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Control of *Neurospora crassa* Morphology by Cyclic Adenosine 3',5'-Monophosphate and Dibutyryl Cyclic Adenosine 3',5'-Monophosphate

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The role of cyclic adenosine 3',5'-monophosphate (cyclic AMP) in the control of the *Neurospora* asexual life cycle was studied. Endogenous cyclic AMP levels were 10 to 20 times higher in strains having the wild-type *cr-1* allele than in those carrying the mutated allele. In a wild-type strain these levels remained constant throughout the entire growth period in shaken liquid cultures, except during a short period at the beginning of the stationary growth phase. In this period a marked increase in the cyclic nucleotide level was observed. The culture of *cr-1* mutant strains in the presence of cyclic AMP or its dibutyryl derivative restores some morphological properties characteristic of wild-type strains. Specifically these cyclic nucleotides stimulated the rate of mycelial elongation, as well as the differentiation of aerial hyphae.

The asexual life cycle in *Neurospora* is characterized by the following steps. (i) The first step is the germination of macroconidia and the formation of the first vegetative hyphae. (ii) Next is the continuous elongation of these vegetative hyphae, which branch and fuse repetitively, leading to the formation of the vegetative mycelial pads. In this latter step the immersed growth is characteristic of cultures in liquid medium. (iii) Differentiation and the development of hyphae that elongate from the surface of the liquid medium, growing outward by continuous elongation and becoming aerial, follows. (iv) The last step is the differentiation of blastoarthrospores from the aerial hyphal tips by a process "characterized by an initial basifugal budding of proconidial elements which are then secondarily separated as maturing conidia by interconidial septa" (18).

Evidence indicates that the development of aerial hyphae and conidiation is inhibited under conditions that prevent a continuous exposition of vegetative mycelia to air or that increase the rate of glycolysis and alcohol fermentation (8, 18, 21). On the contrary, growth over an agar surface leads to a preferential formation of aerial hyphae and conidiation; such a phenomenon is more evident in those zones of the culture having the best aeration (i.e., the slant top or the border of plates).

An interesting family of *Neurospora* morphological mutants is that called "crisp." The growth pattern of these was first described by Lindegren as "short aerial hyphae with tight clusters of more brightly colored conidia than the wild-type" (10). These strains are characterized by a colonial phenotype showing an impairment in the elongation of aerial hyphae and a premature accumulation of conidia (12).

The "crisp" phenotype is determined by mutations at any one of three different loci: *cr-1*, *cr-2*, and *cr-3*. These nonallelic loci are located in the right arm of linkage group I in the following order: centromere, *nic-2*, *cr-1*, *cr-3*, *cr-2*, and *al-2* (4).

Evidence described elsewhere (14, 16) indicates that one type of "crisp" strains, those having mutated the *cr-1* allele, exhibited a reduced adenylate cyclase specific activity. This could suggest that cyclic adenosine 3',5'-monophosphate (cyclic AMP) is one of the factors controlling elongation of aerial hyphae and subsequent conidiation in *Neurospora*. The work described in the present paper was undertaken to prove this hypothesis.

MATERIALS AND METHODS

Strains. Most of the strains were a generous gift from W. N. Ogata (Fungal Genetic Stock Center, Arcata). The following strains were used throughout the work: St.L. 74 (wild type); BAT 9-5, *cot*, *nic-3*; FGSC 2209, *cr-2*, *al-2*; FGSC 2329, *cr-3*; FGSC 810, *os-1*; FGSC 488, *cr-1*; FGSC 806, *cr-1*; FGSC 826, *cr-1*; FGSC 814, *cr-1*; FGSC 487, *cr-1*; FGSC 329, *cr-1*, *arg-1*, *aur*, *os-1*; FGSC 825, *cr-?*. Allele numbers of the genetic markers used were: *arg-1* (arginine): B 369; *cr-1* (crisp): B123, B 74, R 2360, and C-Ex-11-67; *cr-3*:

R2509; *cr-2:* R2445; *cr-?:* B180; *aur* (aurescent): 34508; *os-1* (osmotic): B135; *nic-3* (nicotinic acid): Y 31881; *cot-1* (colonial temperature sensitive): C 102(t). Strain nomenclature was that recommended by Barrat and Ogata (1).

Media and culture conditions. Vogel minimal medium supplemented with 2% sucrose and biotin was used for most experiments. C and M media were prepared according to Westergaard and Mitchell (22) with the modification proposed by Turian (17). Standing liquid cultures were grown in test tubes (10 by 100 mm) each containing 2 ml of medium, or in tubes (10 by 75 mm) containing 1 ml of medium.

Measure of cell growth. Cell growth was measured in mycelial cultures carried out either on Vogel minimal liquid or solid medium supplemented with sucrose and biotin (20). In the former case, the cultures were filtered on a Büchner funnel, and the mycelial pads were washed with water and pressed between several sheets of filter paper. Mycelial mass was expressed either as wet or dry weight or as total protein. Dry weight was measured on the pressed mycelial pads, heated for 16 h at 80 C.

