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Adenosine 3',5'-Cyclic Monophosphate and Morphology in *Neurospora crassa*: Drug-Induced Alterations

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Grown in liquid culture in the presence of a variety of structurally unrelated drugs, mycelia of wild-type *Neurospora* assume a colonial or semicolonial growth habit similar to that of known morphological mutants. Drugs that produce these morphological changes include atropine, theophylline, histamine, and several of the quinoline-containing antimalarials. Each of these drugs decrease the endogenous adenosine 3',5'-cyclic monophosphate (cAMP) concentration of mycelia as a result of their effect on the activity of adenyl cyclase, the cAMP-dependent phosphodiesterase, or both. The evidence indicates a relationship between the degree of morphological abnormality, the degree to which intracellular cAMP is reduced, and the action of the drugs on the adenyl cyclase and phosphodiesterase.

In *Neurospora crassa*, over 100 nonallelic genes are known to influence the mycelial growth habit (3). Mutations at these loci produce morphologically abnormal strains, and several are known to result in defective enzymes of carbohydrate metabolism (15). To accurately describe the adverse consequences of these enzymatic lesions on growth and morphology, an understanding of their pleiotropic effects is necessary (15). Evidence has accumulated suggesting that adenosine 3',5'-cyclic monophosphate (cAMP) has highly specialized functions related to growth and development in lower eukaryotic and prokaryotic organisms. Examples include dimorphism in *Mucor* (8) and aggregation and development in slime molds (1). cAMP therefore appeared to be a likely candidate to mediate at least some pleiotropic effects of the enzymatic lesions in *Neurospora* morphological mutants. In support of this idea, one mutant, *fr*, which carries a defective glucose 6-phosphate dehydrogenase, was found to have reduced cAMP levels (15). The positive response of *fr* to exogenous theophylline suggested the cAMP reduction has an adverse effect on the morphology and growth of this strain (15).

To assess more fully the role of cAMP in *Neurospora*, various drugs, including known effectors of the cAMP economy of other organisms, such as theophylline (13), histamine (13), and quinine (12), were tested for their ability to alter the morphology and cAMP system of *Neurospora* in liquid culture. Described here are the effects of these drugs on the morphology, cAMP levels, and activities of adenyl cyclase and phosphodiesterase of wild-type mycelia. The results are consistent with the idea that cAMP influences growth and shape in *Neurospora* and are also consistent with the previous results obtained with the cAMP-deficient *fr* mutant (15).

MATERIALS AND METHODS

[8-^{3}H]cAMP (20.8 Ci/mmol) and [8-^{3}H]adenosine 5'-triphosphate (ATP) (12 to 15 Ci/mmol) were obtained from Schwarz/Mann and purified by paper chromatography (6). Dowex AG 50W-x8 was purchased from Bio-Rad Laboratories. Sigma Chemical Co. was the source of drugs and other special chemicals.

Neurospora strains and growth conditions. The *Neurospora* wild-type RL3-8A, used throughout this study, was obtained from the Rockefeller stock collection. Mycelia were grown at 25 C on a rotatory shaker (120 to 130 cycles per min) in 125-ml flasks containing 50 ml of minimal medium (21) and 2% sucrose. Inocula consisted of 10^5 conidia per flask or small amounts of homogenized mycelia from a 24-h culture. Drugs were added to media prior to sterilization or were added to sterilized media with the aid of syringes equipped with Swinnex 0.45-μm filters. Cultures that contained light-sensitive compounds were wrapped in aluminum foil. Mycelia were harvested on a Büchner funnel covered with filter paper, washed with cold distilled water, and dried at 100 C overnight before weighing. Large-scale cultures for enzyme isolation were grown in 5-gallon (ca. 18.9-liter) Pyrex carboys with aeration (17). The resulting mycelia were stored at 5 C as a lyophilized powder.

Isolation and assay of cAMP. A sufficient number of cultures to yield between 50 and 200 mg (dry weight) of mycelia were harvested with suction on a Büchner funnel covered with filter paper. Care was

taken to avoid excessive drying or compression of the mycelia pad. The pad was immediately immersed in 25 ml of 5% trichloroacetic acid and allowed to stand for 10 min at 0 C. The time from the beginning of filtration until immersion in trichloroacetic acid was less than 30 s. The precipitate, obtained after centrifugation at 12,000 × *g* for 15 min, was transferred to a tared pan and weighed after drying overnight at 100 C. Each trichloroacetic acid supernatant fluid was extracted at 25 C five times with diethyl ether or until the pH of the aqueous phase was greater than 4.0. The extracted aqueous phase was brought to dryness by lyophilization or flash evaporation. After the residue was dissolved in 0.5 ml of 50 mM sodium acetate (pH 4.0), samples were stored in screw-cap vials at −20 C. Recovery of [^{3}H]cAMP added to trichloroacetic acid prior to extraction of mycelia was 85 to 90%.

