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Adenosine 3′:5′-cyclic monophosphate deficiency in *Neurospora crassa*

(adenylate cyclase/theophylline/morphology/linolenic acid)

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ABSTRACT **Depending on growth conditions, the adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels of the *fr* mutant, a morphologically aberrant strain of *Neurospora crassa*, are reduced 2- to 5-fold. By taking advantage of the differences in phenotype of *fr* in liquid and agar cultures and the positive response of *fr* grown on solid support to exogenous theophylline, a relationship between the degree of morphological abnormality and intracellular cyclic AMP levels of the mutant is observed. Progressive restoration of the *fr* phenotype toward a normal state is paralleled by increases in cyclic nucleotide content. Striking differences in the sedimentation and thermal characteristics of the *fr* and wild-type adenylate cyclases [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] are observed. Approximately 50% of the normal activity sediments at 105,000 × *g* compared to 5% of the mutant enzyme. In addition, the overall stability of the *fr* adenylate cyclase is significantly decreased and its rate of inactivation at 37° in the absence of substrate is 10-fold greater than the wild-type adenylate cyclase. Arrhenius plots also indicated that the Q_{10} (increase in rate per 10° temperature increase) and the temperature of maximal activity of the *fr* enzyme are reduced. Supplementation of *fr* agar cultures with linolenic acid results in an elevated cyclic AMP content and a wild-type-like morphology similar to that obtained with inhibitors of phosphodiesterase (3′:5′-cyclic AMP 5′-nucleotidohydrolase, EC 3.1.4.17). An increased thermostability of the *fr* adenylate cyclase occurs on linolenate enrichment of the mutant. It is concluded that the cyclic AMP deficiency is at least partially responsible for the *fr* phenotype and that this reduction results from a membrane defect that affects adenylate cyclase function.**

Mutations affecting certain steps of carbohydrate metabolism in *Neurospora crassa* lead to morphological abnormalities (1). Recent studies have shown that a knowledge of the enzymatic defects is in itself insufficient for understanding the phenotypic changes of these strains. To fully appreciate the adverse consequences of such mutations on the growth habit of the organism, an accurate description of the pleiotropic effects of the enzymatic lesions is also necessary (1). Various drugs that reduce the intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels of wild-type *Neurospora* produce phenocopies of morphological mutants (2). Because of this finding and the fact that cyclic AMP is a ubiquitous regulatory agent with specialized functions related to growth and development in lower organisms (3–5), it seemed plausible that imbalances of cyclic AMP economy may mediate at least some of the pleiotropic effects of the mutations in the morphologically altered strains of *Neurospora*. In this paper *fr*, a morphological mutant of *Neurospora*, is shown to have reduced intracellular cyclic AMP levels and an altered adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1], which presumably results from a defective plasma membrane. Results obtained by manipulation of growth conditions and growth of *fr* on theophylline-supplemented media are consistent with the idea that cyclic AMP influences growth and shape in *Neurospora*.

Abbreviations: cyclic AMP, adenosine 3′:5′-cyclic monophosphate; ICI 63,197, 2-amino-6-methyl-5-oxo-4-*n*-propyl-4,5-dihydro-*s*-triazolo[1,5-*a*]pyrimidine.

MATERIALS AND METHODS

Cultures. The *Neurospora* strains RL3-8A (wild-type), B110 (*fr*), and alcon (*fl*) were obtained from the Rockefeller University collection. Mycelia were grown at 30° in minimal medium (6) containing 1% glucose or 1% sucrose. Conditions for liquid cultures were described previously (2). Cultures grown on solid support contained 1.5% agar in 10 cm diameter petri plates. To facilitate the harvesting of mycelia, the agar surfaces were overlaid with sterile, water-permeable cellophane (Dupont) prior to inoculation. *Neurospora* mycelia readily grow on the cellophane and can be collected by scraping the cellophane surface with a spatula. Growth rates were estimated in growth tubes (7) on agar media. Where appropriate, phosphodiesterase inhibitors were introduced into media prior to sterilization (theophylline) or were added to sterilized media with the aid of syringes equipped with Swinnex 0.45 μm filters [aminophylline and 2-amino-6-methyl-5-oxo-4-*n*-propyl-4,5-dihydro-*s*-triazolo[1,5-*a*]pyrimidine (ICI 63,197)]. The levels of drugs employed (see *Results*) represent the minimum concentrations that produce phenotypic reversion and maximal increase in the growth rate of *fr*. Linolenic acid was added to sterile agar media maintained at 60°. Provided the media were swirled, no lipid droplets were visible after solidification of the agar.