Growth on solid medium was quantitated as follows: petri dishes (6 cm in diameter) were filled with 5 ml of medium containing 1.5% agar (wt/vol). After solidification, the surface was covered with a circle cut from an open dialysis tubing previously boiled in water for 10 min. Aliquots of a conidial suspension were seeded on the surface of this circle. At the indicated times, the mycelium was scraped off this surface and suspended in 1 ml of 10% trichloroacetic acid. Total protein was assayed with the Folin reagent (11) after treating the acid precipitate with 1 ml of 1 N NaOH for 10 min at 100 C.

The rate of mycelial elongation was measured by the race tube method (13).

Cyclic AMP assay. Intracellular concentration of cyclic AMP was measured in *Neurospora* cultured in shaken liquid media. Mycelia were rapidly filtered and washed with cold water on a Büchner funnel. A piece of about 200 to 400 mg from the wet mycelium was scraped off with a spatula and placed in 2.5 ml of cold 5% trichloroacetic acid containing 0.75 pmol of cyclic [^{3}H]AMP (specific activity, 10 mCi/μmol). The time elapsing between the start of filtration and suspension in the acid solution varied with the volume of the sample culture: 20 to 25 s for a sample of 40 ml and 45 to 55 s for a sample of 200 ml. The time of processing the samples influenced the intracellular level of the cyclic nucleotide. For example, a sample processed in 45 s gave a value of 35 pmol/mg of protein, whereas an equivalent sample processed in 120 s gave a value of 62 pmol/mg of protein.

Samples were purified as follows. The acid mixtures were left in ice for at least 120 min, and then they were centrifuged in a clinical centrifuge for 5 min. The supernatants were transferred to culture tubes (15 by 125 mm) further acidified with 0.1 ml of 2 N HCl and extracted five times with 3 volumes of ethyl ether and lyophilized. Cyclic [^{3}H]AMP recovery in this step was 70 to 80%. After that, the samples were redissolved in 4.5 ml of 0.05 M HCl and passed through columns (0.4 by 15 cm) of Dowex WX 100 to 200 mesh equilibrated with the same acid solution (2). The columns were washed with 4 ml of the same solution. The percolate and washing were discarded. Elution was performed with 14 ml of this acid solution. The eluates received in glass centrifuge tubes (30 by 150 mm) were lyophilized and dissolved in 0.5 ml of 0.05 M sodium acetate buffer, pH 4.

Recoveries from the columns were about 50 to 70%, so that the final recovery was about 35 to 50%.

Recoveries were measured in 0.15-ml aliquots from the latter step dissolved in Bray solution. Cyclic AMP was measured on 0.025-ml aliquots from the same samples, using the procedure of Gilman (5). Incubation mixtures contained 1 pmol of cyclic [^{3}H]AMP. Under these conditions, sensitivity of the method was about 0.25 pmol, equivalent to 10 pmol in the whole sample after correction for recovery.

Column purification seemed to be essential in measuring specifically cyclic AMP with this binding procedure. Samples not purified by column chromatography exhibited cyclic nucleotide levels higher than those chromatographed on Dowex 50 columns (Table 1). Before this purification procedure, a large proportion of the material reacting as cyclic AMP in the binding assay was resistant to cyclic nucleotide phosphodiesterase treatment. This treatment leads to almost complete disappearance of the radioactive cyclic AMP used as the recovery marker, as well as the material reacting as cyclic AMP after purification by column chromatography.

The treatment of 2.5-ml aliquots of 5% trichloroacetic acid solution according to the purificative procedure described above gave, after column chromatography, material reacting as cyclic AMP but resistant to phosphodiesterase treatment. The corresponding values (about 15 pmol) were substracted from those found with the mycelial samples. Experience over several hundred determinations indicated that this substraction makes it unnecssary to correct for material resistant to phosphodiesterase treatment for each mycelial sample. Cyclic AMP levels were expressed as picomoles per milligram of total protein in the trichloroacetic acid precipitate.

Assays were performed in duplicate aliquots from duplicate mycelial samples.

Adenylate cyclase assay. The standard assay for adenylate cyclase activity contained 0.5 mM [α-^{32}P]ATP, 0.5 mM $MnCl_2$, 100 mM PIPES [piperazine *N,N'*-bis(2-ethanesulfonic acid)]-NaOH buffer, pH 6.3, and the enzyme preparation (0.1 to 0.3 mg of protein). The total volume was 0.1 ml. Incubations were carried out for 2.5 to 5 min at 37 C. Reactions were stopped and the radioactive cyclic AMP was purified from these mixtures as previously described (3).