cAMP was determined by the binding protein assay as described by Gilman (4). The cAMP-binding and inhibitor proteins were prepared from fresh bovine muscle (4) and stored in liquid nitrogen. Reaction mixtures, prepared in duplicate, contained in 0.05 ml of sodium acetate/acetic acid (pH 4.0): 1.0 pmol of [^{3}H]cAMP, sufficient binding protein (35 μg) to bind less than 30% of the ^{3}H-labeled nucleotide, and either buffer, cell extract, or authentic cAMP. Reactions were initiated by the addition of binding protein, and the mixture was incubated for 1 h on ice and then filtered through 24-mm cellulose ester (Millipore) filters (0.45 μm). The filters were washed with 10 ml of cold 20 mM potassium phosphate buffer (pH 6.0) and placed in vials containing 1.0 ml of Cellosolve, which dissolves the filters. A scintillation fluid of Cellosolve-toluene (1:3) plus fluors (4) was used; the counting efficiency was approximately 60% (7,000 counts/min per pmol). Blanks consisting of reaction mixtures minus the binding protein were 30 to 60 counts/min. Standard curves for the cAMP assay were constructed from duplicate assays of eight different concentrations of authentic cAMP ranging from 0.25 to 20.0 pmol. These curves, as reported by Gilman (4), are linear throughout the entire concentration range of cAMP. During routine analysis of the cAMP content of cellular extracts, duplicate assays containing 0.5, 2.0, and 10.0 pmol of authentic cAMP were included with each set of determinations as an internal control. cAMP levels are reported as nanomoles per gram (residual weight) of the trichloroacetic acid precipitate. This precipitate represents 80 to 85% of the dry weight of mycelia.

Radioimmunoassays (18) for cAMP were performed by Collaborative Research, Inc., Waltham, Mass. Mycelial extracts were prepared as described for the binding protein assay.

Isolation and assay of phosphodiesterase. The cAMP-dependent phosphodiesterase was partially purified and assayed as described previously (16). Activity is reported as micromoles of adenosine formed per 20 min at 30 C. All reported values are averages of duplicate assays.

Isolation and assay of adenyl cyclase. Lyophilized mycelia were ground in a mortar at 5 C with an equal weight of sea sand. The powdered mycelia, suspended in 20 volumes of 1 mM $NaHCO_3$ per g of tissue, were extracted by 20 min of stirring. Cell debris and unbroken cells were removed by centrifugation at 12,000 × *g* for 15 min. A crude membrane fraction was obtained by centrifugation of the supernatant at 105,000 × *g* for 90 min. The precipitate, resuspended in a minimum volume of 1 mM $NaHCO_3$, was used directly as the source of enzyme.

The assay procedure for the *Neurospora* adenyl cyclase was essentially that of Flawia and Torres (2). Assay mixtures contained in a final volume of 0.1 ml: 0.1 M piperazine-*N*,*N*′-bis(2-ethane-sulfonic acid) buffer, pH 6.35; 3 × 10^6 dpm of [8-^{3}H]ATP (purified); and either 2.5 mM ATP-Mn^{2+} or 0.25 mM ATP-Mn^{2+}. Reactions were initiated by the addition of enzyme (0.2 mg of protein). After 8 min at 37 C, the reaction was stopped by the addition of 0.1 ml of a solution containing 12.9 mM cAMP and 40 mM ATP(14), followed by boiling for 2 min. cAMP was purified by Dowex 50 chromatography and $BaSO_4$ precipitation (7). The amount of [^{3}H]cAMP formed was determined by liquid scintillation counting in Aquasol (New England Nuclear). Blanks for each experiment consisted of complete incubation mixtures stopped immediately after addition of the enzyme. Adenyl cyclase activities are expressed as nanomoles of cAMP formed per 8 min. All assays were carried out in duplicate.

For kinetic analyses, a constant amount of [^{3}H]ATP (2.3 × 10^6 to 3.3 × 10^6 dpm) was added to the incubation mixtures, and the amount of nonradioactive ATP was varied from 0.125 to 5.0 mM. The ATP/Mn^{2+} ratio was maintained at 1. All other ingredients and conditions were as described above.

Adenyl cyclase activity, under the stated conditions, is proportional to the concentration of protein and the incubation time. The reaction product, purified by Dowex 50 chromatography and $BaSO_4$ precipitation, had a mobility identical to that of authentic cAMP on paper chromatography in a 7:3 mixture of 95% ethanol-1 M ammonium acetate (6). Although adenyl cyclase preparations from *Neurospora* contain adenosine triphosphatase activity, as do similar preparations from other sources (13), the inclusion of an ATP-generating system (phosphoenol pyruvate-pyruvate kinase) in the assay mixture did not increase the rate of cAMP production or the V_{max} of the enzyme. Chromatography of whole reaction mixtures (no ATP-generating system) on paper indicated that less than 50% of the ATP was hydrolyzed during the 8-min incubation. These data, together with the finding that substrate inhibition of adenyl cyclase occurs at high ATP concentrations (>5.0 mM), suggest that ATP depletion is not a serious problem in the assay of the *Neurospora* enzyme and furthermore that the observed saturation kinetics of the enzyme are valid. However, the present study is a comparative one. In experiments where drug effects on the activity and kinetic parameters of adenyl cyclase were measured, controls without drugs were included. Therefore, in each case the reported actions of the drugs on adenyl cyclase are relative. Details of the assay and measurement of the kinetic parameters of adenyl cyclase from the slime variant of *Neurospora* were examined previ-

ously (2) and corroborated in large part in this laboratory with the wild-type enzyme.

Protein determination. Protein was determined by the method of Lowry et al. (9) with bovine serum albumin as a standard.

