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The Effect of Cyclic AMP on Morphogenesis and Enzyme Accumulation in *Dictyostelium discoideum*

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When 3′,5′-cyclic AMP is applied externally to migrating slugs and to cell aggregates during particular stages of morphogenesis, it disturbs the normal polarity and dominance relationships among the cells in a specific manner. It also interferes with the developmental programs of at least two enzymes that are needed for fruit construction.

INTRODUCTION

At three different stages of their development, the cell populations of *Dictyostelium discoideum* display the kind of positionally coordinated interactions that fall under the rubric of a morphogenetic field (Child, 1941). These are briefly outlined below.

1. *Aggregation.* Excited by a chemotactic stimulus (Bonner, 1947) the dispersed cells stream radially inward toward aggregative centers, thereby facilitating direct cell contacts, mutual adhesion, and as a consequence, the formation of the characteristic multicellular aggregates. At high population density, potential centers of aggregation compete with one another, the extent of the competition depending on environmental parameters, cell size distributions, and genetic constitution (Sussman and Sussman, 1961).

2. *Slug migration.* The migrating slug represents a transient alternative to fruiting body construction (Newell *et al.*, 1969). The cells at the anterior tip of the slug direct the migration and the more posterior cells follow their lead. The relative positions of the cells within the slug are fixed by the original order of their entry into the aggregate and remain fixed during migration. These positions determine the ultimate fates of the cells in the mature fruiting body, i.e., whether they become spores, stalk cells, or basal disc cells. Appropriate segmentation and fusion experiments have demonstrated the regulative nature of these polarities (Raper, 1941; Farnsworth and Wolpert, 1971).

3. *Fruiting body construction.* The morphogenetic movements during this stage resemble a fountain with reversed flow. The cells move up the outside of the growing stalk. The more apical ones extrude cellulose to build up the outer cylindrical sheath of the stalk and then, as they reach the growing tip, enter its confines and acquire the parenchymatous appearance typical of stalk cells. The more basal cells transform into spores when they reach the top. These vertical oriented, polar relationships are maintained even though the cells moving through the apex are constantly changing as they enter the stalk, transform into vacuolated stalk cells, and are replaced at the apex by the cells immediately behind them (Raper and Fennell, 1952).

The original formulation of the morphogenetic field concept supposed that the maintenance of polarity and dominance relationships depended on gradients and local discontinuities in the concentrations of diffusible extracellular substances (Child, 1941) and this continues to be a reasonable model (Crick, 1970). As already noted, in the case of *D. discoideum* aggregation, it is a fact. Furthermore, recent

data indicate that 3′,5′—cyclic AMP (c-AMP) at very low concentrations can provide this chemotactic stimulus (Konijn *et al.*, 1967).

It had been supposed many years ago that the chemotactic agent active during aggregation might also serve to control polarity at the later morphogenetic stages (Bonner, 1947, 1949). However, casual observations of slug migration and fruit construction in the presence of c-AMP failed to provide evidence that c-AMP exerts any specific effects on these activities other than to inhibit them totally at high concentrations (Chassy *et al.*, 1969; Konijn *et al.*, 1968). A more systematic examination, the results of which are reported here, has revealed that c-AMP does interfere in a specific manner with polarity and dominance relationships during slug migration and fruit construction and has a significant effect on the program of enzyme synthesis that accompanies these processes.

MATERIALS AND METHODS

Culture and experimental conditions. *Dictyostelium discoideum* strain NC-4 (haploid) was grown in association with a prototrophic strain of *Aerobacter aerogenes* on NZCase agar (Newell and Sussman, 1970). Near the end of the exponential growth phase, cells were harvested from the plates, washed three times in cold water by centrifugation and suspended in water at a density of 2×10^8 cells/ml.

When development of fruiting bodies was required, aliquots containing 10^8 cells were dispensed on 42 mm Whatman No. 50 filters resting on absorbent pads in 60-mm plastic petri dishes. The pads were saturated with a well buffered salt solution (lower pad solution: 0.04 *M* phosphate buffer pH 6.4 containing per liter 1.5 g KCl, 0.5 g $MgCl_2 \cdot 6H_2O$, and 0.5 g streptomycin sulfate (Newell *et al.*, 1969). This solution will be referred to as LPS. Incubation was in the dark at 22°C. Under these conditions the migrating slug stage is precluded and approximately 1000 aggregates formed and developed synchronously into fruiting bodies during a 24-hour period.