Cyclic nucleotide phosphodiesterase assay. Incubation mixtures contained 0.25 μM cyclic [^{3}H]AMP, 50 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.4, and 5 mM $MgCl_2$ and enzyme (0.2 to 0.4 mg of protein). The total volume was 0.1 ml. Incubations were performed for 5 min at 34 C. Reactions were stopped by heating at 100 C for 2 min. After that, the mixtures received 0.01 ml of a solution containing 10 mg of *Ophiophagus hannah* venom per ml as the source of 5'-nucleotidase activ-

TABLE 1. *Specificity of the cyclic AMP assay in samples purified or not by Dowex 50 column chromatography*[a]

Purification (column chromatography)	Phosphodiesterase treatment			
	No		Yes	
	Cyclic AMP level	Recovery (%)	Cyclic AMP level	Recovery (%)
Yes	24	35	4	2
	36	35	6	2
No	128	77	84	66
	120	81	100	65

[a] Mycelial samples (50 h of culture; 2.5 g, wet weight) of the St.L. 74 wild-type strain were extracted with 25 ml of cold 5% trichloroacetic acid containing 7.5 pmol of cyclic [^{3}H]AMP for 2 h. After centrifugation, the supernatants were acidified with 0.3 ml of 2 N HCl, extracted five times with 3 volumes of ethyl ether, and lyophilized. The residues were taken with 4.5 ml of water and neutralized with NaOH. Aliquots (0.45 ml) of these solutions were subjected to one of the four following treatments: (i) column chromatography, (ii) no treatment, or (iii) phosphodiesterase treatment, and the samples containing 4 mM $MgCl_2$ and 0.2 mg of beef heart phosphodiesterase per ml and adjusted to pH 8.5 with 2 M tris(hydroxymethyl)aminomethane (free base) were incubated for 2 h at 37 C. The reactions were stopped by heating at 100 C for 1 min. (iv) The fourth was phosphodiesterase treatment followed by column chromatography. After lyophilization, all samples were taken in 0.5 ml of 50 mM sodium acetate buffer, pH 4, and assayed for cyclic AMP. Other conditions were as those indicated in the text. Cyclic AMP levels are expressed as picomoles per milligram of protein after normalization for recoveries. Recoveries were estimated from the tritium radioactivity in the samples to be assayed. Given values correspond to two mycelial samples.

ity and were further incubated for 30 min at 34 C. Reactions were finally stopped by the addition of 0.02 ml of a solution containing 5 mM adenosine and 50 mM ethylenediaminetetraacetic acid. The mixtures received 0.4 ml of water and then were centrifuged, and the supernatants were passed through columns (0.4 by 4 cm) of AG 1 X4 resin, 200 to 400 mesh (chloride form) equilibrated with water. The columns were washed with 4 ml of water. Percolates and washings were saved, pooled, and counted for radioactivity by using Bray solution.

Analytical procedures. Protein was assayed by the method of Lowry et al. (11).

Materials. All chemicals were of analytical grade. Nutrients were purchased from Difco Laboratories. Cyclic [^{3}H]AMP (specific activity, 10 mCi/μmol) was purchased from New England Nuclear Corp. Specific beef heart cyclic nucleotide phosphodiesterase (catalog no. P-0134), cyclic AMP, dibutyryl cyclic AMP, fluoroacetate, and *p*-chloromercuribenzoate were obtained from Sigma Chemical Co., and Dowex 50WX8, 100 to 200 mesh, was from Serva. The resin was washed as described by Butcher et al. (2). AG 1 X4 resin, 200 to 400 mesh, was purchased from Bio-Rad Laboratories.

RESULTS

Adenylate cyclase activity and cyclic AMP levels in different *Neurospora* strains grown up to the stationary phase. As was reported previously (14, 16), *Neurospora* strains having a mutated *cr-1* allele exhibited low levels of adenylate cyclase activity. In Table 2 several *cr-1* mutants from different origins are compared with the wild type. The enzyme deficiency seemed to be specific for the *cr-1* mutation but not for the "crisp" morphology since mutants of the *cr-2* and *cr-3* loci did not show a reduced adenylate cyclase activity.

It is important to emphasize that all of these reported specific activities correspond to preparations obtained from mycelia cultured for 48 h in liquid medium. As will be shown later, adenylate cyclase specific activity changed with the age of the culture.

The data in Table 2 also demonstrate that the adenylate cyclase deficiency in *cr-1* strains was associated with reduced levels of endogenous cyclic AMP; in fact, cyclic AMP levels in mycelia from the St.L. 74 wild-type strain grown for about 40 h in complete medium were about 90 pmol/mg of protein; under identical conditions cyclic AMP levels in mycelia from *cr-1* mutants were 10 to 20 times lower.

In view of these results, we decided to investigate whether the "crisp" phenotype in *cr-1* mutants was a consequence of a reduced intracellular cyclic AMP level.

Cyclic AMP levels, adenylate cyclase, and cyclic nucleotide phosphodiesterase activities

TABLE 2. *Adenylate cyclase activity and cyclic AMP levels in different Neurospora strains*[a]

Strain[b]	*cr-1* allele	Morphological phenotype	Adenylate cyclase activity (pmol/min per mg of protein)	Cyclic AMP level (pmol/mg of protein)
St.L. 74	Wild type	Wild type	53.0	94
BAT 9-5	Wild type	Wild type	33.0	75
FGSC 2209	Wild type	Crisp[b]	53.0	49
FGSC 2329	Wild type	Crisp[b]	63.0	38
FGSC 810	Wild type	Osmotic	28.0	40
FGSC 488	B-123	Crisp	0.3	5
FGSC 826	B-74	Crisp	0.1	5
FGSC 806	R-2360	Crisp	0.5	12
FGSC 814	C-Ex-11-67	Crisp	0.1	5

[a] Mycelia obtained from liquid cultures performed in complete medium incubated at 28 C for 40 to 50 h were processed as indicated in the text.