RESULTS

Drug effects on mycelial morphology and growth rate. The drugs listed in Table 1 are representative of a group of compounds that alter the morphology and reduce the growth rate of *Neurospora* mycelia in liquid shake cultures. In the absence of such agents, mycelia grow as a loose filamentous mat (Fig. 1A). Grown in their presence, cultures are phenotypically similar to those of known morphological mutants (3). The drugs fall into two classes based on their phenotypic and inhibitory effects. The first class, which includes atropine and the antimalarial drugs (quinine, quinidine, and chloroquine), produces marked morphological changes. As shown in Fig. 1B and C for quinidine and atropine, these drugs cause wild-type mycelia to assume a restricted colonial growth habit reminiscent of the colonial mutants (3) and also of the wild type grown in the presence of sorbose, a well-known paramorphogenic agent of *Neurospora* (20). Concentrations of atropine and the antimalarials that result in morphological abnormalities severely inhibit growth. This is illustrated in Fig. 2 for quinidine. Growth inhibition increases sharply at 1 mM quinidine. The transition from wild-type to colonial morphology as a function of drug concentration is also abrupt and is coincident with the greatly decreased growth rate. The organism assumes a colonial phenotype at quinidine levels ranging from 1 to 4 mM. Although the growth rate is reduced at these concentrations of quinidine, cultures continue to grow until the carbon source is exhausted and maintain an abnormal morphology. This behavior is typical of the other antimalarials and atropine. The lower concentration of each drug listed in Table 1 that produces morphological aberrations corresponds to the inflection point of the growth curve as with quinidine. The upper concentration is the maximum drug level that permitted a measurable amount of growth in 72 h.

The second group of drugs, typified by theophylline and histamine, produces a less restricted phenotype than the antimalarials or atropine. Colonies from theophylline cultures (Fig. 1D) have irregular shapes and are similar to those of the semicolonial or spreading morphological mutants (3). Growth inhibition by theophylline is substantially less than that produced by atropine or the antimalarials

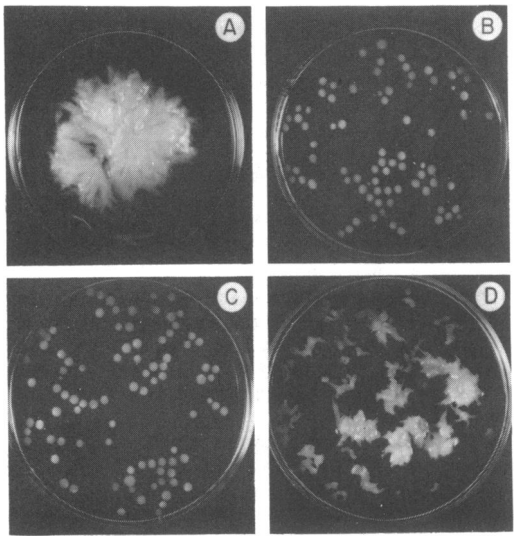

FIG. 1. *Drug-induced morphological changes of Neurospora mycelia grown in liquid shake cultures. (A) Minimal medium, 48 h of growth; (B) 2.5 mM quinidine, 72 h of growth; (C) 10 mM atropine, 72 h of growth; and (D) 10 mM theophylline, 50 h of growth. Because of the greater growth inhibition produced by quinidine and atropine (Table 1), cultures containing these compounds were grown for longer periods of time than theophylline cultures. Prior to photography, contents of cultures were transferred to 10-cm diameter petri plates.*

TABLE 1. *Effects of various drugs on the morphology and growth rate of Neurospora mycelia in liquid culture*

Drug	Concn[a] (mM)	Morphology[b]	% Reduction of growth rate[c]
Quinine	2.5 (1-5)	Colonial	85-95
Quinidine	2.5 (1-5)	Colonial	85-95
Chloroquine	2.5 (1-5)	Colonial	85-95
Atropine	10 (7.5-15)	Colonial	85-95
Theophylline	10 (10 or greater)	Semicolonial	10-15
Histamine	10	Semicolonial (transient)	

[a] Indicated in parentheses is the approximate concentration range of each drug that produces morphological aberrations.

[b] Quinine, quinidine, chloroquine, and atropine cultures were grown in the presence of the indicated concentration of the drug for 72 h, and the morphology was scored. The same procedure was followed for theophylline and histamine cultures, except growth was for 48 h.

[c] Expressed as percent dry weight (after 48 to 50 h of growth) of controls that contained no drug.

(Table 1). In addition, complete inhibition of growth was not obtained within the limits of theophylline solubility. The phenotypic effects of histamine (and also imidazole, not listed in Table 1) resemble those of theophylline but are transient, presumably because histamine is readily metabolized by the organism. During the first 2 to 3 days of growth, histamine cultures are morphologically abnormal and then gradually become more wild type in appearance. Growth inhibition by histamine is similar to that produced by theophylline although more difficult to measure because of the transitory nature of the histamine effects. For many of the comparative studies presented, drug concentrations that gave significant and reproducible morphological changes were chosen (Tables 1 and 2).

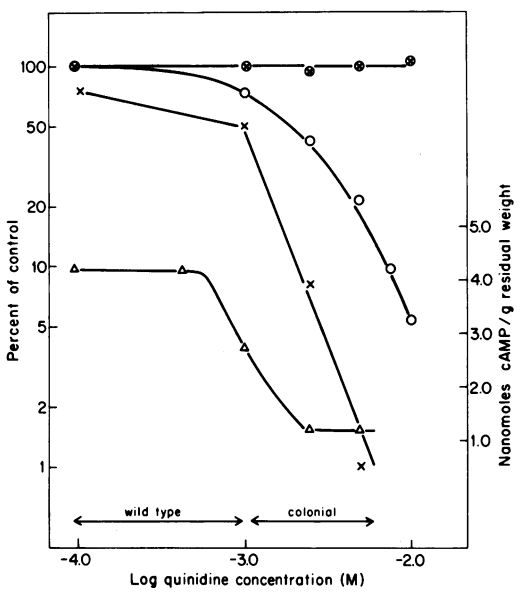

FIG. 2. *Growth inhibition, morphology, and endogenous cAMP of Neurospora mycelia and activities of the isolated adenyl cyclase and phosphodiesterase as a function of quinidine concentration. Morphologies of cultures grown in the presence and absence of quinidine were scored after 48 h of growth. At this time, contents of four identical cultures were pooled for dry weight determinations. Similarly, 48-h cultures were harvested and extracted with trichloroacetic acid for cAMP analyses. The appropriate concentrations of quinidine were added to standard assay mixtures of the two enzymes. Adenyl cyclase and phosphodiesterase were isolated from mycelia grown in minimal medium. Culture morphologies are indicated at the bottom of the graph. Symbols: ×, mycelial dry weight; ○, adenyl cyclase activity; ⊗, phosphodiesterase activity; Δ, endogenous cAMP. Mycelial dry weights and adenyl cyclase and phosphodiesterase activities are plotted as log percentage of controls that contained no drugs.*