Migrating slugs were prepared according to the procedure of Ellingson *et al.* (1971). Aliquots of 3×10^7 cells were dispensed in a thin line along one edge of a 12-cm² dish containing 2% nonnutrient unbuffered agar. Black Millipore filters were placed on the agar about 2 cm from the line of cells. The dishes were wrapped in aluminum foil, and a small horizontal slit was cut opposite the line of cells to provide a light gradient. Cell aggregates developed almost exclusively into slugs which migrated toward the light and onto the filters at an average speed of 2 mm/hour.

When these migrating slugs were exposed to overhead light (fluorescent ceiling fixtures), they immediately stopped migration and constructed fruiting bodies over the next 7 hours (Newell *et al.*, 1969). The synchrony of this reponse was improved by transferring the slugs to an absorbent pad saturated with LPS just prior to exposure to overhead light.

For studies of the effect of cyclic-AMP and other chemicals on slime mold development, filters containing slugs or aggregates were transferred to absorbent pads saturated with a buffered solution of the desired chemical. Incubations were at 22°C with overhead light.

Enzyme assays. Developing cell aggregates were harvested from the filters in cold 0.1 *M* Tricine pH 7.5, containing 20% (v/v) glycerol (Telser and Sussman, 1971). Cells were lysed by addition of the detergent Cemulsol NPT-12 at a final concentration of 0.15%.

Enzyme assays were performed immediately after preparation of the extracts. The assays for UDP-galactose 4-epimerase (EC 5.1.3.2) and UDP-glucose pyrophosphorylase (EC 2.7.7.9) have been described previously (Telser and Sussman, 1971; Newell and Sussman, 1969).

These enzymes will be referred to as the epimerase and pyrophosphorylase.

Cyclic AMP phosphodiesterase was assayed by a modification of the procedure of Reidel and Gerisch (1971). This assay measures the decrease in absorbance at 265 nm upon conversion of 5′-adenylic acid to inosine monophosphoric acid by the enzyme 5′-adenylate deaminase (Rossomando and Sussman, manuscript in preparation). A Gilford model 2400 recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a Haake circulating water bath was used for all enzyme assays.

Proteins were determined by the method of Lowry *et al.* (1951) using a crystalline bovine serum albumin standard.

Tricine (*N*-tris(hydroxymethyl)methylglycine) was purchased from Calbiochem, Los Angeles, California; streptomycin sulfate from Nutritional Biochemical Corp., Cleveland, Ohio; black Millipore filters 47 mm (AABP004700) from the Millipore Corp., Bedford, Massachusetts. Absorbent pads are Whatman No. 17, W. & R. Balston, Ltd., England. The nonionic detergent Cemulsol NPT-12 was a gift of Melle-Bezons, 29 Rue Emile Zola, 95 Benzons, France. Adenosine 3′,5′—cyclic monophosphoric acid (sodium salt) and 5′-adenylate deaminase were obtained from Sigma Chemical Co., St. Louis, Missouri. All other chemicals were of reagent grade.

RESULTS

Effect of c-AMP on Fruiting Body Construction

Washed cells were dispensed on filter paper circles and incubated as described in Methods. Aggregation of the cells and fruit construction occurred in the normal temporal sequence (Newell *et al.*, 1969) as shown in Fig. 1. At intervals, filters containing the aggregates were switched to fresh pads saturated with a solution containing 3 m*M* c-AMP in LPS and the subsequent morphogenetic events were observed during further incubation at 22°C.

Striking morphogenetic changes were observed when the aggregates were exposed to c-AMP between 16 and 18 hours' development. The subsequent development of the aggregates was altered, terminating in aberrant structures such as those illustrated in Fig. 2A,B. The height of the stalk was only about one-third that of untreated controls although the stalk cells were vacuolated, had rigid walls, and appeared to be normal. The apical spore mass was either absent or greatly diminished in size and most of the cells were concentrated in a mound at the base. When the cells were exposed to c-AMP at 16 hours, the apical structure remained in an immature state as shown in Fig. 2A. When the exposure started at 18 hours, the stalk, though still reduced in height and in the number of component cells, attained a more normal terminal appearance (Fig. 2B). In both cases as many as 10% of the cells at the base were found to have transformed into apparently normal spores. It should be noted that basal spores are never observed in untreated populations.

Cell aggregates exposed to c-AMP after 18 hours' development were unaffected by the treatment and completed fruit construction within the usual 24-hour period.

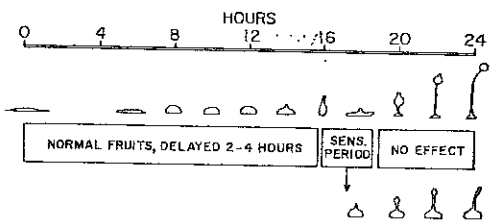

FIG. 1. The c-AMP sensitive period of fruiting body construction. Cell aggregates were prepared on filter paper circles as described in Methods. At hourly intervals filters were switched to fresh pads saturated with a solution containing 3 m*M* c-AMP in LPS buffer and were incubated until no further morphogenetic changes occurred.

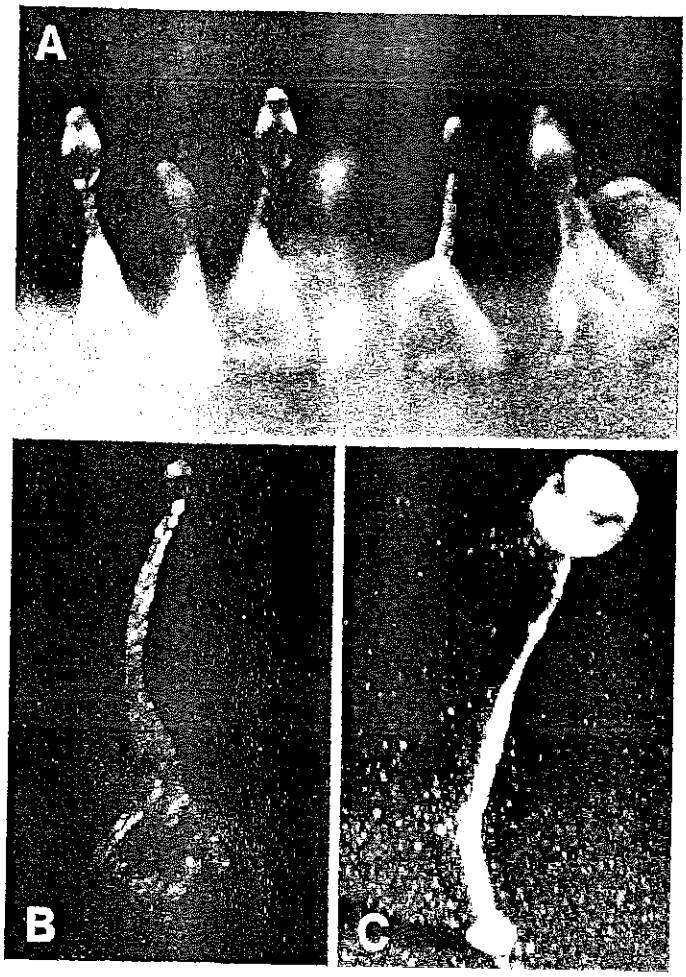

FIG. 2. The effect of c-AMP on fruiting body construction. Cell aggregates were prepared on filter paper circles as described in Methods. After 16–18 hours' development, filters containing the aggregates were transferred to absorbent pads containing 3 m*M* c-AMP in LPS buffer and were incubated until no further morphogenetic changes occurred. The terminal morphogenetic structures were photographed. (A) Filter transferred to c-AMP at 16 hours' development. × 50. (B) Filter transferred to c-AMP at 18 hours' development. × 70. (C) Normal fruiting body. × 25.

The ability of the apical cells to continue their morphogenetic movements after exposure to c-AMP might be taken to mean that they are resistant to its effects at these later stages of development. However, it is also possible that the cells remain susceptible but that c-AMP cannot penetrate the upper reaches of the aggregate fast enough or accumulate to a high enough concentration to affect them.

When cell aggregates were exposed to c-AMP starting at any time before 16 hours, they constructed normal fruiting bodies but consistently required 26–28 hours to complete the process. The later the time of exposure to c-AMP, the greater the delay in completion of morphogenesis.

The failure of cells which were transferred before 16 hours to respond to c-AMP during the 16–18 hour period was further investigated. Aggregates were ex-

posed to c-AMP starting at 0–15 hours and transferred to fresh pads containing c-AMP at 16–18 hours. Under these conditions, aberrant structures were formed, similar to those seen in Fig. 2, suggesting that the c-AMP originally present had been removed before the 16–18 hour period of sensitivity. No change was observed in the total c-AMP content of the pads, however. It is possible that a product such as 5′-AMP accumulates within 2 hours and counteracts the effects of c-AMP. Experiments in which 5′-AMP was added along with c-AMP failed to support this possibility. However, a local depletion in the steady-state concentration of c-AMP immediately around the aggregates could occur through the action of the phosphodiesterase which is normally secreted by aggregating cells (Chang, 1968) without materially reducing the total concentration. Both intracellular and extracellular phosphodiesterase activities were assayed throughout morphogenesis in normal and c-AMP treated aggregates. No differences were found between the two, and in both cases the activity was constant throughout morphogenesis. These results suggest that in aggregates exposed to c-AMP before 16 hours, normal levels of phosphodiesterase are sufficient to reduce local concentrations of c-AMP enough to allow normal morphogenesis to proceed after a short delay.

In another experiment, cells were exposed to c-AMP starting at zero time and were switched to fresh pads containing c-AMP every 2 hours. Under these conditions, aggregation was very much delayed but the cells eventually attained a morphology corresponding to the 16-hour stage observed in untreated controls. Additional exposure to c-AMP at this stage produced aberrant structures similar to those shown in Fig. 2.

The concentration dependence of c-AMP is shown in Table 1. Aggregates exposed to 12 m*M* c-AMP at 16 hours stopped further morphogenesis within 2 hours and remained at the stage characteristic of 18 hours' development. Intermediate concentrations (1.5–6 m*M*) yielded the typical inverted fruit structures shown in Fig. 2. At 0.3 m*M* c-AMP, the fruiting structures appeared normal.

Effect of c-AMP on Two Developmentally Regulated Enzymes during Fruit Construction

Because of the striking morphogenetic alterations produced by c-AMP during the developmental sequence, we examined its effect on two enzymes whose activities undergo characteristic changes during morphogenesis.

UDPG pyrophosphorylase is a key enzyme for cellular slime mold development. Its product UDPG serves as a precursor for the synthesis of one disaccharide and at least three polysaccharides which are

TABLE 1

CONCENTRATION OF c-AMP REQUIRED TO ALTER THE NORMAL MORPHOGENETIC SEQUENCE[a]

c-AMP concentration (*mM*):	12	6	3	1.5	0.3
Terminal morphogenetic structure						

[a] Cell aggregates were prepared on filters as described in Methods. At 16 hours' development, filters were transferred to absorbent pads saturated with the indicated concentration of c-AMP in LPS buffer and incubated at 22°C until no further morphogenetic changes occurred. The terminal morphogenetic structure is indicated in the diagram.

involved in fruiting body construction. One of these is a mucopolysaccharide that is uniquely associated with spores and is probably part of the outer spore coat. Its synthesis requires UDPGalactose which is produced from UDPG by UDPGal 4-epimerase.

As seen in Fig. 3, pyrophosphorylase and epimerase activities begin to increase at different stages of fruiting body construction, and reach peak levels at 19–20 hours of development. Pyrophosphorylase activity subsequently declines to about 50% of its peak value (Ashworth and Sussman, 1967), and epimerase activity is completely lost (Telser and Sussman, 1971).

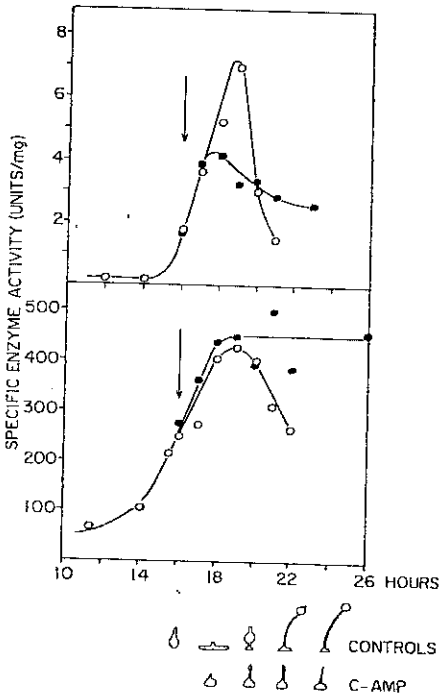

FIG. 3. The effect of c-AMP on epimerase and pyrophosphorylase specific activities during morphogenesis. Cell aggregates were prepared as described in Methods. At 16 hours' development, filters supporting the aggregates were transferred to absorbent pads saturated with LPS buffer (open symbols) or buffer containing 3 m*M* c-AMP (solid symbols) and incubated at 22°C. At the time intervals and morphogenetic stages indicated along the abscissa, cells were harvested from single filters and enzyme assays and protein determinations were performed. Upper: epimerase. Lower: pyrophosphorylase.

The addition of c-AMP at 16 hours altered both of these patterns. For about 2 hours, enzyme accumulation continued unabated. By this time, epimerase activity had reached about half the normal peak level. Subsequent increase was aborted and the level very slowly declined thereafter in contrast to the extremely rapid disappearance in the controls. The pyrophosphorylase activity which by the same time had reached the normal peak level remained constant thereafter instead of declining.

Effect of c-AMP on the Construction of Fruiting Bodies by Migrating Slugs

Cell aggregates of *D. discoideum* respond to a combination of environmental parameters (pH, buffer concentration, ionic strength, and the presence of a volatile, alkaline metabolic product) and either (a) construct a fruiting body directly at the site of aggregation or (b) transform into a migrating slug and move away (Newell *et al.*, 1969). The slug can migrate for many days, randomly in the dark or directly toward a horizontal dark-light gradient (Bonner *et al.*, 1950). If at any time it is shifted to the environmental conditions which promote fruiting, it immediately stops migrating and constructs a fruit over a 7-hour period. If the slug is exposed to overhead light even for a short time (15–30 minutes), the same effect is produced; it stops migration and constructs a fruit over the same time period. This light signal can override all the other environmental parameters (Newell *et al.*, 1969).

Cells were incubated under the conditions described in Methods in order to produce migrating slugs. These were allowed to migrate 48 hr in a horizontal dark-light gradient until they had crawled onto Millipore filters that had previously been placed in their paths. The filters were then transferred to absorbent pads saturated with LPS containing various concentrations of c-AMP and were exposed to overhead light. Figure 4A shows the initial ap-

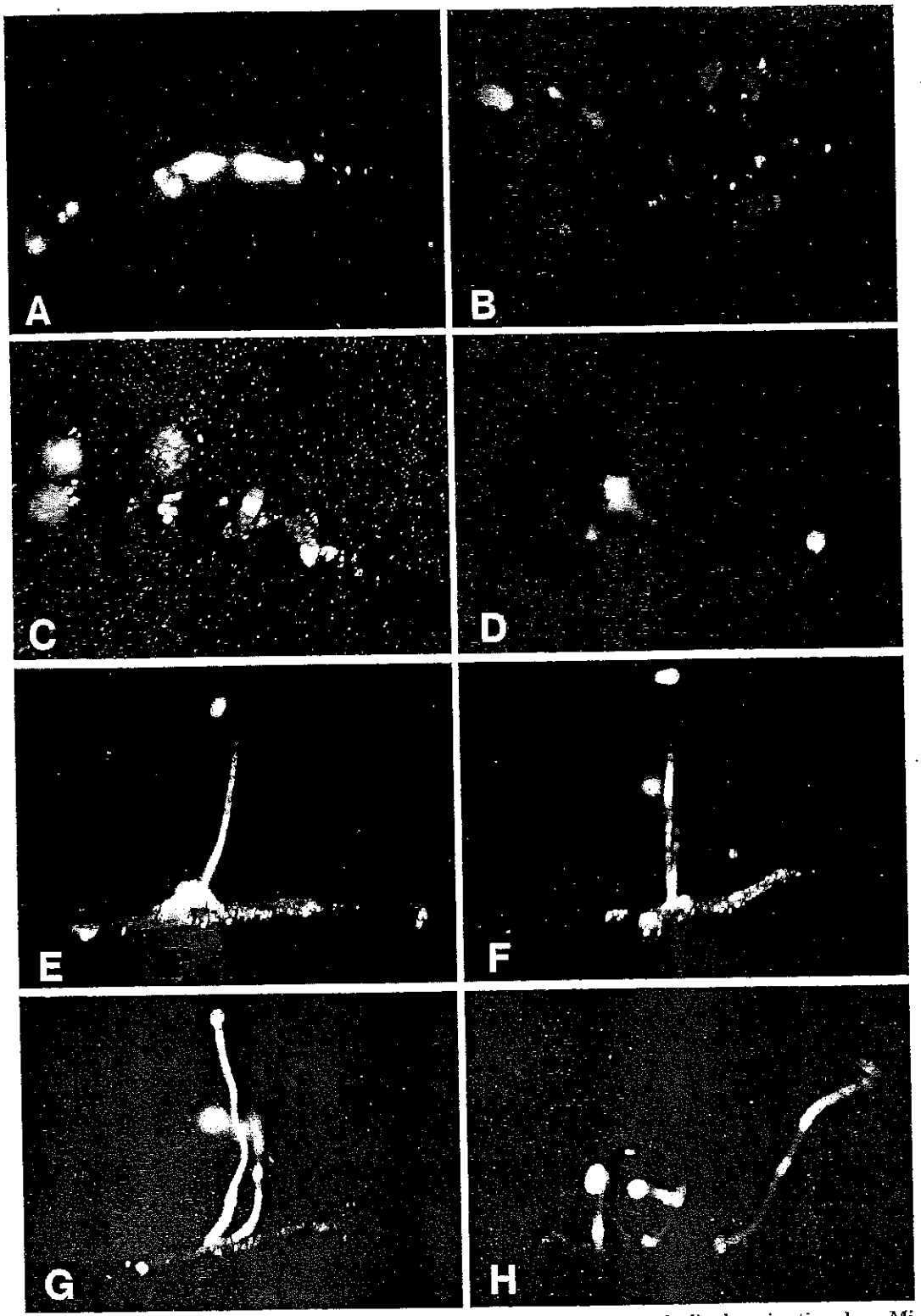

FIG. 4. The effect of c-AMP on the light-induced construction of fruiting bodies by migrating slugs. Migrating slugs were prepared on black Millipore filters as described in Methods. After about 48 hours' migration, the filters supporting the slugs were removed from the light gradient and placed on absorbent pads saturated with LPS buffer containing the indicated concentration of c-AMP. The slugs were exposed to overhead light until no further morphogenetic changes took place. Except for (A), photographs were taken of the terminal morphogenetic structures. (A) Migrating slug, untreated. × 40. (B) and (C) 6 m*M* c-AMP. × 40. (D) 0.6 m*M* c-AMP. × 40. (E) and (F) 3 m*M* c-AMP. × 30. (G) 1.5 m*M* c-AMP. × 30. (H) 0.15 m*M* c-AMP. × 20.

pearance of the slugs, and Figs. 4B–H show their terminal appearances in the presence of c-AMP. Without c-AMP, each slug produced a normal fruiting body which included all the cells (Fig. 2C). In the presence of high c-AMP (6 m*M*) the slugs developed "poly heads" and split into many small slugs. The slugs stopped migration, but fruits did not form. At lower concentrations of c-AMP the head of the slug dispersed and the individual cells fanned out into the deltalike pattern seen in Figs. 4D–G. Some of the posterior cells formed one or more fruiting bodies generally of the aberrant types shown in Fig. 2A, B but not quite as extreme.

In normal migrating slugs, the pyrophosphorylase accumulates much more slowly than it does in aggregates engaged in the construction of fruiting bodies, but it reaches approximately the same level of specific activity. This level remains constant; the enzyme does not disappear. The epimerase does not accumulate at all. Given the signal to stop migration and to start fruit construction, the cells synthesize a second complete round of pyrophosphorylase and a complete first round of epimerase (Newell and Sussman, 1970) as shown in Fig. 5. Both of these latter responses were completely inhibited by c-AMP.

The Specificity of c-AMP Inhibition

Filters bearing migrating slugs were transferred to absorbent pads saturated with LPS containing various concentrations of the substances listed below. None had any observable effect on the construction of fruiting bodies by the slugs after exposure to overhead light. The substances are adenosine; 5′-GMP; 2′,3′- or 5′- AMP; GTP; ADP; dibutyryl cyclic AMP; ATP; hypoxanthine.

DISCUSSION

When a cell aggregate just beginning fruiting body construction is exposed to high enough concentrations of c-AMP, the morphogenetic movements of all the cells except those in the apical tip appear to be disrupted. The result is that the apical cells continue their upward movement and construct a normal lower stalk while the more basal cells remain at the base, some of them transforming into apparently normal spores. The failure of apical tip cells to respond to c-AMP may be due to their elevation off the filter. If the exposure to c-AMP occurs slightly earlier, when more cells are in contact with the filter surface, even the apical cells are affected and their morphogenesis is halted before the lower stalk can be completed and even

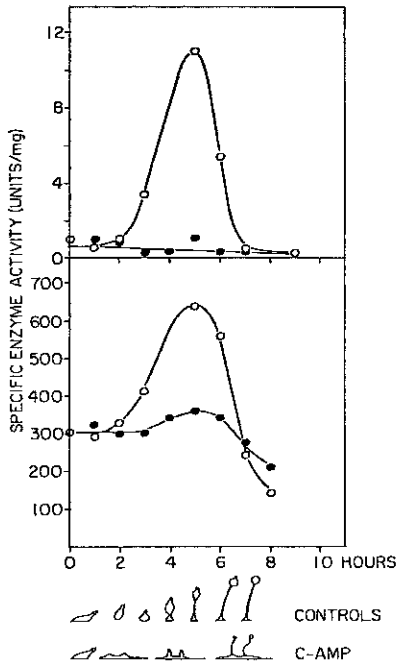

FIG. 5. The effect of c-AMP on epimerase and pyrophosphorylase specific activities during the light-induced construction of fruiting bodies by migrating slugs. Migrating slugs were prepared as described in Methods. After about 48 hours' migration, the filters supporting the slugs were transferred to absorbent pads containing LPS buffer (open symbols) or buffer containing 0.3 m*M* c-AMP (solid symbols) and were exposed to overhead light at 22°C. At the time intervals and morphogenetic stages indicated along the abscissa, cells were harvested from single filters and enzyme assays and protein determinations were performed. Upper: epimerase. Lower: pyrophosphorylase.

fewer spores are observed among the cells clustered at the base.

The data suggest that c-AMP may affect some critical event occurring sometime between 16 and 18 hours. When c-AMP is added at earlier times, the aggregates can recover after a delay of 2–4 hours and go on to construct normal fruiting bodies. Presumably this is due to destruction of c-AMP in the area immediately surrounding the aggregate by the c-AMP phosphodiesterase.

The existence of a period of maximum sensitivity to c-AMP explains the failure of previous investigators (Konijn *et al.*, 1968; Chassy *et al.*, 1969) to observe the morphogenetic changes reported here. Development beginning in the presence of low concentrations of c-AMP would appear normal due to its destruction in the immediate environment of the aggregate by the phosphodiesterase. Very high concentrations of c-AMP would be unaffected by the phosphodiesterase and would thus cause complete inhibition of development.

As shown in Fig. 4, c-AMP also interferes drastically with the movement of and polarity relationships within the migrating slug, and it disrupts the normal course of fruit construction by slugs which have been induced to stop migrating and start fruiting after exposure to overhead light. All these observations are consistent with (though by no means validate) the supposition that c-AMP is continuously synthesized at (a) the apical tip of the cell aggregate which is engaged in fruit construction and (b) the anterior tip of the migrating slug. Thus c-AMP might play a role in directing the vertical movement of cells engaged in fruit construction and the horizontal movement of cells in migrating slugs. The addition of external c-AMP would flood the system and disrupt these polarity relationships.

In addition, c-AMP was observed to interfere with the regulation of at least two enzymes employed in normal fruit construction. After a brief time lag, their further synthesis and/or subsequent disappearance were halted. This is consistent with the possibility that the flow of morphogenetic events provides feedback signals that appropriately alter the course of enzyme accumulation and disappearance. The results of previous experiments (Newell and Sussman, 1970; Ellingson *et al.*, 1971; Newell *et al.*, 1971, 1972) have argued compellingly in favor of this relationship and have indicated that such controls may operate both at the levels of transcription and translation.

The capacity of some cells to differentiate into normal stalk cells and spores despite the drastic changes in the normal topography of the aggregate has been observed previously in mutant strains of *D. discoideum* (Sussman and Sussman, 1969). It is also consistent with the recent observation that when stationary phase cells are incubated at low population density in the presence of high concentrations of c-AMP, they do not aggregate, and yet a small proportion acquire a swollen, rigid-walled, vacuolated condition reminiscent of normal stalk cells (Bonner, 1970).

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