[b] FGSC 2209 and 2329 strains are mutants of the *cr-2* and *cr-3* genes, respectively.

as a function of growth time. Cyclic AMP levels were measured in the wild-type St.L. 74 strain and in a *cr-1* mutant at different times of development in liquid medium. It was observed that the nucleotide level remained unchanged in the wild-type strain, except for a period elapsing between 35 and 45 h where a marked increase in endogenous cyclic AMP was observed. Figure 1A and B show the results of two experiments illustrating such a phenomenon. In these experiments each point corresponds to an individual culture; therefore, the increase in cyclic AMP levels observed in Fig. 1A at the time of 40 h could be attributable to an artifact resulting from abnormal culture conditions, defects in the manipulation of the sample, etc. However, after processing one culture every hour from 35 to 45 h (Fig. 1B), it became evident that such elevation was not artifactual. A full synchrony in the development of cultures performed in different flasks could not be expected. This fact might explain the sudden rises and falls of cyclic AMP levels observed in Fig. 1B. To avoid such irregularities, samples from a single large-scale culture were taken; two different cultures were run in parallel. Under these conditions two successive elevations of cyclic AMP levels were observed (Fig. 1C). Such a phenomenon resembles that previously described in *Dictyostelium* (6).

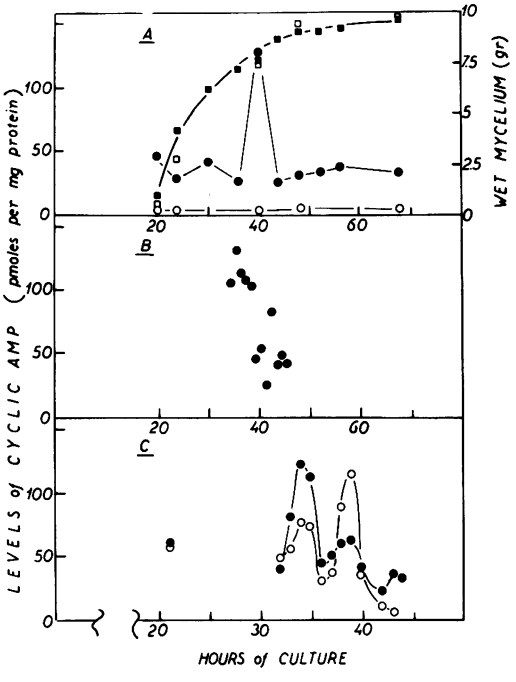

FIG. 1. *Cyclic AMP levels in a wild-type and a cr-1 mutant as a function of growth time. (A and B) Cultures were made in 1,000-ml Erlenmeyer flasks containing 200 ml of medium. A culture was sampled every 4 to 5 h from 20 to 68 h in (A) and every hour from 35 to 45 h in (B). Symbols: FGSC 488: ○, cyclic AMP level; □, wet mycelium weight. St.L. 74: ●, cyclic AMP level; ■, wet mycelium weight. (C) Two cultures of the St.L. 74 strain were run in parallel in 6,000-ml Erlenmeyer flasks containing 1,200 ml of medium. At the indicated times 40-ml aliquots were taken from each culture. All cultures were made in Vogel minimal medium and incubated at 30 C with agitation. Other conditions were as indicated in the text.*

Figure 1A also shows the results obtained with a *cr-1* mutant. It was evident that in this mutant cyclic AMP levels remained below 5 pmol/mg of protein throughout the entire period of growth studied.

Some attempts were made to correlate the changes of cyclic AMP levels during the growth of wild-type *Neurospora* with the activities of adenylate cyclase and phosphodiesterase assayed in "crude mycelial extracts." The results are shown in Fig. 2. Adenylate cyclase specific activity increased during the exponential phase of growth, reaching a maximum and then declining. Highest specific activities were coincident with the marked increase in cyclic AMP levels observed between 35 and 45 h of culture. On the other hand, phosphodiesterase specific activities measured at a low cyclic AMP concentration (0.25 μM) did not show major changes during growth.

On the other hand, adenylate cyclase specific activity in the *cr-1* mutant remained low throughout the entire growth period studied (Fig. 2).

Effects of dibutyryl cyclic AMP on *Neurospora* cultures in liquid or solid media. Figures 1A and 3A show that the total mycelial growth in liquid medium of cultures of wild-type or *cr-1* strains was roughly similar. In addition, the presence or absence of dibutyryl cyclic AMP in the growth medium did not exert a major influence on the growth pattern of these strains (Fig. 3A). These results indicate that, although an increase in the cyclic nucleotide levels was characteristic of wild-type *Neurospora* reaching the stationary phase of growth, cyclic AMP is not a factor affecting the growth rate of vegetative mycelia in liquid media.