The acondial strain alcon (*fl*) was employed as the wild-type strain for agar cultures. *Neurospora* produces abundant conidia on solid media, which interfere with cyclic AMP and adenylate cyclase determinations. Apparently conidia contain low levels of cyclic AMP and adenylate cyclase compared to mycelia. Whether this is actually the case, however, remains to be determined, since incomplete extraction and breakage of the spores may contribute to the low values. In control experiments the cyclic AMP levels and properties of the adenylate cyclase from young wild-type (RL3-8A) cultures were determined prior to conidiation. Both parameters were indistinguishable from those of the alcon strain.

Cyclic AMP Determinations. Cyclic AMP levels of *Neurospora* were estimated as described (2).

Phosphodiesterase and Adenylate Cyclase. The preparation and assay of the *Neurospora* cyclic AMP phosphodiesterase (3′:5′-cyclic AMP 5′-nucleotidohydrolase, EC 3.1.4.17) and adenylate cyclase have been described in detail (2, 8). Heat inactivation of adenylate cyclase was measured by incubating the enzyme at 37° for the appropriate time intervals. The enzyme was immediately cooled in ice and assayed under standard conditions (2). The temperature dependence of adenylate cyclase activity was monitored by varying the assay tempera-

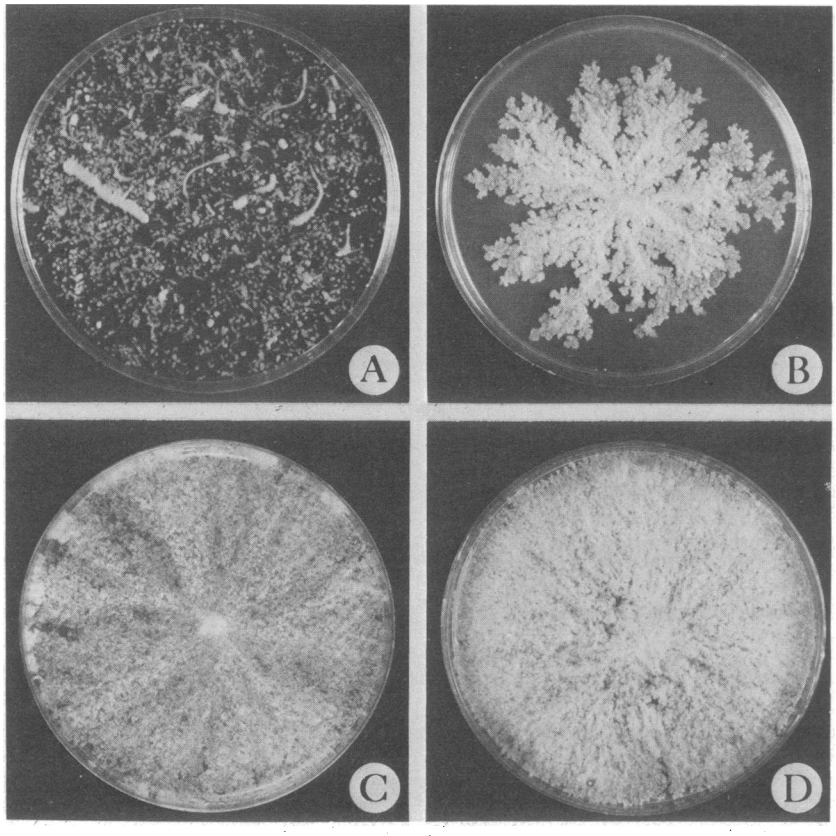

FIG. 1. Influence of culture conditions on the morphology of *fr*. (A) Liquid culture, minimal medium. Contents of the culture were transferred to a petri plate prior to photography. (B) Agar culture, minimal medium. (C) Agar culture supplemented with 10 mM theophylline. (D) Agar culture containing 0.1 mM linolenic acid. All cultures were grown at 30° for 3–5 days.

ture. Assays were conducted at temperatures ranging from 0° to 50° with the aid of a Lauda circulating water bath. The variation in temperature at any given setting was less than 0.1°. Data are presented as Arrhenius plots.

