THE EFFECT OF ACTINOMYCIN D ON CELLULAR SLIME MOLD MORPHOGENESIS[1]

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Previous studies (Sussman et al, 1965; Loomis et al, 1966; Ashworth et al, 1966) have demonstrated that in Dictyostelium discoideum the synthesis of at least two enzymes, UDP-galactose: polysaccharide transferase and UDP-glucose pyrophosphorylase is inhibited by actinomycin D when this agent is present early enough in the developmental sequence. However neither these publications nor a recent study by Pannbacker and Wright (1966) provided a detailed description of the effect of actinomycin D on the multicellular organization and cytodifferentiation that accompany normal morphogenesis. Because such information is crucial to a meaningful evaluation of the role played by RNA synthesis in slime mold development, the present investigation was undertaken. The results show that under conditions where actinomycin drastically depresses RNA synthesis and confines ^{32}P-phosphate incorporation to 4S RNA, normal slime mold development, in both gross and cytological aspects, is deranged. The type of derangement depends upon the time at which actinomycin is added. Further, no metabolic consequences have been detected which would suggest that the primary site of actinomycin action under these circumstances is anything other than at the level of RNA synthesis.

D. discoideum strain NC-4 (haploid) was grown on nutrient agar in assoc-

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iation with Aerobacter aerogenes (Sussman, 1966). The vegetative amoebae were harvested at the start of the stationary growth phase, washed by centrifugation, and samples of 10^8 cells were dispensed on 2" Millipore filters resting on support pads saturated with pad fluid containing salts - .01M phosphate-streptomycin solution (pH 6.4) (Sussman, 1966). At intervals after deposition of the cells, the filters were shifted to fresh support pads containing 125ug/ml actinomycin D in the pad fluid. Incubation was continued and subsequent gross development was scored under a dissecting microscope; cytodifferentiation was scored by examining squashed wet mounts under 400x magnification. Terminal morphogenetic stages of control and actinomycin poisoned cells were scored after 24 and again after 48 hours of incubation at 22°C.

The poisoned (and control) cells were incubated in the dark to avoid inactivation of actinomycin and were discarded after a single examination. The results are summarized in Fig. 1.

The normal development sequence is illustrated schematically at the top of the figure. The transitional forms are placed over the times at which

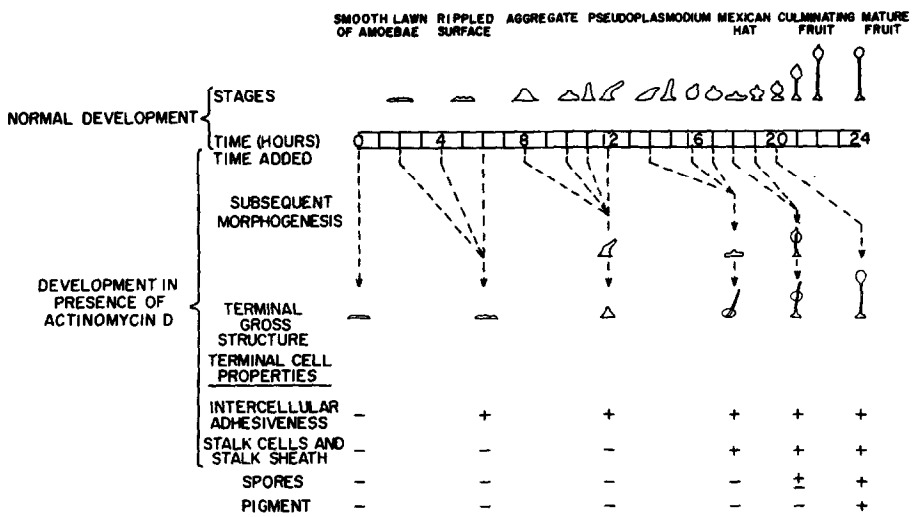

Fig. 1. A schematic summary of the effects of actinomycin D on slime mold morphogenesis. See text for details.

they appear after deposition of the cells on the filters. When actinomycin was added at zero time or shortly thereafter, the amoebae remained in a smooth lawn. When added between and 2 and 6 hours, aggregation began, but stopped at an early stage. When added between 8 and 12 hours aggregation was completed and the aggregates became normal pseudoplasmodia. Further development ceased at this point and the pseudoplasmodia subsided into loose conical masses of amoebae which nevertheless retained the adhesiveness acquired during aggregation. When the drug was added between 14 and 17 hours, the pseudoplasmodia continued developing normally up to the Mexican hat stage observed at 18 hours. From this point a significant developmental diversion occurred, i. e., a normal stalk was constructed, the component cells assuming the typical stalk cell appearance, but the prespore cells instead of rising on the stalk remained at its base and retained their amoeboid character. Thus, a differential inhibition was observed at the cytological level at this time. When the drug was added between 18 and 19 hours, normal fruit construction ensued until the prespore cell mass had risen halfway up the stalk. Further development ceased, only incomplete spores (possessing the characteristic ellipsoid shape but with thin spore walls and residual internal granulation) appeared, and no pigment was produced. Addition of actinomycin at 20 hours or later did not prevent the appearance of normal mature fruits with typical viable spores and the characteristic yellow pigment. In parallel experiments, the cells were removed from the filter supports after as long as 14 hours incubation, dissociated by mild trituration (Loomis et al, 1966), redeposited on millipores, and then exposed to actinomycin. No change in the pattern of inhibition by actinomycin was observed, i.e., the dissociated cells reaggregated rapidly and regained the stage of development which they had reached at the time of dissociation. Their subsequent development stopped at the same point as had that of undissociated amoebae exposed to actinomycin as described above.

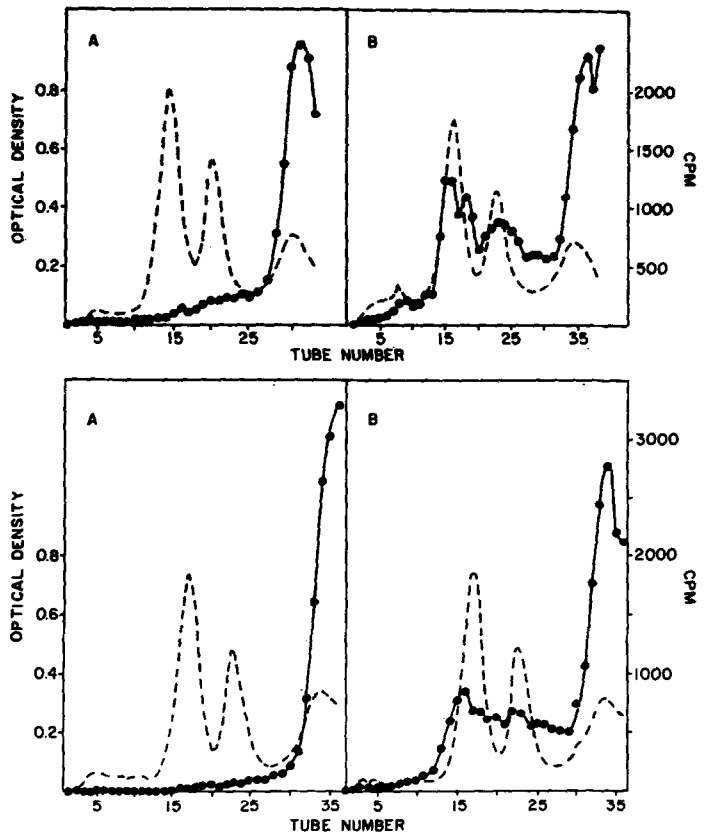

Fig. 2. Effect of actinomycin D on RNA synthesis.

Top:

Cells were harvested from growth plates and pretreated by shaking at 22°C for one hour in streptomycin-nutrient broth (Inselburg et al, 1966) before deposition on Millipore filters with and without actinomycin (125 ug/ml). After 2 hours all received ^{32}P-phosphate (sterile, neutralized, carrier-free at 30 uc/ml of phosphate-free pad fluid). Two hours later the cells from single Millipore filters were harvested, washed, frozen, thawed in 1 ml 1% SDS and spun through a 15-30% sucrose-0.2% SDS gradient in a Spinco Model L SW-25 rotor for 17 hours at 18°C. The tubes were emptied by a finger pump through a Gilford recording spectrophotomater and 1 ml fractions containing 200 ug carrier DNA were precipitated with 15% TCA, washed on a

Millipore filter, and counted in a gas flow counter.

A-actinomycin treated; B-controls

Bottom:

Same as above except that exposure to actinomycin 125 ug/ml occurred after 14 hours incubation on millipores and ^{32}P-phosphate between 16 and 18 hours. Under these conditions the viable count of contaminating A. aerogenes was less than 200 per millipore. The purified, labeled ribosomal RNA yielded a single peak which cochromatographed with D. discoideum carrier r-RNA (Inselburg et al, 1966).

The rate of ^{3}H-uridine incorporation into trichloracetic acid insoluble material by the actinomycin poisoned cells was depressed to a level 20-25% of that observed in the controls, but this residual incorporation continued for at least four hours, in approximate agreement with data previously obtained using a mutant strain of D. discoideum (Sussman et al, 1965). The residual incorporation probably reflects exchange at the CCA end of transfer RNA as observed previously in animal cells (Franklin, 1963) since, as indicated in Fig. 2 RNA pulse labeled with ^{32}P-phosphate in the presence of actinomycin either early or late in the developmental sequence is almost exclusively 4S. It should be noted that the pretreatment of the amoebae in nutrient-streptomycin broth, described in the legend of Fig. 2, is vital in order to reduce the level of contamination by the bacterial associate to a point where the latter do not contribute to the incorporation of isotope. Adequate controls, including analysis of the labeled RNA by methylated albumin-kieselguhr column chromatography (Inselburg et al, 1966) to distinguish between slime mold and bacterial RNA must be run before incorporation data can be evaluated.

The question arises as to whether under these conditions the primary site of actinomycin action can be placed uniquely at the level of RNA synthesis. Three considerations are relevant here:

a. The amoebae were deposited on Millipore filters and ^{14}C-amino acids (New England Nuclear amino acid mixture: 1.5 uc/mg; 0.5 uc/ml) were added to the support pad fluid with and without actinomycin. The rate of amino acid incorporation into TCA insoluble material remained unaffected by the drug for periods of 6 hours after exposure. The periods of exposure covered the entire 24 hours sequence of development. This result is in agreement with a similar experiment carried out with a mutant strain of D. discoideum (Sussman et al, 1965). Since the rate of amino acid incorporation must of necessity be the resultant of many metabolic pathways both anabolic and catabolic, it may be expected to serve as a sensitive indicator of nonspecific inhibition.

b. The synthesis of UDP-galactose: polysaccharide transferase and UDP-glucose pyrophosphorylase both start 12 hours after deposition of the cells on Millipore filters and peak specific activities are reached at 21 - 22 hours (Sussman et al, 1965; Ashworth et al, 1966). When actinomycin was added at 14 hours or later, both enzymes were synthesized at rates equal to or even somewhat greater than those of the control cells and ultimately reached peak levels of activity 100 - 110% of those attained by the controls. Hence, neither the rate nor the extent of synthesis of at least two specific proteins were adversely affected over a period as long as 7 hours after addition of actinomycin.

c. Where the amoebae could accomplish at least a part of the normal developmental sequence after actinomycin D addition as indicated in Fig. 1, this occurred at about the same rate as observed in control cells. Moreover the treated cells at no time displayed signs of generalized damage, i.e., excessive vacuolation, nuclear degeneration, lysis, etc.

In summary, actinomycin D inhibits RNA synthesis in D. discoideum wild

type as well as in at least one mutant strain (Sussman et al, 1965) without adversely affecting their capacity to carry out many other activities for a considerable period after exposure to the agent. Under the same experimental conditions it can inhibit morphogenesis and cytodifferentiation and, if added early enough, prevent the synthesis of at least two enzymes (Sussman et al, 1965; Loomis et al, 1966; Ashworth et al, 1966). Thus the conclusion that RNA synthesis does play a major role in slime mold morphogenesis appears warranted. This conclusion is not unreasonable in view of previous demonstrations that RNA synthesis is intimately involved in the development of: sea urchin and amphibian embryos (Gross et al, 1964; Glisin et al, 1966; Whitely et al, 1966; Brown et al, 1966), lily anthers (Hotta & Stern, 1963), mouse pancreatic rudiments (Wessels, 1964), chick lens rudiments (Reeder & Bell, 1965) and chick retinal rudiments (Kirk & Moscona, 1963).

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