The absence of effects of dibutyryl cyclic AMP on *Neurospora* growing in shaken liquid media cannot discount the possibility that the cyclic nucleotide could play a role in the control of the development of some specialized structures. In fact, in shaken liquid cultures only steps (i) and (ii) of the asexual life cycle (see above) take place.

Some recent observations performed by

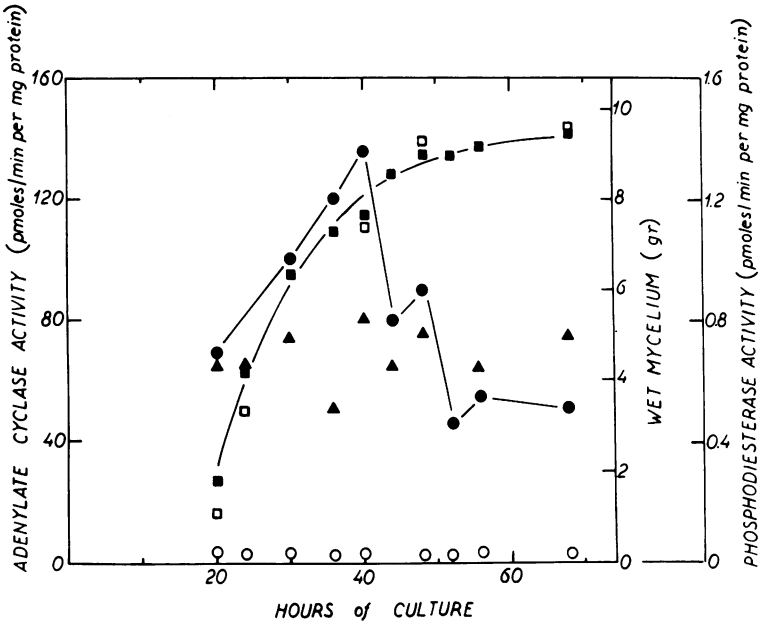

FIG. 2. *Adenylate cyclase and phosphodiesterase activities in a wild-type and a cr-1 mutant as a function of growth time. The data were obtained from the same experiment shown in Fig. 1A. Symbols: FGSC 488:* ○, *adenylate cyclase specific activity;* □, *wet mycelium weight. St.L. 74:* ●, *adenylate cyclase specific activity;* ■, *wet mycelium weight;* ▲, *phosphodiesterase specific activity. The enzymatic assays were as indicated in the text.*

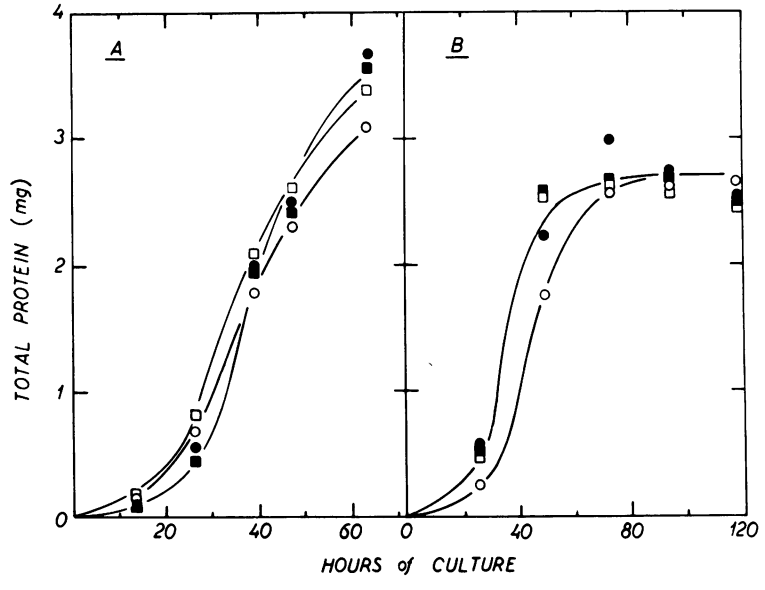

FIG. 3. *Effect of dibutyryl cyclic AMP on the growth of strains having wild-type or mutated cr-1 alleles. Growth was expressed in terms of the total protein of the cultures. (A) Liquid cultures of BAT 9-5 (closed symbols) and FGSC 488 (open symbols) were made in 10-ml Erlenmeyer flasks containing 2 ml of minimal medium supplemented with nicotinamide. Cultures were incubated at 27 C with agitation. (B) Cultures on dialysis tubing of St.L. 74 (closed symbols) and FGSC 488 (open symbols) were made on petri dishes (6 cm in diameter) containing 2 ml of solid Vogel minimal medium. The culture media were supplemented (squares) or not (circles) with 5 mM dibutyryl cyclic AMP. Other conditions were as indicated in the text.*

Harding on wild-type *Neurospora* grown in solid media containing cyclic AMP indicated that the nucleotide influences conidiation and pigment synthesis (7).