Protein. Protein was determined by the method of Lowry *et al.* (9).

RESULTS

Morphology and Cyclic AMP Levels of the *fr* Strain. The phenotype of the *fr* mutant is dependent on growth conditions. The loose filamentous appearance of wild type in liquid cultures (2) contrasts with the growth of *fr* as tight pellets (Fig. 1A), a behavior reminiscent of the most restricted class of *Neurospora* morphological mutants, the colonials (10). On agar media *fr* exhibits a spreading growth habit (Fig. 1B) characteristic of the less restricted spreading colonial mutants that tend to cover the surface of the medium during growth, but retain a morphology distinct from that of wild type (10). Thus, the growth habit of *fr* can be manipulated between two distinct phenotypes by a simple change in growth conditions. This morphological difference in liquid and agar cultures is atypical; morphological mutants of *Neurospora* in general exhibit a similar degree of phenotypic abnormality regardless of the method of growth.

Cyclic AMP measurements indicated that the cyclic nucleotide content of *fr* is reduced and that the extent of the reduction is correlated with the degree of phenotypic abnormality. Wild-type mycelia grown in liquid cultures contain 4.2 nmol of cyclic AMP per g dry weight (2). Under the same culture conditions, the intracellular cyclic AMP level of the *fr* strain is 0.9 nmol per g dry weight or approximately one-fifth the wild-type value (Table 1). The cyclic AMP level of *fr* grown on agar media increases to 50% of the wild-type content (Table 1). Of interest is the finding that the cyclic nucleotide levels of both strains grown on solid support are substantially higher than in mycelia from liquid media (Table 1). The 11-fold increase in the *fr* cyclic AMP content on shifting from liquid to agar cultures, compared to the 4.8-fold increase for wild type, accounts for the greater relative cyclic AMP levels of *fr* cultures grown on agar. The increase of the mutant cyclic AMP content relative to that of wild type, therefore, parallels the reversion of the *fr* morphology (Fig. 1).

Phosphodiesterase Inhibitors. To test whether the cyclic AMP deficiency contributes to the morphological abnormalities of *fr*, agar cultures were supplemented with phosphodiesterase inhibitors. Exogenous theophylline (10 mM), aminophylline (5 mM), or ICI 63,197 (3 mM, ref. 11) increase the *fr* cyclic AMP content to 20 nmol per g dry weight, a level comparable to that of wild type grown on glucose alone (Table 1). Characteristically, the phenotypes of *fr* cultures containing phosphodiesterase inhibitors more closely resemble those of wild type than do the phenotypes of cultures grown on minimal medium (compare Fig. 1B with Fig. 1C). The supplemented cultures tend to be confluent and lack the "snowflake" appearance of the mutant. Phosphodiesterase inhibitors also have a beneficial effect on the growth of *fr*. Hyphal extension proceeds at 1.4 cm/day on minimal medium. In contrast, on media supplemented with theophylline or the other phosphodiesterase inhibitors, the growth rate of *fr* is increased 3-fold (4.2 cm/day). Exogenous cyclic AMP and the dibutyryl and 8-bromo derivatives at millimolar concentrations have none of the positive effects of the phosphodiesterase inhibitors on *fr*, presumably because of the inability of actively growing *Neurospora* to

Table 1. Influence of growth conditions on the cyclic AMP levels and adenylate cyclase of *Neurospora crassa*

Strain	Growth conditions	Cyclic AMP level*		Adenylate cyclase					
				Half-life at 37°‡		Activity in 105,000 × *g* precipitate§		Temperature of maximal activity¶ (°C)	
		Value (nmol/g)	Ratio†	Value (min)	Ratio†	Value (%)	Ratio†	Value	Difference
Wild type	Liquid	4.2	4.8	10	10	50	10	44.1	6.7
fr	Liquid	0.9		1		5		37.4	
Wild type	Agar	20	2.0	10	10	31	1.4	38.8	1.4
fr	Agar	10		1		23		37.4	
Wild type	Agar linolenate	20	1.0	2.5	1.0	25	1.0	37.0	0.1
fr	Agar linolenate	20		2.5		24		36.9	

* Values are given as nmol per g dry weight of mycelium.
† Ratio of the wild-type to *fr* values.
‡ Time for 50% inactivation of the enzyme in the 105,000 × *g* precipitate (see Fig. 2).
§ Percent of the total activity of the crude extract.
¶ Data from representative experiments are given. Repetitive determinations on different preparations of each enzyme varied less than 2%.

transport phosphorylated compounds (2). As a result, it has not been possible to demonstrate the cyclic AMP–morphology relationship directly by supplementation means. Comparable levels (above) of phosphodiesterase inhibitors have no significant effect on the growth rate (11 cm/day) or morphology of wild type, and produce but slight increases in cyclic AMP levels of the strain.

Adenylate Cyclase. To determine the molecular basis for the cyclic AMP reduction of *fr*, the properties of the mutant and wild-type adenylate cyclases and phosphodiesterases were compared. The phosphodiesterases of the two strains isolated from liquid and agar cultures are indistinguishable by the criteria outlined below. The *fr* adenylate cyclase, however, is heat labile and has unusual sedimentation characteristics. In the absence of substrate, the mutant activity from liquid cultures decays with a half-life of 1.0 min at 37° (Fig. 2 and Table 1). Inactivation kinetics of the wild-type adenylate cyclase under the same conditions are biphasic and 50% loss of activity occurs in 9–11 min. The inactivation rates of mixtures are intermediate between those of the mutant and wild-type enzymes (Fig. 2). The relative half-life of each mixture is that expected based on the proportions of the two preparations. In addition, no synergism with respect to activity is apparent in that the activities of the mixtures are the sum of the contributions of the individual enzymes.

In addition to being thermolabile, the *fr* adenylate cyclase is significantly less stable overall than the wild-type enzyme. Complete inactivation of the isolated *fr* activity occurs within 20 hr at 5°, whereas the wild-type enzyme retains full activity for 3–4 days. Measurements of adenylate cyclase activity at different temperatures indicated that the maximal temperature of the *fr* enzyme from liquid cultures is reduced 7° (Table 1 and Fig. 3) and its Q_{10} (the relative increase in reaction rate for a 10° temperature increase) is decreased 40%. The wild-type and *fr* adenylate cyclases also differ with respect to sedimentation properties. The activity of the normal enzyme distributes equally between the 105,000 × *g* supernatant and precipitate, whereas only 5% of the *fr* activity sediments under the same conditions (Table 1). As a result, the specific activity of the *fr* enzyme in the 105,000 × *g* precipitate is one-tenth that of wild type, since the total activity (under conditions of substrate saturation) and protein content of both strains are comparable. Substitution of Tris·HCl (pH 7.4) for $NaHCO_3$ as the isolation buffer, variation of the ionic strength from 1 to 100 mM, increased centrifugation time from 90 to 120 min, or the presence of divalent cations (2.5 mM) has no influence on the sedimentation characteristics of the enzyme from either strain.

The properties of the *fr* and wild-type adenylate cyclases isolated from liquid and agar cultures were compared to determine whether the properties of the *fr* enzyme parallel its functional capacity as indicated by the cyclic AMP levels of the mutant. As is shown in Table 1 and Fig. 3, the sedimentation behavior and maximum temperature of activity of the *fr* enzyme from agar cultures closely resemble those of the wild-type adenylate cyclase. Nevertheless, differences between the two adenylate cyclases from agar cultures exist. The half-life of the *fr* adenylate cyclase is one-tenth that of the normal enzyme and is therefore unaffected by the change in culture conditions. Furthermore, the Arrhenius plots of the mutant and wild-type activities clearly differ (Fig. 3). Although the Q_{10} of the mutant enzyme from agar cultures is increased (Fig. 3), the profile of the wild-type adenylate cyclase contains a break point at 15° not evident for the *fr* activity.