Morphological differences between drug and control cultures are also evident at the microscopic level. In minimal medium, *Neurospora* mycelia are composed of long hyphal filaments which are branched at irregular intervals behind the growing tip (11). Drug-grown hyphae are similar in appearance to those of known morphological mutants as is the mycelial growth pattern. Drug-grown hyphae in general have irregular-shaped lateral walls and an increased amount of branching, especially at the hyphal tip.

The phenotypic effects of all drugs listed in Table 1 are reversible, as illustrated for quinidine (Fig. 3). Transfer of mycelial colonies from drug cultures to minimal media results in a resumption of a normal growth habit. This finding suggests that these drugs have no permanent effect on the organism.

cAMP levels and mycelial morphology. cAMP was estimated in mycelia grown in the presence and absence of the drugs listed in Table 1. Levels were determined at various points along the growth curve for both types of cultures. Only actively growing mycelia were samples; cultures in which the carbon source had been exhausted were excluded in these experiments. In general, cAMP was measured in duplicate 5- and 10-μl aliquots from two identical extracts (diluted 1:5) by the binding protein assay (4). Each data point, therefore, represents a minimum of eight determinations. A fivefold dilution of the concentrated mycelial extracts was sufficient in most cases to eliminate any interference from substances in the extract with the binding assay. This was determined by comparing cAMP levels before and after treatment of diluted extracts with Dowex 50. Passage of mycelial trichloroacetic acid extracts through Dowex 50 prior to cAMP determination removes the drugs (Table 1) as well as the bulk of nucleosides and nucleotides that compete with cAMP for the binding protein. Material determined to be cAMP by the binding assay was also examined for its sensitivity to cyclic nucleotide phosphodiesterase (Sigma Chemical Co.). Treatment with phosphodiesterase by the method of Makham and Sutherland (10) destroyed the cAMP-binding activity of extracts to the same extent (70 to 80%) as that of authentic cAMP. To further validate the binding reaction, known amounts of authentic cAMP were added to diluted extracts. In this manner, it could be shown that varying amounts of cAMP can be accurately measured in the presence of these extracts. From these observations, it was concluded that the material detected by the binding protein assay in the extracts is indeed cAMP.

TABLE 2. *Summary of the effects of various drugs on the cAMP system and morphology of Neurospora*

Addition	Concn (mM)	Intracellular cyclic AMP[a]	% Activity[b]			Morphology
			Adenyl cyclase[c]		Phosphodiesterase[d]	
			0.25 mM ATP	2.5 mM ATP		
None		4.2 ± 0.3	100	100	100	Wild type
Quinidine	2.5	1.2 ± 0.2	54	100	102	Colonial
Quinine	2.5	1.2 ± 0.2	60	102	105	Colonial
Atropine	10	1.2 ± 0.2	63	97	106	Colonial
Histamine	10	2.5 ± 0.2	100	105	180	Semicolonial (transient)
Chloroquine	2.5	1.2 ± 0.2	55	106	34	Colonial
Theophylline	10	2.5 ± 0.2	49	108	30	Semicolonial

[a] Expressed as nanomoles of cAMP per gram of residual weight.

[b] Data from representative experiments. Comparable results were obtained for each drug in at least two separate experiments with different enzyme preparations. Expressed as percent activity of controls that contained no drug.

[c] At 0.25 and 2.5 mM ATP the reaction is substrate limiting and in substrate excess, respectively (see Fig. 5). See footnote *b* for other comments.

[d] All assays were carried out at saturating levels of substrate (cAMP). See footnote *b* for other comments.

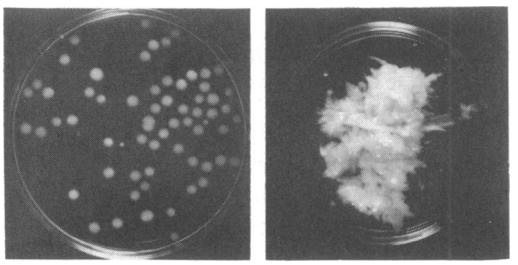

FIG. 3. *Morphology of a quinidine (2.5 mM) culture before and after transfer to minimal medium. (Left) Quinidine culture after 72 h of growth. (Right) Culture obtained by transfer of a single colony of the quinidine culture to minimal medium and an additional 48 h of growth. As shown, the morphology of the culture on transfer is indistinguishable from that of wild type (compare with Fig. 1A). Contents of cultures were emptied into petri plates (10 cm in diameter) for photography.*

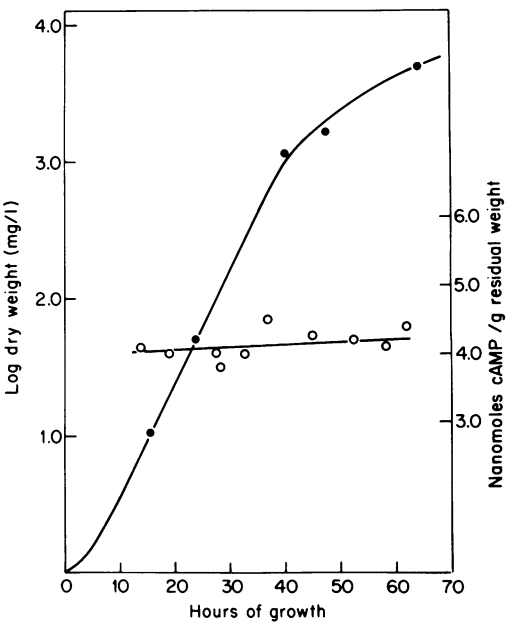

FIG. 4. *Intracellular cAMP levels of mycelia during log-phase growth in minimal medium. Dry weights (●) and cAMP (○) were estimated from duplicate cultures.*

Mycelia grown in minimal medium contain approximately 4.2 nmol of cAMP per g (dry weight). No cAMP was detected in culture supernatant fluids (with and without drugs). Spent media were found to contain phosphodiesterase-like activity. *Neurospora* may therefore release cAMP into the growth medium which is subsequently degraded. This possibility has not been ruled out.

cAMP levels of mycelia showed little change during log-phase growth (Fig. 4) as did the levels of mycelia grown in the presence of atropine, theophylline, and the antimalarials. Because of the transient phenotypic effects of histamine, endogenous cAMP was measured only when the morphology of the culture was most restricted. The cAMP contents of mycelia grown in the presence and absence of drugs are compared in Table 2. Typically a reduction in endogenous cAMP was observed with any one of the drugs in the culture medium. Atropine and the antimalarials reduce cAMP levels approxi-

mately 70 to 75% compared to 40 to 50% for histamine and theophylline. These results were confirmed by the radioimmunoassay for cAMP (18). The ratios of values obtained for control and drug cultures were found to be comparable to those determined by the binding protein assay, although the absolute amounts of cAMP detected by the radioimmunoassay were 30 to 40% lower in all cases.

A correlation between cAMP levels and the phenotype produced by the various drugs is suggested. Compounds that result in a restricted colonial growth (atropine and the antimalarials) decrease endogenous cAMP to a greater extent than do compounds such as histamine and theophylline that cause a less restricted semicolonial growth habit (Table 2). The relationship between cAMP levels and phenotype is also indicated from a comparison of both parameters within a concentration series of one drug. The change from a wild-type to a colonial or semicolonial morphology occurs within a narrow range of drug concentrations, as indicated above. However, mycelial morphologies are intermediate between that of wild type and the typical drug-induced phenotype at drug concentrations which correspond to the transition point of the phenotypic change. For example, at 1 mM quinidine cultures consist of loose fluffy colonies in contrast to the tight restricted growth observed at higher quinidine levels (Fig. 1). The cAMP levels at 1 mM quinidine are intermediate between those at 0.5 mM and 2.5 mM drug concentrations which produce no morphological abnormalities and a tight colonial growth habit, respectively (Fig. 2).

Exogenous cAMP, dibutyryl cAMP, and 8-bromo-cAMP were ineffective in overcoming the morphological effects of the drugs (Table 1). Mycelia grown in the presence of drugs (at concentrations given in Table 2) and various amounts of either cAMP or the two cAMP analogues (1 to 10 mM) were phenotypically identical to mycelia grown in the presence of the drug alone. Addition of [^{3}H]cAMP or [^{3}H]dibutyryl cAMP to media (with and without drugs) indicated that little if any of either [^{3}H]cyclic nucleotide penetrated actively growing mycelia. This result, however, is preliminary. More detailed uptake studies are necessary to determine the extent to which cAMP and cAMP analogues are transported by *Neurospora* under these growth conditions.

Phosphodiesterase and adenyl cyclase. No significant differences between the specific activities of phosphodiesterase and adenyl cyclase, the enzyme systems responsible for the breakdown and synthesis of cAMP, respectively, were observed in extracts of mycelia obtained from control and drug cultures. This suggests that the drugs (Table 1) do not result in altered levels of these enzymes. To determine whether drug-mediated inhibition or stimulation of these activities can account for the observed changes in endogenous cAMP levels of drug cultures, the effects of each drug on the isolated adenyl cyclase and phosphodiesterase were measured. Enzyme activities were compared in standard assay mixtures in the presence and absence of these various compounds. The drug concentrations employed were identical to those that reduce cAMP levels and produce morphological abnormalities in vivo. The results of these experiments are summarized in Table 2. Based on their effects on the two enzymes, the drugs can conveniently be divided into three groups (Table 2): those that inhibit adenyl cyclase, those that stimulate phosphodiesterase, and those that inhibit both enzymes.

Quinine, quinidine, and atropine belong to the first group; all three inhibit adenyl cyclase. Inhibition occurs at low substrate (ATP-Mn^{2+}) levels but is overcome at saturating concentrations of ATP-Mn^{2+} (Table 2 and Fig. 5A). Kinetic analysis suggested that these drugs decrease the substrate affinity of adenyl cyclase. The value for K is increased approximately threefold in the presence of these drugs, as shown for quinidine in Fig. 5B. Quinine, quinidine, and atropine, however, have no effect on phosphodiesterase activity regardless of the substrate (cAMP) concentration.

The second group of drugs, those that stimulate phosphodiesterase activity, includes histamine (and also imidazole [16]). Histamine (Table 2; reference 16) increases the V_{max} of the enzyme (data not shown) but has no effect on the substrate binding constant of phosphodiesterase or on the kinetic parameters of adenyl cyclase.

The third group of drugs, theophylline and chloroquine, inhibits both enzyme activities (Table 2). The effect of these compounds on adenyl cyclase is similar to that described for quinidine (Fig. 5) in that each increases the value for K two- to threefold. Phosphodiesterase inhibition by chloroquine and theophylline is more complex. As previously described (16), the *Neurospora* phosphodiesterase consists of two forms that have different cAMP-binding constants. Chloroquine and theophylline reduce the substrate affinity as well as the V_{max} of both activities. Velocity-versus-substrate curves in the presence and absence of 10 mM theophylline are shown in Fig. 6. For the sake of

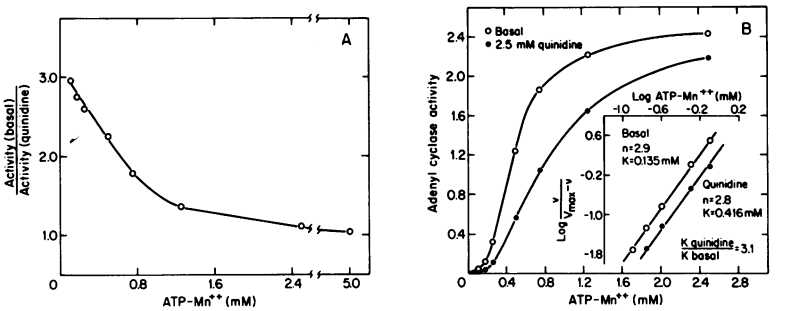

FIG. 5. *Effect of 2.5 mM quinidine on the kinetic parameters of adenyl cyclase. (A) Ratio of basal activity to that in 2.5 mM quinidine as a function of ATP-Mn^{2+} concentration. (B) Velocity of adenyl cyclase versus substrate concentration in the presence and absence of 2.5 mM quinidine. K is the equilibrium constant of the Hill equation (5) and n is the Hill coefficient. Note that the abscissa in (A) extends to 5.0 mM ATP-Mn^{2+} compared to only 2.5 mM ATP-Mn^{2+} for that in (B).*

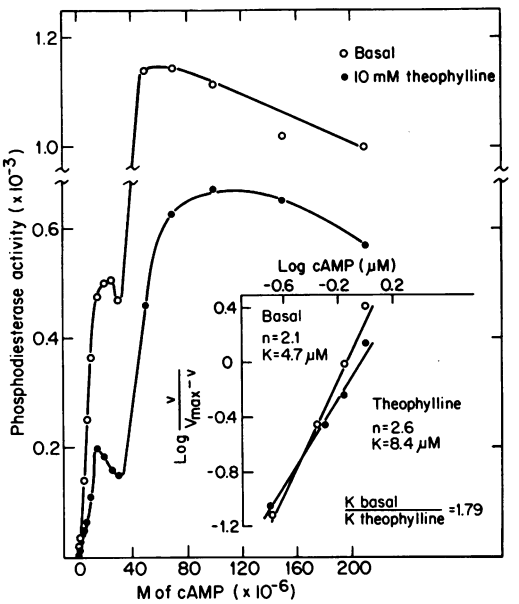

FIG. 6. *Effect of 10 mM theophylline on the kinetic parameters of phosphodiesterase. The inset is a Hill plot (5) of data from the lower portion of the substrate versus velocity curve. These points correspond to the phosphodiesterase activity with the higher cAMP-binding constant.*

simplicity, only the Hill plot for the activity with the higher cAMP-binding constant is presented. As shown for theophylline, both drugs increase the value for *K* approximately twofold and reduce the maximal activity 50 to 70%. The magnitude of the effects of theophylline and chloroquine on the phosphodiesterase with the lower cAMP-binding constant is similar.

Although chloroquine and theophylline inhibit adenyl cyclase and phosphodiesterase activities in vitro, several lines of evidence suggest that the in vivo actions of the two drugs are different. No significant uptake of [^{14}C]chloroquine by mycelia could be demonstrated at concentrations (2.5 mM) that produce morphological abnormalities. Chloroquine, therefore, may reduce cAMP levels in vivo by inhibiting only the adenyl cyclase since the enzyme is bound to the plasma membrane (2). On the other hand, comparative studies of the methylxanthines, including theophylline and caffeine, suggested that these compounds reduce adenyl cyclase and phosphodiesterase activities in vivo. Simultaneous reduction of both enzyme activities should decrease the rate of degradation as well as the rate of synthesis of cAMP. As a result, cAMP levels could either increase or decrease depending on the relative degree of inhibition of the two enzymes. Such is the case with the methylxanthines. The cAMP content of mycelia grown in the presence of 10 mM caffeine, in contrast to that in theophylline, is elevated from 4.2 to 6.6 nmol/g of dry weight (compare with the values for theophylline in Table 2).

The theophylline-mediated reduction in cAMP levels of mycelia in liquid cultures is unusual. Theophylline increases the intracellular cAMP levels of most organisms (13). The observed reduction in the cAMP content of *Neurospora* cannot be attributed to an artifact, such as theophylline interference of cAMP assays, since the drug is removed from trichloroacetic acid extracts by passage through Dowex 50. Theophylline inhibits the *Neurospora* adenyl cyclase in addition to the phosphodiesterase, and the drug has no effect on the synthesis of either enzyme. Under these conditions, a net reduction in cAMP content can occur only if the inhibition of adenyl cyclase is the predominant effect in vivo. The differential effects of theophylline on mycelia grown in liquid and solid media suggest this possibility.

Addition of theophylline (10 mM) to agar cultures of *Neurospora* results in increased (40%) cAMP levels, which is the opposite effect obtained in liquid culture. Kinetic studies similar to those shown in Fig. 6 indicated that theophylline inhibits the phosphodiesterase isolated from agar cultures in the same manner as the enzyme from liquid cultures. However, the adenyl cyclase from agar cultures is insensitive to the drug. The effects of theophylline on mycelial cAMP levels, which are reversed by growth conditions, correlate with the sensitivity of adenyl cyclase to the methylxanthine.

Significant inhibition (or stimulation of phosphodiesterase in the case of histamine) of adenyl cyclase and phosphodiesterase occurs only at drug (Table 1) concentrations that result in reduced cAMP levels and morphological abnormalities in vivo. This is evident from measurements of adenyl cyclase and phosphodiesterase activities as a function of drug concentration as illustrated in Fig. 2 for quinidine. Quinidine (0.1 to 10 mM), as stated above, has no effect on phosphodiesterase activity. Significant inhibition of adenyl cyclase, in contrast, begins at approximately 1 mM quinidine and increases sharply at higher drug concentrations. Comparable levels of quinidine in vivo produce a reduced cAMP content and an abnormal morphology (Fig. 2). For example, at 2.5 mM quinidine, the concentration chosen for kinetic studies of adenyl cyclase, mycelial cAMP levels are 25% of control cultures without the drug, adenyl cyclase activity is decreased 45 to 55%, and cultures exhibit a colonial morphology. Similar correlations between drug concentration and endogenous cAMP, morphology, and the activities of adenyl cyclase or phosphodiesterase were observed for all compounds listed in Table 1. With theophylline, inhibition of both enzymes paralleled cAMP reductions and morphological changes.

DISCUSSION

The results presented in this paper demonstrate that a variety of structurally unrelated drugs (Table 1) including the quinoline-containing antimalarials (quinine, quinidine, and chloroquine), atropine, histamine, and theophylline alter the morphology of *Neurospora* in liquid shake cultures (Fig. 1). The morphological aberrations induced by these compounds are similar to those caused by known mutations in *Neurospora* (3). For example, the restricted growth produced by atropine and the antimalarials resembles that of the colonial strains, whereas theophylline and histamine cultures are phenotypically similar to the semicolonial or spreading colonial mutants. Although several of these compounds are structurally related to known mutagens, under the conditions employed in this study, each results only in phenocopies of morphological mutants (Fig. 3).

In addition to their morphogenic properties, each of these drugs reduces intracellular cAMP levels (Table 2 and Fig. 2). The data indicate a relationship between the degree of morphological abnormality and the degree to which endogenous cAMP is reduced. A comparison of the effects of individual drugs indicated that atropine and the antimalarials reduce cAMP levels to a greater extent and produce more striking morphological abnormalities than do theophylline and histamine. Likewise, within a concentration series of a single drug, there is a mutual relation between the degree of morphological restriction and the decrease in cAMP levels. Where the drug in question acts only on adenyl cyclase or phosphodiesterase, reductions in endogenous cAMP levels can be further correlated with the in vitro action of the drug on one of these two enzymes. Quinine, quinidine, and atropine lower the substrate-binding constant (increase in K) of adenyl cyclase. Presumably endogenous cAMP is reduced by decreasing the rate of cAMP synthesis. On the other hand, histamine apparently increases the rate of cAMP breakdown by activating (increase in V_{max}) phosphodiesterase. Because theophylline and chloroquine inhibit both enzymes in vitro, the correlation between the action of the drugs on the two enzymes and cAMP levels is less clear at first glance. The in vivo effects of theophylline and chloroquine on the phenotype and cAMP levels of the organism differ quantitatively (Table 2). Chloroquine results in a colonial morphology and reduces cAMP levels 70 to 75% in contrast to the semicolonial morphology and 40 to 50% reduction of endogenous cAMP produced by theophylline. Compounds within the other two groups of drugs influence the activity of only one of the two enzymes and have identical consequences on the mycelial phenotype and cAMP levels. Therefore the behavior of chloroquine and theophylline is unusual in this respect.

Chloroquine does not penetrate mycelia (see Results) to any significant extent. It is plausible that in vivo the drug inhibits only the adenyl cyclase which is located on the plasma membrane. This assumption seems reasonable since the effects of chloroquine on morphology and endogenous cAMP are similar to those of the other antimalarials that affect adenyl cyclase but not phosphodiesterase (Table 2). The fact that theophylline lowers, and caffeine increases,

endogenous cAMP suggests that the methylxanthines reduce the activities of adenyl cyclase and phosphodiesterase in mycelia grown in liquid culture. The dissimilar effects of caffeine and theophylline are assumed to reflect in vivo differences in the relative inhibition of the two enzymes. The nonidentical effects of theophylline on mycelia grown in liquid and solid media substantiate this idea and indicate that the sensitivity of adenyl cyclase to theophylline determines the action of the drug on *Neurospora*. This point can also be demonstrated with the *fr* strain, a morphological mutant that has a reduced cAMP content (15). Growth of *fr* on solid media supplemented with 10 mM theophylline increases the mycelial growth rate threefold, increases endogenous cAMP to a wild-type value, and produces a partial reversion of mutant phenotype toward a more wild-type state (15). In contrast, theophylline supplementation of *fr* growing in liquid cultures results in none of these positive effects. Inhibition of the *fr* phosphodiesterase by theophylline is similar to that of the wild-type enzyme. In addition, the sensitivity of the *fr* adenyl cyclase to theophylline is influenced by the growth conditions in an identical fashion to that of the wild-type enzyme (W. A. Scott, unpublished data). The dependence of the theophylline sensitivity of the membrane adenyl cyclase on growth conditions also presents the interesting possibility that the state of the membrane enzyme is different in mycelia grown in liquid and solid media.

The inability of exogenous cAMP or cAMP analogues to block the phenotypic effects of the drugs is expected, if indeed cyclic nucleotides fail to penetrate mycelia as suggested by the preliminary uptake studies. These compounds are also ineffective (Scott, unpublished data) in reversing the phenotype of *fr*, although exogenous theophylline results in some morphological reversion (15). Based on these observations, it seems likely that actively growing *Neurospora* is impermeable to cAMP and its derivatives.

As a whole the results presented here are consistent with the idea that cAMP plays a role in the determination of *Neurospora* morphology. This conclusion is based on the observed relationship among morphology, cAMP levels, and the capacity of the drugs to inhibit the synthesis or stimulate the breakdown of cAMP. Taking into account that these drugs may have pleiotropic effects in vivo and that the mechanism of action of several is unknown, the fact that similar results are obtained by either stimulation of phosphodiesterase activity or inhibition of adenyl cyclase nevertheless is striking. The idea that alterations of cAMP economy influence *Neurospora* morphology does not imply that morphological abnormalities occur only as a consequence of changes in the cAMP system. The fact that the cAMP content of most *Neurospora* morphological mutants is normal (Scott, unpublished data) clearly indicates this is not the case. Considering the role of cAMP in regulating carbohydrate metabolism (19) and the influence of carbohydrate metabolism on *Neurospora* morphology (15), it is not surprising that compounds which either reduce or increase endogenous cAMP result in morphological abnormalities. The finding that exogenous theophylline partially restores the phenotype and cAMP levels of *fr* growing on solid medium complements the results of this study. The proposed relationship between morphology and cAMP would appear to hold whether cAMP levels are artificially decreased in wild type, thereby producing morphological abnormalities, or are artificially increased in cAMP-deficient strains, thereby producing some phenotypic reversion.

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LITERATURE CITED

1. Bonner, J. T. 1973. Aggregation and differentiation in the cellular slime molds. Annu. Rev. Microbiol. **25**:75–92.
2. Flawia, M. M., and H. N. Torres. 1972. Adenylate cyclase activity in *Neurospora crassa* I. General properties. J. Biol. Chem. **247**:6873–6879.
3. Garnjobst, L., and E. L. Tatum. 1967. A survey of new morphological mutants in *Neurospora crassa*. Genetics **57**:579–604.
4. Gilman, A. G. 1970. A protein binding assay for adenosine 3',5'-cyclic monophosphate. Proc. Natl. Acad. Sci. U.S.A. **67**:305–312.
5. Hill, A. J. 1913. XLVII. The combinations of haemoglobin with oxygen and with carbon monoxide. I. Biochem. J. **7**:471–480.
6. Hirata, M., and O. Haysishi. 1967. Adenyl cyclase of *Brevibacterium liquaefaciens*. Biochim. Biophys. Acta **149**:1–11.
7. Krishna, G., B. Weiss, and B. B. Brodie. 1968. A simple, sensitive method for the assay of adenyl cyclase. J. Pharmacol. Exp. Ther. **163**:379–385.
8. Larsen, A. F., and P. S. Sypherd. 1974. Cyclic adenosine 3',5'-monophosphate and morphogenesis in *Mucor racemosus*. J. Bacteriol. **117**:432–438.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
10. Makham, R. S., and E. W. Sutherland. 1965. Adenosine 3',5'-cyclic phosphate in *Escherichia coli*. J. Biol. Chem. **240**:1309–1314.
11. Murray, J. C., and A. M. Srb. 1962. The morphology and genetics of wild type and seven morphological mutants of *Neurospora crassa*. Can. J. Bot. **40**:337–349.
12. Price, S. 1973. Phosphodiesterase in tongue epithelium: activation by bitter taste stimuli. Nature (London) **241**:54–55.

13. Robinson, G. A., R. W. Butcher, and E. W. Sutherland. 1971. Cyclic AMP, p. 342-389. Academic Press Inc., New York.
14. Rodbell, M. 1967. Metabolism of fat cells. V. Preparation of "ghosts" and their properties; adenyl cyclase and other enzymes. J. Biol. Chem. **212**:5744-5750.
15. Scott, W. A., N. C. Mishra, and E. L. Tatum. 1973. Biochemical genetics of morphogenesis in *Neurospora*. Brookhaven Symp. Biol. **25**:1-18.
16. Scott, W. A., and B. Solomon. 1973. Cyclic 3',5'-AMP phosphodiesterase of *Neurospora crassa*. Biochem. Biophys. Res. Commun. **53**:1024-1030.
17. Scott, W. A., and E. L. Tatum. 1971. Purification and partial characterization of glucose 6-phosphate dehydrogenase from *Neurospora crassa*. J. Biol. Chem. **246**:6347-6352.
18. Steiner, A. L., D. M. Knipis, R. Utiger, and C. W. Parker. 1969. Radioimmunoassay for the measurement of adenosine 3',5'-cyclic phosphate. Proc. Natl. Acad. Sci. U.S.A. **64**:367-373.
19. Sutherland, E. W., and G. A. Robinson. 1969. The role of cyclic AMP in the control of carbohydrate metabolism. Diabetes **18**:797-819.
20. Tatum, E. L., R. W. Barratt, and V. M. Cutter, Jr. 1949. Chemical induction of colonial paramorphs in *Neurospora* and *Syncephalastrum*. Science **109**:509-511.
21. Vogel, H. J. 1964. Distribution of lysine pathways among fungi: evolutionary implications. Am. Nat. **98**:435-446.