The addition of dibutyryl cyclic AMP to solid medium cultures of *cr-1* mutants decreased the accumulation of conidia and stimulated the elongation of hyphae. This was evident in cultures performed in petri dishes (Fig. 4). Under these conditions the cyclic nucleotide slightly inhibited the growth of wild-type cultures. The effect of the cyclic AMP derivative on the growth pattern of *cr-1* mutants was a consequence of a preferential stimulation of hyphae elongation but not of an increase in total mycelial mass. This was shown in cultures performed in petri dishes on cellophane sheets. Figure 3B shows that dibutyryl cyclic AMP did not modify the increase of cell protein in a *cr-1* mutant and slightly inhibited that of a wild-type strain.

The specific effect of dibutyryl cyclic AMP on hyphae elongation was measured by using the procedure of Ryan et al. (13). Table 3 shows that the mycelium elongation rate in strains having wild-type alleles was about 0.45 cm/h at 30 C. "Crisp" strains gave values below 0.19 cm/h, corresponding to *cr-1* mutants, with the lowest value being 0.015 to 0.057 cm/h. The presence of dibutyryl cyclic AMP on the agar cultures increases severalfold the rate of hyphae elongation of strains having any one of three different mutations of the *cr-1* locus and only slightly that of a *cr-2* mutant. On the other hand, the cyclic nucleotide did not influence the rate of hyphae elongation of a *cr-3* strain and slightly inhibited those of wild-type strains.

Effect of cyclic nucleotides and some metabolic inhibitors on standing liquid cultures. Standing liquid cultures offer the possibility of distinguishing between two modalities of mycelial growth: submerged below the liquid surface (step ii) and aerial, above this surface (step iii). Figure 5 shows that dibutyryl cyclic AMP stimulated aerial growth in four different *cr-1* strains. No effect of the cyclic nucleotide was observed on *cr-2* or *cr-3* mutants, whereas in a wild-type strain the compound slightly inhibited the development of aerial hyphae.

The effect of growing a wild-type or a *cr-1* mutant in the presence of cyclic nucleotides and/or different metabolic inhibitors was studied. The mycelium growing above and below the liquid surface was collected separately, and its protein content was measured. The results of two experiments of this type are shown in Table 4. The data indicate that the wild-type and the *cr-1* strains grow to the same extent in terms of their total protein content. The latter, however, formed exclusively submerged myce-

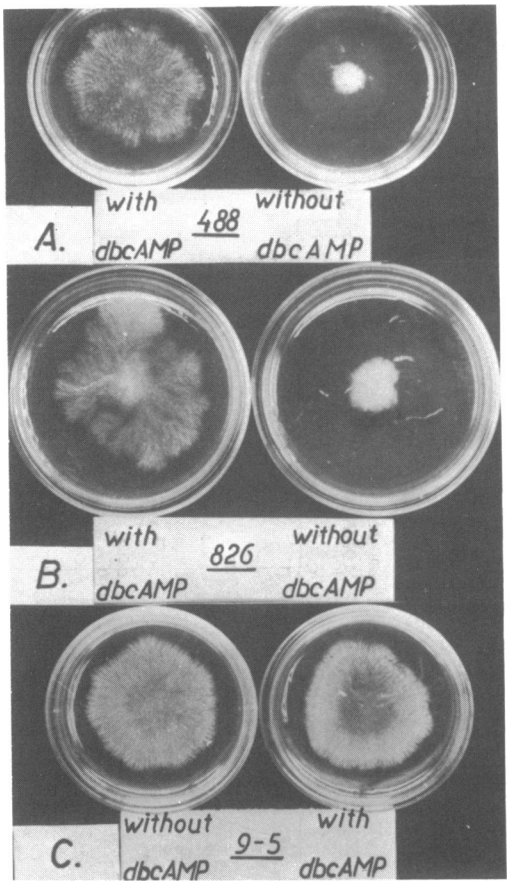

FIG. 4. *Effect of dibutyryl cyclic AMP on Neurospora morphology. The cultures were made in solid Vogel minimal medium supplemented with nicotinamide. In addition, the growth media were supplemented or not with 5 mM dibutyryl cyclic AMP. Incubations were carried out at 27 C for 72 h. (A, B, and C) FGSC 488, FGSC 826, and BAT 9-5 strains, respectively. Other conditions were as indicated in the text.*

TABLE 3. *Effect of dibutyryl cyclic AMP on the rate of mycelial elongation in solid medium*[a]

Strain	Type of crisp mutation	Allele	Mycelial elongation (cm/h)	
			No addition	Dibutyryl cyclic AMP (5 mM)
FGSC 806	*cr-1*	R-2360	0.07	0.33
FGSC 826	*cr-1*	B-74	0.015	0.38
FGSC 487	*cr-1*	B-123	0.045	0.20
FGSC 329	*cr-1*	B-123	0.057	0.21
FGSC 2209	*cr-2*	R-2445	0.19	0.30
FGSC 2329	*cr-3*	R-2509	0.14	0.18
FGSC 825	*cr-?*	B-180	0.19	0.19
St.L. 74	Wild type	Wild type	0.455	0.416
FGSC 810	*os-1*	Wild type	0.40	0.35

[a] Conditions were as indicated in the text.

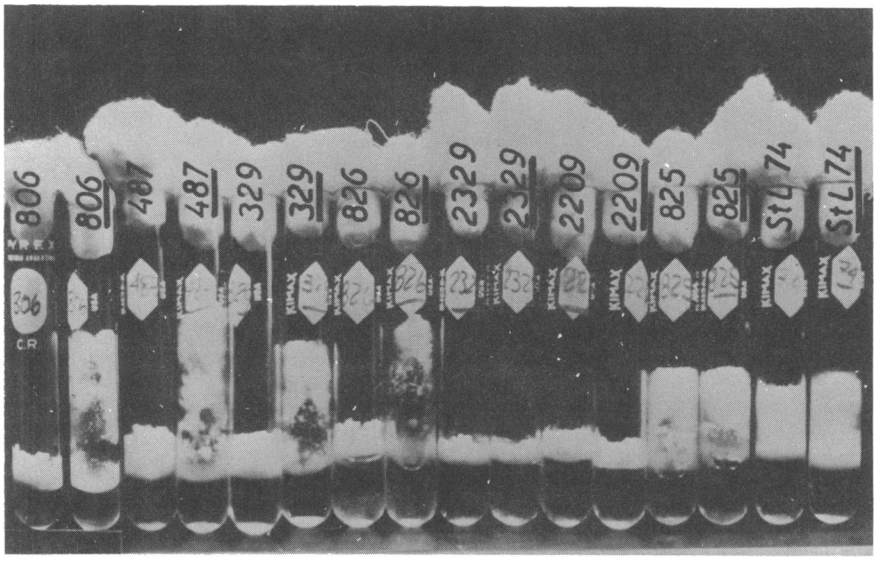

FIG. 5. *Effect of dibutyryl cyclic AMP on aerial hyphae formation in standing liquid cultures of the following Neurospora strains: FGSC 806, FGSC 487, FGSC 329, and FGSC 826 (cr-1 mutants); FGSC 2329 (cr-3 mutant); FGSC 2209 (cr-2 mutant); FGSC 825 (cr-? mutant); and St.L. 74 (wild type). Cultures were made in Vogel minimal medium. The right hand tube of each pair was supplemented with 5 mM dibutyryl cyclic AMP; arginine was added to FGSC 329 culture media. Incubations were carried out at 30 C during 4 days. Other conditions were as indicated in the text.*

lium. The addition of cyclic AMP or dibutyryl cyclic AMP did not influence largely the total growth in these strains, but clearly stimulated the formation of aerial hyphae in the *cr-1* mutant.

Evidence obtained by Weiss and Turian (21) indicates that some metabolic inhibitors which act at the level of the glycolytic pathway or the Krebs cycle cause alterations in the morphology of standing liquid cultures of *Neurospora* by affecting the formation of conidia and the distribution of aerial and submerged mycelium; similar effects were obtained by these authors by controlling the nitrogen source of the medium using the C (oxidative or conidiogenous medium) and M (glycolytic or mycelial medium) modifications of the Westergaard basal salt solution (17). A *cr-1* mutant and the wild-type strain were tested under these growth conditions (Table 4, experiment 1). The data demonstrate that the relative extent of submerged and aerial growth can be modified in the wild-type strain, as was expected from the observations of Turian. On the other hand, none of the compounds tested induced the formation of aerial hyphae in the *cr-1* mutant cultures. This indicates that the effect of the cyclic nucleotides in promoting the development of aerial mycelium in the *cr-1* strains was specific. In a similar experiment (Table 4, experiment 2), it was observed that the morphogenetic effects of dibutyryl cyclic AMP and cyclic AMP are counteracted by fluoroacetate and are somewhat enhanced by *p*-chloromercuribenzoate and iodoacetate.

DISCUSSION

Cyclic AMP levels in *Neurospora*. The marked and transient increase in the cyclic AMP level observed in wild-type *Neurospora* reaching the stationary phase suggests that the cyclic nucleotide acts as a starting signal for the initiation of some important events. The work of Urey (19) indicates that aerial hyphae can differentiate at the expense of vegetative mycelium, in the absence of a carbon source. On this basis it is reasonable to suppose that such differentiation results from a marked change in cyclic nucleotide levels. The sequence of events leading to these changes is not understood. Maximal adenylate cyclase levels were detected during the growth period coincident with the highest cyclic AMP levels. The nature of the primary signal responsible for these changes is also unknown; however, one of two facts could be a good alternative: exhaustion of some substance in the growth medium or, in turn, an increase in the level of some hormone-like compound.

Cyclic AMP and *Neurospora* morphology. The availability of adenylate cyclase-deficient strains facilitated the studies on the role of cyclic AMP in the control of the asexual life cycle in *Neurospora*. According to the evidence

TABLE 4. *Effect of cyclic nucleotides and some metabolic inhibitors on the development of submerged and aerial mycelia of cr-1 mutant and the wild-type strains grown in standing liquid cultures*

Growth medium[a]	Addition[b]	Mycelial growth after 72 h, measured as total protein content (mg)[c]			
		St.L. 74		FGSC 488	
		Submerged	Aerial	Submerged	Aerial
Expt 1					
Vogel minimal	None	0.96	0.85	1.95	
	5 mM dibutyryl cyclic AMP	0.96	0.68	0.48	0.92
	25 mM fluoroacetate	1.02	0.06	0.92	
	2×10^{-6} M *p*-chloromercuribenzoate	1.02	0.58	1.26	
	5 mM iodoacetate	1.20	0.30	0.96	
	10 mM sodium bisulfite	0.24		0.92	
	5 mM 3',5'-cyclic AMP	1.47	0.45	0.92	1.06
M modified Westergaard	None	0.30	0.10	0.50	
	25 mM fluoroacetate	0.58	0.06	0.65	
	2×10^{-6} M *p*-chloromercuribenzoate	0.06	0.10	0.24	
C modified Westergaard	None	0.27	0.17	0.78	
	25 mM fluoroacetate	0.72		0.72	
	2×10^{-6} M *p*-chloromercuribenzoate	0.34	0.25	0.78	
Expt 2					
Vogel minimal	None	0.87	1.13	1.45	
	25 mM fluoroacetate	0.97		0.78	
	2×10^{-6} M *p*-chloromercuribenzoate	0.90	1.14	1.09	
	5 mM iodoacetate	0.78	0.66	1.37	
Vogel minimal + 5 mM dibutyryl cyclic AMP	None	0.97	0.34	0.42	0.94
	25 mM fluoroacetate	0.94		0.85	
	2×10^{-6} M *p*-chloromercuribenzoate	0.84	0.77	0.58	0.80
	5 mM iodoacetate	1.02	0.60	0.80	0.46
Vogel minimal + 5 mM cyclic AMP	None	1.14	0.44	0.96	0.68
	25 mM fluoroacetate	1.06	0.18	0.96	
	2×10^{-6} M *p*-chloromercuribenzoate	0.96	0.97	0.96	1.02
	5 mM iodoacetate	0.89	0.34	0.66	0.61
Vogel minimal + 5 mM 5'-AMP	None	1.09	0.92	1.37	
	25 mM fluoroacetate	1.02		0.92	
	2×10^{-6} M *p*-chloromercuribenzoate	0.92	0.68	1.14	
	5 mM iodoacetate	0.96	0.36	1.20	

[a] Conidia were inoculated in test tubes (10 by 100 mm) containing 2 ml of minimal medium supplemented as indicated.

[b] Additions were made at the indicated final concentration 24 h after inoculation of the cultures.

[c] Submerged and aerial mycelia were collected separately and taken in 10% trichloroacetic acid. Total protein was determined in the trichloroacetic acid-insoluble residue after alkaline treatment, as described in the text.

depicted in this paper, cyclic AMP seems to stimulate the transition from step (ii) to step (iii), that is, from vegetative mycelia to conidiating aerial hyphae. At higher concentrations, however, the cyclic nucleotide inhibits the development of conidia (unpublished data).

Studies carried out in *Neurospora* (8, 21) and in *Mucor* (9, 15) have stressed the significance of the balance between fermentative and oxidative pathways for carbohydrate utilization in controlling fungal morphogenetic events. Our own observations using metabolic inhibitors tend to indicate that, whatever the site of action cyclic AMP may have in the morphogene-

sis of *Neurospora* or *Mucor*, its effect is conditioned by the inferred metabolic balance.

Some other effects of cyclic AMP on the life cycle events of *cr-1* mutants were observed by us and are now under study. Two of these are relevant: the first is the acceleration of conidia germination (16); the second is the cyclic AMP requirement for the formation of melanized protoperitecia (unpublished data). All of these facts would suggest that in *Neurospora* the nucleotide regulates the conversion from conidiogenesis to the development of sexual elements.

Specificity of the effects of cyclic AMP and dibutyryl cyclic AMP. As discussed above, morphological effects of cyclic AMP and its dibutyryl derivative were observed on cultures of *Neurospora cr-1* mutants performed basically under two different conditions, solid medium and standing liquid cultures. Maximal effects of these compounds were attained at rather high concentrations, 5 mM or higher (16). The possibility that other compounds could mimic the action of cyclic AMP or its dibutyryl derivative was explored in both growth conditions. To test this possibility, the effects of 5'-AMP or sodium butyrate, if any, were assayed on the cultures. The former compound did not show any effect in solid medium (14) or in standing liquid cultures (Table 4). Sodium butyrate slightly inhibited total mycelial growth in both solid and standing liquid cultures (14). In addition, under most of the circumstances dibutyryl cyclic AMP seemed to be more efficient than cyclic AMP in bringing about morphological changes in *Neurospora* cultures.

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