Polyunsaturated Fatty Acids. Agar cultures of *fr* are known to respond to Tween 80. The growth rate and phenotype of cultures grown on Tween-supplemented media resemble those on media supplemented with phosphodiesterase inhibitors. The *fr* strain is reported to have a reduced content of linolenic acid (12). The mutant therefore may utilize Tween as a source of polyunsaturated fatty acids to overcome its linolenic acid deficiency. The specificity of the response of *fr* to pure fatty acids supports this idea. Exogenous polyunsaturated fatty acids (0.03–0.12 mM) increase the mutant growth rate approximately 3-fold and produce a wild-type phenotype (Fig. 1D). Qualitatively, linolenic acid is somewhat more effective than linoleic acid. Other polyunsaturated fatty acids (γ-linolenic and arachidonic acids) produce phenotypic changes similar to those of linolenic acid. No response, however, is obtained with similar concentrations of fatty acids that contain a single double bond (oleic or palmitoleic acids) or with saturated fatty acids of chain length C_{16} to C_{20}. Direct evidence for the uptake and incorporation of polyunsaturated fatty acids by *fr* cultures has been demonstrated by employing radioactive fatty acids.

Supplementation of agar cultures of *fr* with polyunsaturated

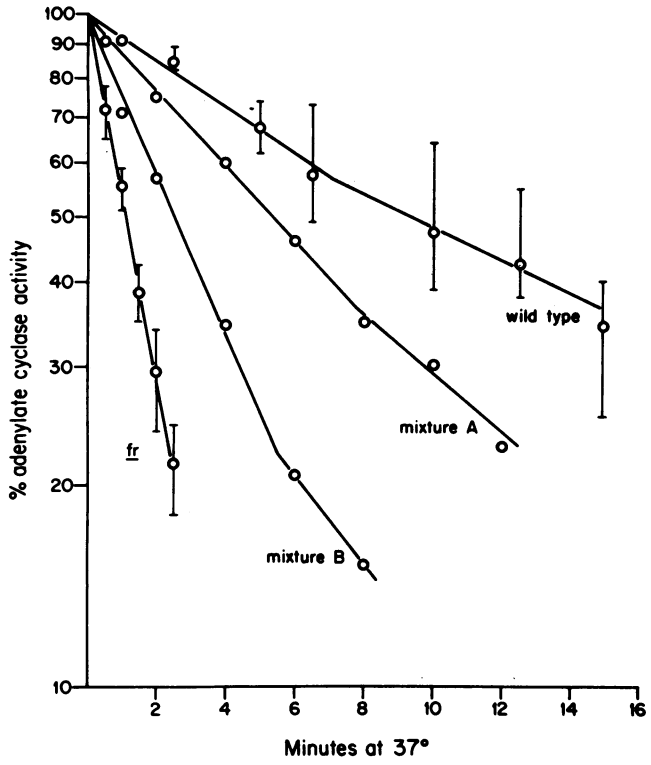

FIG. 2. Time course of adenylate cyclase inactivation at 37°. The wild-type and *fr* preparations were diluted to 20 mg of protein per ml with 1 mM $NaHCO_3$. The proportions of each enzyme in the mixtures were calculated on a protein basis with a final protein concentration of 20 mg/ml. Mixture A (33% *fr* plus 67% wild type) contained 6.6 mg and 13.4 mg of *fr* and wild-type protein, respectively. The mixture catalyzed the formation of 3200 cpm of cyclic [^{3}H]AMP per 8 min. Assays of the separate enzyme preparations indicated that 13.4 mg of wild-type protein formed 3300 cpm of cyclic AMP and 6.6 mg of the *fr* protein 187 cpm. In mixture B (67% of *fr* plus 33% wild type), the amounts of protein of the two preparations were reversed. Mixture B formed 1315 cpm of cyclic AMP per 8 min. The *fr* protein (13.4 mg) represented 402 cpm or 28% of the activity and the wild-type protein (6.6 mg) the remainder or 1047 cpm (72%). All data are plotted as percent residual activity. The *fr* and wild-type adenylate cyclase curves are presented as the mean and standard deviations from five different enzyme preparations of each strain grown in liquid cultures. The mixing experiments represent duplicate determinations. The source of the enzyme was the 105,000 × *g* precipitate of mycelial extracts.

