Reports

Mitosis and Differentiation in Roots Treated with Actinomycin

Abstract. *In the presence of actinomycin D (90 μg/ml), mitosis in root meristem of* Allium cepa *L. ceases after a delay of 36 hours. The block occurs at interphase. Differentiation of prevascular tissue is suppressed, and the evidence suggests it begins to fail before mitosis stops. Autoradiograms of roots treated with tritiated uracil show that synthesis of ribonucleic acid is blocked after mitosis fails. The effects of the antibiotic are reversible.*

The antibiotic actinomycin D blocks or depresses DNA-dependent RNA synthesis, both in vivo and in vitro (*1*). It has already proved to be a useful tool in the analysis of cell function as directly controlled by gene products. The drug depresses mitosis in tissue cultures (*2*), stops all RNA synthesis in some bacteria (*3*), and interferes with development while permitting continued division in fertilized sea urchin eggs (*4*). We now report experiments in which roots of *Allium cepa* L. were exposed to high concentrations of the antibiotic.

Bulbs were grown in Knop's medium, which contained either 2 percent ethanol or 2 percent ethanol plus actinomycin D (*5*) at a final concentration of 90 μg/ml. The ethanol was used to prepare a stock solution and was therefore added to the control media in the same final concentration as that in the actinomycin medium. Roots were collected at intervals of several hours and fixed in acetic acid and alcohol (1:3). Mitotic indices were calculated from counts made on acetic-orcein squash preparations; ten microscopic fields (about 1000 cells) were counted for each interval sampled. Root tips were embedded in paraffin and sectioned for histological study. Some of these specimens were from experiments in which the media contained tritiated uracil (3 μc/ml); such sections were covered with Kodak AR10 stripping film for autoradiography.

Figure 1 summarizes the results of the mitotic-index determinations. For the first 36 hours after immersion of the roots in Knop's solution and ethanol or in Knop's solution, ethanol, and actinomycin, respectively, the control and experimental mitotic indices did not differ appreciably. The mitotic index in both systems seems to have been somewhat reduced compared with that expected in ordinary Knop's solution, and probably resulted from the added ethanol. Then, at 36 hours, the mitotic indices fell precipitously in the actinomycin-treated roots, and reached zero 44 hours after the first contact with the actinomycin. No concomitant change took place in the control roots. Figures 2 and 3 are photomicrographs of typical control and experimental squash preparations. Figure 2, the control, shows a number of mitoses distributed normally among the various stages of the cycle. There are, on the other hand, no mitoses at all in the actinomycin-treated cells, represented in Fig. 3; all of these appear to be at interphase, although there are more than the normal number of very large and very small nuclei, and some of the latter stain with abnormal intensity.

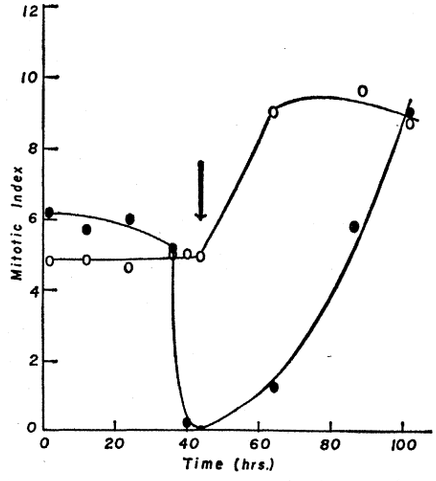

Fig. 1. Mitotic indices of root tip squashes. Each point represents a count of approximately 1000 cells. Open circles, controls in Knop's medium containing 2 percent ethanol. Filled circles, actinomycin D (90 μg/ml) in same medium. The vertical arrow indicates the time of transfer of the bulbs to fresh normal Knop's medium.

Elongation in the actinomycin-treated roots stops well before the mitotic index falls. While it is not possible, with the methods used thus far, to fix the exact time when elongation stops, this quite certainly occurs sometime during the first 12 hours.

Forty-four hours after first immersion in actinomycin D or in the control medium, the bulbs were removed to fresh Knop's solution, and the roots were sampled for observation as before. There was a rapid rise of about 40 percent in the mitotic index of the control, followed by stabilization at the new value. This rise was almost certainly a result of the removal of the roots from contact with the ethanol in both control and experimental media. The mitotic indices rose more slowly in the actinomycin-treated roots, but reached those of the control after 3 days. These roots then grew normally. The mitotic indices calculated from counts made during the recovery period are plotted in Fig. 1.

Figures 4 and 5 show longitudinal sections from control and experimental root tips, respectively, fixed at 44 hours after the beginning of an experiment. A central cylinder of prevascular cells is visible in the control section; it represents a characteristic mode of differentiation of root meristem, evidence of which is normally seen even in the youngest roots. The tip regions in the actinomycin-treated roots show no such organization. This inhibition of differentiation must have occurred earlier in the treatment period, while mitosis was still in progress; otherwise at least some beginnings of prevascular differentiation would have been seen in material fixed after the rapid decline in mitotic index.

Figures 6 and 7 are autoradiograms, photographed at high magnification, of sections from control and experimental root tips, respectively, which were exposed to tritiated uracil for eleven hours after the mitotic indices in the actinomycin-treated roots had fallen to

zero. In these experiments, the bulbs were not removed to fresh Knop's medium. The control autoradiograms show the expected incorporation of the labeled precursor into acid-insoluble polymer, presumably RNA. But in the roots which were in actinomycin D, both mitosis and differentiation had ceased, and the roots showed no incorporation at all. The grain counts for preparations like that seen in Fig. 7 are not higher than background.

These results recall those of Reich *et al.* (*2*), who found that actinomycin D blocks cell division in mouse fibroblasts. The concentrations required for mitotic blockade in their system were, however, very much lower than those employed by us for the plant material. In the cultured mammalian cells actinomycin D (0.1 μg/ml) produced a selective suppression of RNA syntheses and cell division, while at much higher concentrations (2.0 μg/ml), protein and DNA synthesis were also depressed. We have found that in our system 5 μg/ml have no observable effect, whereas at 50 μg/ml essentially the full spectrum of growth changes we report can be seen. Gross and Cousineau (*4*) have recently observed the failure of sea urchin embryos treated with actinomycin D to become differentiated. Curiously, mitosis in the eggs was very much less affected even at concentrations like those used in the experiments with onion roots. In the eggs, DNA synthesis was only slightly affected while RNA synthesis stopped; protein synthesis was unaffected for long periods after the cessation of RNA turnover. Mattingly (*6*) has recently reported that actinomycin D inhibited the elongation of roots in *Vicia faba*. Here, a retardation of protein synthesis was observed, but the incorporation of thymidine into DNA remained quite vigorous. It thus seems likely, although it is not proved by the data presented, that the growth-inhibiting effects in the roots result primarily from interference with RNA synthesis.

Evidence from several cases now suggests that messenger RNA's (*7*) function with varying stability in all cells. Detailed studies have been reported not only for bacteria (*3*, *6*), but for yeasts (*8*), mammalian nuclei (*9*), invertebrate eggs (*4*), and for other cell types. The discovery and analysis of DNA-dependent RNA synthesis (*10*) has, of course, strengthened all such studies, for it is by this mechanism that messenger RNA, and possibly all RNA except that of certain viruses (*2*), is made. The relatively selective depression of DNA-primed RNA synthesis by actinomycin D adds interest to its effects on mitosis and differentiation. Our histological findings suggest that the protein-code message needed for the differentiation of meristematic tissue cells must have stopped coming, or lost function, long before the mitotic indices fell.

The period during which the mitotic index for the experimental roots did not fall, or in which it declined very slowly, is longer than the normal mitotic cycle for plant tissues of this sort, for example, 26 hours for roots of *Vicia faba* (*11*). There is a possibility that some stages of the cell cycle may have been lengthened by the ethanol used in our media; but in any case, the observed lag in mitotic response to actinomycin represents something less than two intermitotic periods. The mitotic index of zero at 44 hours means that the condition of a blocked cell was interphase, unless the small num-

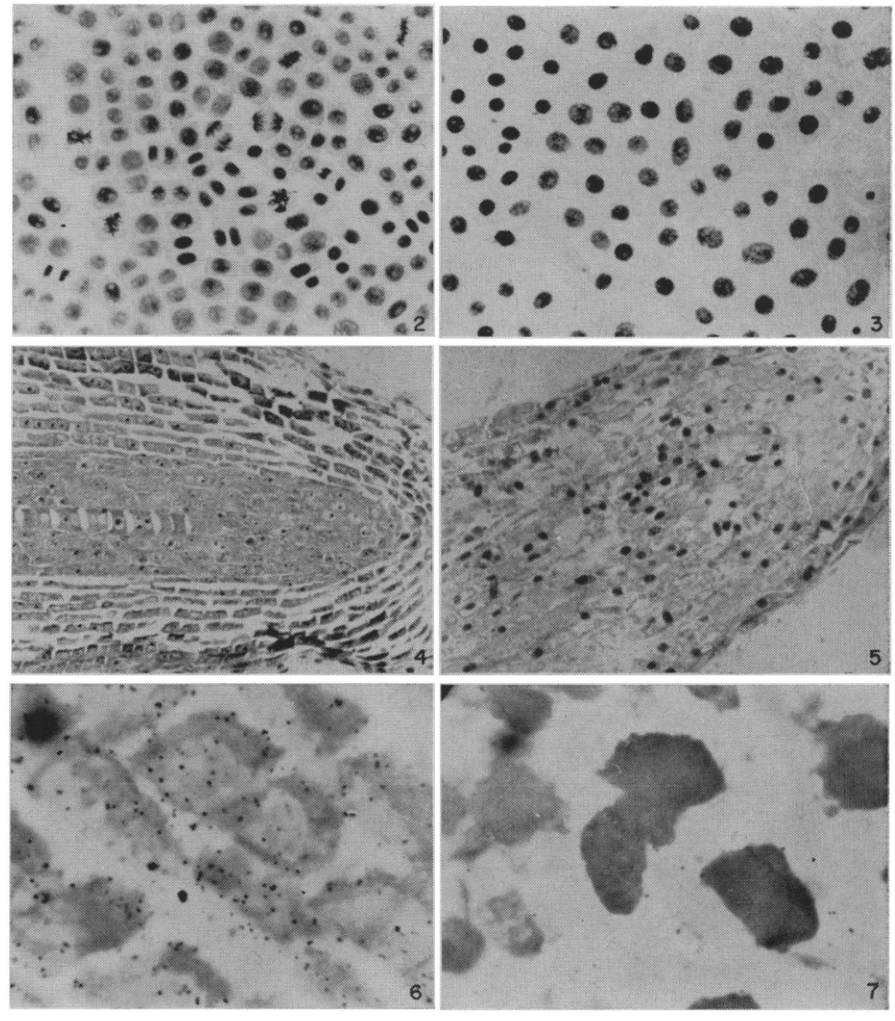

Fig. 2. Control squash preparation of root tip, 44 hours after beginning of an experiment. Stained with aceto-orcein. Fig. 3. Experimental preparation, to parallel that in Fig. 2, 44 hours after immersion in medium containing actinomycin D. Note absence of mitotic figures. Fig. 4. Longitudinal section of control root tip, fixed 44 hours after beginning of an experiment. Azure B stain. Fig. 5. Longitudinal section of experimental root tip, to parallel that in Fig. 4, fixed 44 hours after immersion in actinomycin D medium. Note absence of central prevascular elements. Fig. 6. