I and those obtained by HPLC were not due to omission of Tween 80 from the media destined for HPLC analysis (see "Materials and Methods" for details). Tween 80 is a detergent which is often added to the medium of *Neurospora* grown in standing culture to suppress the growth of aerial hyphae and conidiation.

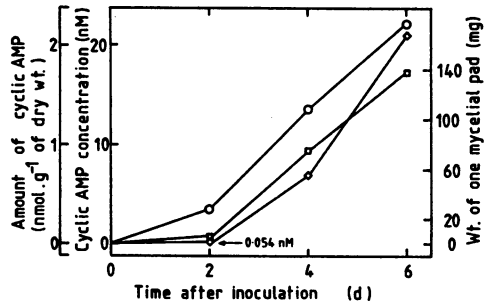

FIG. 1. Extracellular cyclic AMP in the medium of the St.L.74A wild-type of *N. crassa* during growth in the dark at 18°C. Pooled growth medium from 5 mycelial pads was purified and assayed for cyclic AMP by the competitive binding protein method as described in "Materials and Methods." The amount of cyclic AMP (nmol per g dry weight of mycelia, O) and the corresponding concentrations (nM, ◇) of cyclic AMP in the growth medium together with the dry weight of one mycelial pad (□) after 2, 4, and 6 d growth are shown. Each point is the mean of two determinations.

The absence of cyclic AMP and cyclic GMP from the growth medium of the *cr-1* mutant (Table I) is consistent with the absence of adenylate cyclase (26) and possibly guanylate cyclase in this strain (see "Discussion").

We attribute the small peak on the HPLC chromatogram (Fig. 2c) corresponding to cyclic AMP in the *cr-1* mutant as baseline noise, since the competitive binding protein assay gave a zero result for both extracellular (Table I) and intracellular cyclic AMP (Table II) in this strain.

Intracellular Cyclic Nucleotides. Cyclic AMP (and presumably cyclic GMP) levels can change rapidly in fungi (24), and changes may occur during sampling of cultures. Mechanical agitation is one stimulus known to elevate the level of cyclic AMP in *Neurospora* (23) and this, together with our knowledge of extracellular cyclic nucleotides, which could interfere with the measurement of intracellular levels if not excluded, made it necessary to pay particular attention to tissue sampling. Therefore, we compared rapid filtration methods with freeze-clamping (see "Materials and Methods" for details). The mycelial pads act as a 'sponge' when resuspended in 2 ml of fresh medium. When subsequently freeze-clamped, a proportion of this medium is squeezed out, but some remains frozen with the pad. Consequently, any extracellular cyclic nucleotides in this trapped medium would contribute to the values for the intracellular cyclic nucleotides. Therefore, we concluded that rapid filtration and freezing by immersion in liquid N_2 would give the best absolute values for intracellular cyclic nucleotides, but freeze-clamping would be useful when mycelia had to be sampled rapidly after very short periods of irradiation (see below) and should provide good comparative values.

The amounts and calculated concentrations of intracellular cyclic AMP and cyclic GMP for various *Neurospora crassa* strains are shown in Table II. Cyclic AMP levels in the two wild-type strains were in the same range as those previously reported (23, 25). The values for cyclic AMP and cyclic GMP in the *wc* mutants were not considerably different from those in the wild-type strains. However, the Em5297a wild-type generally had the highest levels of both intracellular and extracellular cyclic AMP and extracellular cyclic GMP. In contrast to the results for cyclic

Table I. *Cyclic AMP and Cyclic GMP in the Extracellular Media of two Wild-type and Three Mutant Strains of N. crassa*

The mean value from two determinations for each experimental condition is presented. For each set of determinations, the individual values were always within 20% of the mean value. The cyclic AMP levels were determined by the competitive binding protein assay.

Strain	Nucleotide	Experimental Conditions			
		6 d, 18°C	6 d, 18°C + 2 h, 25°C	6 d, 18°C + 2 h, 18°C	6 d, 18°C + 2 h, 6°C
		nmol per g dry wt of mycelia and corresponding concentration (nM)			
St.L.74A	Cyclic AMP	2.19[a] (22.4)	0.41 (28.8)	0.28 (19.6)	0.13 (8.9)
	Cyclic GMP	0.06 (0.6)			
Em5297a	Cyclic AMP	5.83 (49.2)	0.74 (52.6)		0.27 (19.5)
	Cyclic GMP	0.16 (1.3)			
wc-1	Cyclic AMP	1.38 (10.1)	0.26 (15.7)		0.05 (2.9)
	Cyclic GMP	0.07 (0.5)			
wc-2	Cyclic AMP	1.10 (9.2)	0.28 (15.3)		0.11 (5.9)
	Cyclic GMP	0.06 (0.5)			
cr-1	Cyclic AMP	0.0 (0.0)			
	Cyclic GMP	0.0 (0.0)			

[a] Mean value from 6 determinations.

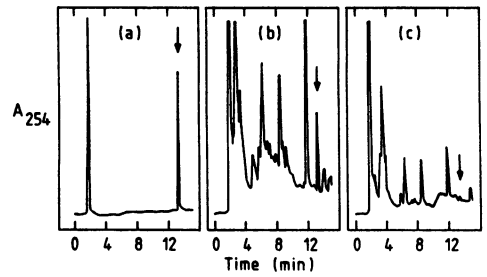

FIG. 2. HPLC analysis of cyclic AMP in *N. crassa* growth medium. Peaks corresponding to cyclic AMP are indicated by the arrows. (a), Authentic cyclic AMP, 156.0 pmol. Purified extracts of (b) St.L.74A and (c) *cr-1* growth media (no Tween 80) after 6 d at 18°C plus 2 h at 25°C. The amount of purified extracts injected corresponded to 1.6 ml of medium. The cyclic AMP peak in (b) was 86.0 pmol.

AMP, we found that the level of intracellular cyclic GMP in the wild-type and the *wc* mutant strains was more than 50 times lower than the value reported previously for the St.L.74A wild-type strain (25). Overall, there was no consistent effect of temperature on the intracellular levels of cyclic AMP or cyclic GMP. We could detect neither cyclic AMP nor cyclic GMP in mycelia of the *cr-1* mutant.

For the results presented in Table II, mycelial pads were sampled by filtration, and cyclic AMP was measured by the competitive binding protein method. Extracts from freeze-clamped mycelial pads of the St.L.74A wild-type strain were also assayed for cyclic AMP by both the competitive binding protein method and HPLC (Fig. 3). The results for each sample by the two assay methods were always within 30% of one another, and cyclic AMP levels in samples that had been freeze-clamped were closely similar to those that had been filtered (results not shown).

Cyclic Nucleotides in the Interstitial Spaces of the Cell Wall. Comparison of the results in Table II with those in Table I shows that the intracellular concentrations of cyclic AMP are considerably higher than the corresponding extracellular concentrations.

Note that the concentration values in Table II include cyclic AMP and cyclic GMP which may be trapped in the interstitial spaces of the cell wall since the cells were not rinsed before sampling. To calculate these concentration values, the intracellular cell volume was calculated as described in Slayman and Tatum (27), and this volume was corrected to include the cell wall. The cell wall occupies 20% of the total cell volume and is permeable to small molecules (27). For the purposes of the calculation, we assumed that the total volume of the cell wall was occupied by water and solutes. This assumption may result, if anything, in a slight underestimation of the intracellular concentration.

To attempt to measure cyclic AMP trapped in the cell wall, a multiple rinse procedure similar to that in (27) was used. The results (Fig. 4) showed that cyclic AMP was rapidly washed from mycelia of *Neurospora*, since the level of the nucleotide in each rinse was about the same, *i.e.* about 15 to 20% of the total intracellular level in each rinse. Therefore, either the plasma membrane of *Neurospora* is freely permeable to cyclic AMP, or cyclic AMP is rapidly released from the mycelia by some other mechanism.