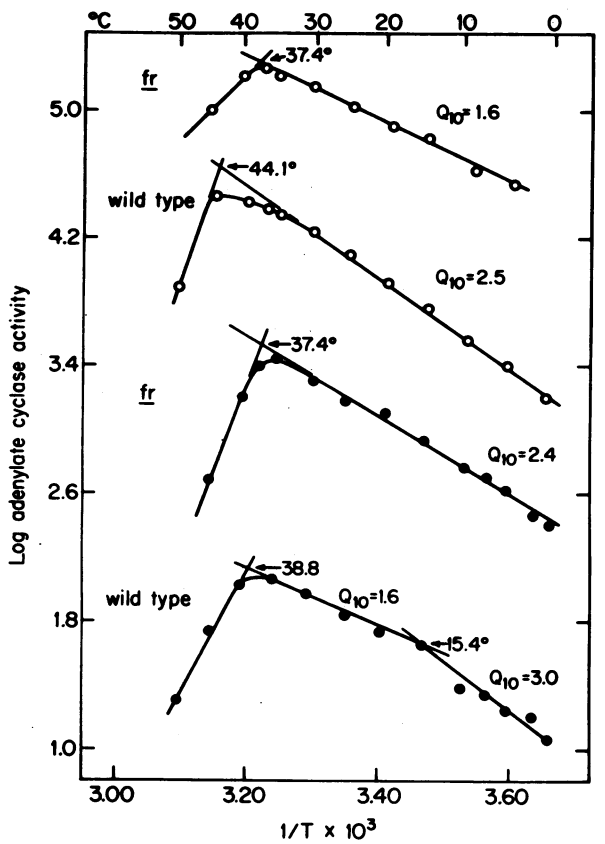

FIG. 3. Arrhenius plots for the membrane-associated adenylate cyclase isolated from liquid (○) and agar (●) cultures. The activity units are arbitrary so that the four profiles can be compared. The source of the enzyme was the 105,000 × *g* precipitate of mycelial extracts.

fatty acids increases the cyclic AMP content of the mutant to normal levels, as is shown for linolenic acid in Table 1. Thus, the effects of polyunsaturated fatty acids on cyclic AMP levels of *fr* are also similar to those produced by phosphodiesterase inhibitors. The data in Table 1 indicate that supplementation of *Neurospora* cultures with polyunsaturated fatty acids has a direct effect on the adenylate cyclase. Compared to that from unsupplemented cultures, the stability of the *fr* activity from agar cultures containing 0.12 mM linolenate is increased 2.5-fold at 37°. Furthermore, the wild-type adenylate cyclase from linolenate-containing cultures closely resembles the *fr* enzyme. The amount of wild-type activity in the 105,000 × *g* precipitate is decreased from 31% to 25% and, in addition, its half-life at 37° is reduced from 10 to 2.5 min. Fatty acid supplementation does not alter the Arrhenius profile of the enzyme from either strain. As a result, the only discernible difference between the adenylate cyclases from polyunsaturated fatty acid containing cultures is the presence of an inflection point at 15° in plots of the normal activity (see Fig. 3). Supplementation with polyunsaturated fatty acids has no significant effect on the growth rate, phenotype, or cyclic AMP content of wild type. Clearly, supplementation of wild type with polyunsaturated fatty acids has no gross effect on the *in vivo* function of the adenylate cyclase, but results in an activity similar to that of preparations from *fr* grown in the presence of polyunsaturates.

DISCUSSION

Previous work has shown that cyclic AMP deficiencies in *Neurospora* result in morphological abnormalities (2). Structurally unrelated drugs, including the methylxanthines, reduce the cyclic AMP levels of mycelia by inhibiting the adenylate cyclase or by stimulating the phosphodiesterase. Drugs that result in a colonial morphology lower cyclic AMP levels 70–75% compared to 40–50% for those that produce a spreading colonial morphology. The present experiments show that the *fr* strain, a morphological mutant, is cyclic AMP deficient. By manipulation of culture conditions, the phenotype and cyclic AMP content of the mutant can be partially restored (Table 1 and Fig. 1). The increase in the cyclic AMP levels of *fr*, relative to those of wild type, parallels the phenotypic changes from a restricted colonial growth in liquid cultures to a spreading colonial phenotype on solid support. The cyclic AMP level associated with a particular phenotype (colonial or spreading colonial) is in excellent agreement with the comparable drug-induced morphology of wild type. Supplementation of *fr* grown on solid support with phosphodiesterase inhibitors produces a further reversion in morphology to a nearly wild-

type state (Fig. 1) and an elevation in cyclic AMP content to normal levels. Thus, a positive correlation between cyclic AMP levels and the mutant phenotype is evident. These results together with the drug studies suggest that the intracellular cyclic AMP concentration is a major determinant of cell shape in *Neurospora*.

The abnormal properties of the membrane-bound adenylate cyclase suggest a reduced rate of synthesis as a plausible explanation for the reduced cyclic AMP levels of *fr*. The finding that the sedimentation and thermal characteristics of the *fr* adenylate cyclase are partially restored in agar cultures, the same conditions that result in increased mutant cyclic AMP levels, indicates these properties can provide an indirect measure of the functional status of the enzyme. Since the mutant response to polyunsaturated fatty acids is similar to that obtained with phosphodiesterase inhibitors, and supplementation with polyunsaturated fatty acids produces measurable changes in the thermal stability of both the wild-type and *fr* adenylate cyclases, it is thought that the *fr* adenylate cyclase defect is a reflection of an altered membrane structure that may result from a partial deficiency of the mutant for linolenic acid (12). A reduced affinity of the membrane site (13) for the enzyme or an increased fragility of the membrane are likely explanations for the nonsedimentation of the *fr* adenylate cyclase.

Experiments from several laboratories have indicated that lipids are necessary for the normal functioning of the mammalian adenylate cyclase (14–16). Several observations of this study suggest a determining role of membrane structure in the adenylate cyclase function of *Neurospora*. Arrhenius plots of the enzyme from agar cultures of wild type contain an inflection point at 15° not evident in profiles of the enzyme from liquid cultures (Fig. 3). Environmental factors are known to affect the fatty acid composition of lower eukaryotes (17). Discontinuities in Arrhenius plots of adenylate cyclase have been reported (18, 19), but their causes remain unknown. Transition temperatures and/or activation energies of several membrane enzymes including the $(Na^+ + K^+)$-ATPase (20) are altered by the degree of lipid unsaturation. The decreased Q_{10} of the *fr* adenylate cyclase from liquid cultures is consistent with a reduction in polyunsaturated fatty acyl groups intimately associated with the enzyme complex. The increase in this parameter of the mutant activity, together with the appearance of the transition temperature of the wild-type adenylate cyclase in agar cultures and the increased cyclic AMP content of both strains, suggest (*a*) the enzyme exists in a more fluid environment in agar than liquid cultures, and (*b*) this leads to an increased enzyme function. Clearly, the relationship of adenylate cyclase and membrane lipid composition requires further investigation. The restoration of the *fr* adenylate cyclase properties and function produced by the changes in growth conditions and fatty acid supplementation, however, strongly argue for a defective membrane rather than a defective enzyme.

The reported linolenate deficiency of *fr* is presumed to result from a defective glucose-6-phosphate (Glc6P) dehydrogenase (1) via a reduction in pyridine nucleotide content (12) produced by the decreased activity of the pentose phosphate shunt. Two other unlinked Glc6P dehydrogenase mutants, *col-2* and *bal*, are deficient in both metabolites, but neither strain responds to exogenous polyunsaturated fatty acids or has reduced cyclic AMP levels. Not all morphological abnormalities in *Neurospora*, therefore, are mediated by the cyclic AMP system, as noted previously (2). The morphological and metabolic consequences of the fatty acid reduction in *bal* and *col-2* are unknown.

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