The Effect of Blue Light on Intracellular Cyclic Nucleotide Levels. We could detect no effect of blue light on the intracellular levels of cyclic AMP or cyclic GMP in the St.L.74A wild-type (Fig. 5, a–c). Figure 5a shows the result of an experiment in which mycelia were irradiated immediately after removal from the flasks in which they had been grown. The gentle agitation required to remove the mycelial pads from the flasks was sufficient to result in a transient increase in cyclic AMP in both the irradiated and dark control mycelia. Nevertheless, irradiation produced no additional changes in cyclic AMP. To circumvent the problem caused by agitation, a second experiment was carried out at 18°C (Fig. 5b). Before irradiation, mycelial pads were removed from their growth flask and incubated for 2 h, a period determined to be long enough to allow cyclic AMP to return to its normal dark level. Again, blue light had no appreciable effect. Irradiation times from 7.5 s to 3.0 h were used to encompass the initial rapid light reaction that signals the switching on of carotenogenesis, the period of synthesis of enzymes required for carotenogenesis, and the time (about 3 h) at which the rate of accumulation of carotenoid pigments is at a maximum at this temperature (14). A third experiment was carried out at 6°C in which the mycelia were irradiated for periods of up to 1 min and then freeze-clamped (Fig. 5c). The rationale for using 6°C was 2-fold. First, it was possible that a transient change in cyclic nucleotide levels operating over a very short period after irradiation may have been slowed down sufficiently at 6°C to enable us to measure it, and second, it seemed pertinent to measure cyclic

Table II. *Intracellular Cyclic AMP and Cyclic GMP in Two Wild-type and Three Mutant Strains of N. crassa*

The mean value from two determinations for each experimental condition is presented. For each set of determinations, the individual values were always within 20% (cyclic AMP) or 60% (cyclic GMP) of the mean value. At the end of each treatment, the mycelial pads were filtered by suction and then frozen with liquid nitrogen. The cyclic AMP levels were determined by the competitive binding protein assay.

Strain	Nucleotide	Experimental Conditions		
		6 d, 18°C	6 d, 18°C + 2 h, 25°C	6 d, 18°C + 2 h, 6°C
		nmol per g dry wt of mycelia and corresponding estimated intracellular concentrations (nM)		
St.L.74A	Cyclic AMP	0.78[a] (245.7)	0.95 (299.2)	0.73 (229.9)
	Cyclic GMP	0.005 (1.6)		
Em5297a	Cyclic AMP	1.14 (359.1)	1.48 (466.1)	1.46 (459.8)
	Cyclic GMP	0.005 (1.6)	0.005 (1.6)	0.005 (1.6)
wc-1	Cyclic AMP	0.62 (195.3)	0.62 (195.3)	0.61 (192.1)
	Cyclic GMP	0.003 (1.0)		
wc-2	Cyclic AMP	0.81 (255.1)	0.89 (280.3)	0.63 (198.4)
	Cyclic GMP	0.002 (0.7)		
cr-1	Cyclic AMP	0.0 (0.0)		
	Cyclic GMP	0.0 (0.0)		

[a] Mean value from 6 determinations.

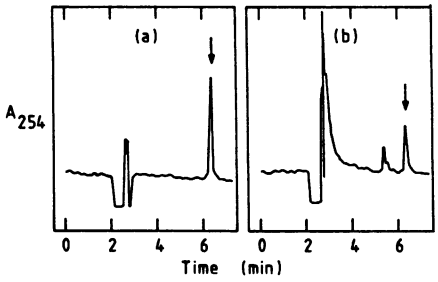

FIG. 3. HPLC analysis of intracellular cyclic AMP from *N. crassa*. Peaks corresponding to cyclic AMP are indicated by the arrows. (a), Authentic cyclic AMP, 31.0 pmol; (b), extract of St.L.74A mycelia after 6 d at 18°C plus 1 h at 6°C. The mycelia were freeze-clamped at the end of the incubation at 6°C. The cyclic AMP peak was 14.6 pmol. A similar amount of the same extract to that injected for HPLC was assayed by the competitive binding protein method and a value of 18.7 pmol obtained.

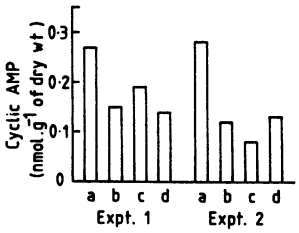

FIG. 4. Cyclic AMP in the distilled H_2O rinses from mycelial pads of *N. crassa* St.L.74A wild-type. Mycelial pads (2 for each determination) were grown for 6 d at 18°C in the dark, then filtered and rinsed with 10 ml of distilled H_2O. The rinse was allowed to stand for about 5 s, then collected by suction filtration. The process was repeated to give an initial filtrate plus three rinses, each of which was purified and assayed for cyclic AMP by the competitive binding protein method. The experiment was carried out in duplicate (initial filtrate, a; rinse 1, b; rinse 2, c; rinse 3, d).

nucleotide levels after incubation at the temperature known to induce the maximum amount of carotenogenesis. Blue light had no appreciable and consistent effect on cyclic AMP or cyclic GMP levels up to 1 min after the onset of irradiation at 6°C. We note that the range of values was quite large, which was possibly due to the freeze-clamping technique.

DISCUSSION

The measurement of cyclic nucleotides in biological extracts can be very difficult, due to their low and often rapidly changing concentrations, and the frequent presence of compounds which interfere with assays. For example, extensive investigations were required to positively identify cyclic AMP and cyclic GMP in plant tissues (2, 21). Furthermore, unpurified tissue extracts from *Phycomyces blakesleeanus* contained a component which inhibited the binding of cyclic AMP to its antibody in the cyclic AMP assay (20). We found that unpurified tissue extracts from *Neurospora* gave erroneous values for cyclic AMP when assayed by the competitive binding protein method (results not shown). Consequently, we paid particular attention to sampling methods and used the two column purification procedure detailed in "Materials and Methods" followed by assay by both the competitive binding protein method and HPLC to unequivocally facilitate positive identification and quantitation of intracellular cyclic AMP. We also subjected intracellular cyclic GMP to a similar purification before measurement by radioimmunoassay. We found that inclusion of Tween 80 in the growth medium made purification by the two column procedure necessary before

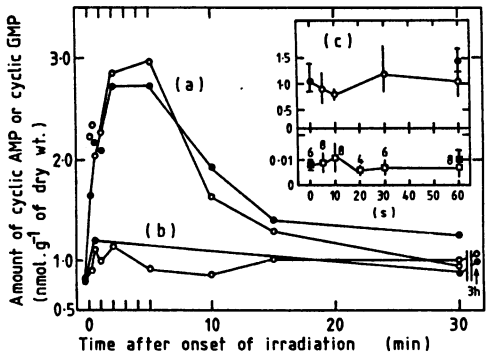

FIG. 5. The effect of blue light on the intracellular cyclic nucleotide levels in the St.L.74A wild-type of *N. crassa*. Mycelial pads were grown for 6 d at 18°C in the dark; (a), excess growth medium was decanted, the pads placed on filter paper on a Büchner funnel at 18°C, irradiated immediately at 18°C, rapidly filtered by suction, and frozen in liquid N_2; (b), the 6 d old mycelial pads were filtered by suction, placed in 9 cm plastic Petri dishes, 2 ml of fresh medium added, the pads incubated in the dark at 18°C for 2 h, irradiated in the dishes with the lids removed, rapidly filtered by suction, and frozen in liquid N_2; (c), as for (b), except that incubation of the mycelial pads for 2 h in fresh medium was at 6°C, and the pads were sampled following irradiation by freeze-clamping. Cyclic AMP was measured by the competitive binding protein method. See "Materials and Methods" for details. Cyclic AMP: dark controls (●), irradiated samples (○). Cyclic GMP: dark controls (■), irradiated samples (□). Each determination was on pooled material from either 2 (experiments a and b) or 5 (experiment c) mycelial pads. Experiments a and b: each point represents the mean value from at least two determinations. Individual values were always within 30% of the mean value. Experiment c: cyclic AMP: each point represents the mean value from 3 determinations. The bars represent the range of values. Cyclic GMP: each point represents the mean ±SD. The number of determinations for each point is indicated next to that point on the figure.

measurement of extracellular cyclic AMP by the competitive binding protein assay. If Tween 80 were omitted, cyclic AMP could be measured accurately without any column purification steps by the competitive binding protein method (results not shown), but for HPLC assays, a one-column purification procedure was necessary to remove constituents of the concentrated medium which obscured the cyclic AMP peak. Extracellular cyclic GMP could be assayed by radioimmunoassay without any column purification steps, with or without Tween 80 in the medium.

Previously the intracellular ratio of cyclic AMP to cyclic GMP in *Neurospora* was reported to be approximately 1:1 (25). However, by using the purification procedures described in the present investigation, we have determined ratios of between 100 and 400 to 1 (intracellular) and between 15 and 40 to 1 (extracellular). These values were more in agreement with those found in animals (*e.g.* [19], cyclic GMP reviewed in [12]) and higher plants (2, 21).

We could detect no cyclic GMP in the *cr-1* mutant strain. This result suggests two possibilities. The first is that adenylate cyclase in *Neurospora* catalyzes the synthesis of both cyclic AMP and cyclic GMP, and the *cr-1* mutant, which lacks adenylate cyclase activity (26), thus has a low level of both of these compounds. The second is that separate enzymes, *i.e.* adenylate and guanylate cyclase catalyze the synthesis of cyclic AMP and cyclic GMP, respectively, and that the *cr-1* mutation results in a deficiency in the activity of both enzymes.

Another interesting aspect of the present investigation was the detection of extracellular cyclic nucleotides in all strains tested except for *cr-1*. The presence of extracellular cyclic AMP in *Neurospora* cultures has been reported previously for a *leu-4*

mutant (7). In that investigation, the effect of amino acid deprivation on the synthesis of macromolecules and nucleotides was determined. Extracellular levels of cyclic AMP were calculated as the difference between that in mycelia plus culture media *versus* mycelia alone.

There is considerable evidence that the egress of cyclic AMP from metazoan cells is energy mediated, presumably by a specific carrier (1). The plasma membrane of *Neurospora* is generally impermeable to small, charged molecules (27). We do not know whether there was a specific transport system across the cell membrane of *Neurospora* for cyclic nucleotides, or if efflux was due to a passive diffusion process. Whatever the mechanism, efflux of cyclic AMP was very rapid, and the results of our experiments where mycelia were repeatedly rinsed with distilled H_2O would favor the hypothesis that the plasma membrane is permeable to cyclic AMP. The results in Figure 1 show that cyclic AMP is released from young and actively growing mycelia and therefore support the hypothesis that cyclic nucleotide release was not just from old or dying mycelia by cell lysis.

Cyclic AMP efflux into the media of all the strains was greater after 2 h at 25°C than at 6°C. This could be due to either increased membrane fluidity at the higher temperature, which would facilitate the diffusion of cyclic AMP across the cell membrane, or if an active transport process were involved, the higher temperature could increase its activity, again resulting in an increased efflux of cyclic AMP.

A possible function for extracellular cyclic nucleotides in *Neurospora* is not known. In *Dictyostelium discoideum*, cyclic AMP functions in cellular aggregation (9). One possible role of the *Neurospora* extracellular cyclic AMP would be to act as a signal for limiting mycelial density. It could diffuse into the medium until intra- and extracellular levels were equal, at which point intracellular levels would rise and signal a halt to growth. A second possibility is that the efflux of cyclic nucleotides serves to maintain intracellular levels.

Recently, it has been reported (17) that *Neurospora* secretes phosphate repressible cyclic nucleotide phosphodiesterases into the growth medium. These enzymes could, under certain growth conditions, provide one possible method of control of the extracellular cyclic nucleotide levels.

In *Neurospora* high concentrations of exogenously added cyclic AMP inhibit blue light induced carotenogenesis (13) and can restore *cr-1* mutants to wild-type morphology (25, 28). It is doubtful whether the levels of naturally occurring extracellular cyclic AMP that we measured are involved in this process since they were about 10^6 times lower than that added in the above experiments. The N^6-monobutyryl derivative of cyclic AMP, added exogenously to *Neurospora* cultures, restores wild-type morphology to *cr-1* mutants at a lower concentration than cyclic AMP (25). This cyclic AMP derivative is thought to be more effective than cyclic AMP because it may be able to cross cell membranes more easily or may be a poor substrate for intracellular cyclic nucleotide phosphodiesterase in *Neurospora* (24, 25).

In the present investigation, we attempted to determine whether light-induced carotenoid biosynthesis in *Neurospora* is regulated by cyclic nucleotide levels. The optimum temperature for maximum photoinduced carotenoid biosynthesis in *Neurospora* is about 6°C (14). Suboptimal responses are observed between 6 and 37°C, and there is no response at 0°C. Therefore, we examined dark grown *Neurospora* strains after incubation at 6 or 25°C to determine whether there was a relationship between cyclic nucleotide levels and potential carotenoid accumulation. There was no correlation.

Since the wild-type strains and *wc* mutants had levels of both intracellular and extracellular cyclic AMP and cyclic GMP of the same order of magnitude, we concluded that the *wc* mutations did not affect the enzymes of cyclic nucleotide metabolism or the mechanism by which cyclic nucleotides were exported into the growth medium of *Neurospora crassa*.

Blue light has been reported to cause a rapid transient decrease in cyclic AMP in the fungus *Phycomyces blakesleeanus* (minimum level reached in 1 min) (4), and to activate both the phosphodiesterase (5) and adenylate cyclase (6) in this organism. In *N. crassa*, a similar transient decrease in cyclic AMP, although over a longer period (minimum level reached in 10–15 min) and in response to white light, has been reported (18). The size of the decrease was shown to be correlated with the subsequent accumulation of carotenoid pigment. Leutwiler and Brandt (20) have failed to repeat the results reported in Cohen (4), and we were unable to show an effect of blue light on cyclic AMP levels in *N. crassa*, possibly because our experimental procedures were different from those of Kritsky *et al.* (18).

However, our results do not completely rule out a blue light effect on cyclic AMP levels, or consequently the involvement of cyclic nucleotides in the control of photoinduced carotenogenesis. For example, although cyclic GMP has been conclusively shown to be involved in vertebrate vision, the demonstration of changing levels of this cyclic nucleotide has proved difficult (29) because its physiologically important form may be bound to other molecules (cyclic GMP assays measure total cellular cyclic GMP), or since both guanylate cyclase and phosphodiesterase may be activated by light, increased turnover of cyclic GMP in response to light may be more important than its absolute level. Thus, a study of possible effects of blue light on the activities of such enzymes in *N. crassa* might prove fruitful.

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LITERATURE CITED

1. BARBER R, RW BUTCHER 1983 The egress of cyclic AMP from metazoan cells. Adv Cyclic Nucleotide Res 15: 119–138
2. BROWN EG, RP NEWTON 1981 Cyclic AMP and higher plants. Phytochemistry 20: 2453–2463
3. CHEN S-C, PR BROWN, DM ROSIE 1977 Extraction procedures for use prior to HPLC nucleotide analysis using microparticle chemically bonded packings. J Chromatogr Sci 15: 218–221
4. COHEN RJ 1974 Cyclic AMP levels in *Phycomyces* during a response to light. Nature 251: 144–146
5. COHEN RJ, MM ATKINSON 1978 Activation of *Phycomyces* adenosine 3′,5′-monophosphate phosphodiesterase by blue light. Biochem Biophys Res Commun 83: 616–621
6. COHEN RJ, JL NESS, SM WHIDDON 1980 Adenylate cyclase from *Phycomyces* sporangiophore. Phytochemistry 19; 1913–1918
7. DE CARLO RR, EW SOMBERG 1974 The effect of essential amino acid deprivation on macromolecular synthesis and nucleotide pool sizes in *Neurospora crassa*. Arch Biochem Biophys 165: 201–212
8. DEGLI-INNOCENTI F, VEA RUSSO 1984 Isolation of new white collar mutants of *Neurospora crassa* and studies on their behavior in the blue light-induced formation of protoperithecia. J Bacteriol 159: 757–761
9. DEVREOTES PN 1983 Cyclic nucleotides and cell—cell communication in *Dictyostelium discoideum*. Adv Cyclic Nucleotide Res 15: 55–96
10. FRANCKO DA 1983 Cyclic AMP in photosynthetic organisms: recent developments. Adv Cyclic Nucleotide Res 15: 97–117
11. GANCEDO JM, MJ MAZON, P ERASO 1985 Biological roles of cyclic AMP: similarities and differences between organisms. Trends Biochem Sci 10: 210–212
12. GOLDBERG ND, MK HADDOX 1977 Cyclic GMP metabolism and involvement in biological regulation. Annu Rev Biochem 46: 823–896
13. HARDING RW 1973 Inhibition of conidiation and photoinduced carotenoid biosynthesis by cyclic-AMP. *Neurospora* Newsl 20: 20–21
14. HARDING RW 1974 The effect of temperature on photo-induced carotenoid biosynthesis in *Neurospora crassa*. Plant Physiol 54: 142–147
15. HARDING RW, S MELLES 1983 Genetic analysis of phototropism of *Neurospora crassa* perithecial beaks using white collar and albino mutants. Plant Physiol 72: 996–1000
16. HARDING RW, RV TURNER 1981 Photoregulation of the carotenoid biosynthetic pathway in albino and white collar mutants of *Neurospora crassa*. Plant Physiol 68: 745–749
17. HASUNUMA K 1983 Repressible extracellular phosphodiesterases showing cyclic 2′,3′ and cyclic 3′,5′-nucleotide phosphodiesterase activities in *Neurospora crassa*. J Bacteriol 156: 291–300
18. KRITSKY MS, VY SOKOLOVSKY, TA BELOZERSKAYA, EK CHERNYSHEVA 1982

Relationship between cyclic AMP level and accumulation of carotenoid pigments in *Neurospora crassa*. Arch Microbiol 133: 206–208
19. KUEHL FA JR, EA HAM, ME ZANETTI, CH SANFORD, SE NICOL, ND GOLDBERG 1974 Estrogen-related increases in uterine guanosine 3':5'-cyclic monophosphate levels. Proc Natl Acad Sci USA 71: 1866–1870
20. LEUTWILER LS, M BRANDT 1983 Absence of significant light-induced changes in cAMP levels in sporangiophores of *Phycomyces blakesleeanus*. J Bacteriol 153: 555–557
21. NEWTON RP, EC KINGSTON, DE EVANS, LM YOUNIS, EG BROWN 1984 Occurrence of guanosine 3',5'-cyclic monophosphate (cyclic GMP) and associated enzyme systems in *Phaseolus vulgaris*. Phytochemistry 23: 1367–1372
22. PACE U, E HANSKI, Y SALOMON, D LANCET 1985 Odorant-sensitive adenylate cyclase may mediate olfactory reception. Nature 316: 255–258
23. PALL ML 1977 Cyclic AMP and the plasma membrane potential in *Neurospora crassa*. J Biol Chem 252: 7146–7150
24. PALL ML 1981 Adenosine 3',5'-phosphate in fungi. Microbiol Rev 45: 462–480
25. ROSENBERG G, ML PALL 1979 Properties of two cyclic nucleotide-deficient mutants of *Neurospora crassa*. J Bacteriol 137: 1140–1144
26. ROSENBERG GB, ML PALL 1983 Reconstitution of adenylate cyclase in *Neurospora* from two components of the enzyme. Arch Biochem Biophys 221: 254–260
27. SLAYMAN CW, EL TATUM 1964 Potassium transport in *Neurospora* I. Intracellular sodium and potassium concentrations and cation requirements for growth. Biochim Biophys Acta 88: 578–592
28. TERENZI HF, MM FLAWIA, MT TELLEZ-INON, HN TORRES 1976 Control of *Neurospora crassa* morphology by cyclic adenosine 3',5'-monophosphate and dibutyryl cyclic adenosine 3',5'-monophosphate. J Bacteriol 126: 91–99
29. VINES G 1985 New light on vision. New Sci 107: 40–43
30. VOGEL HJ 1964 Distribution of lysine pathways among fungi: evolutionary implications. Am Nat 98: 435–446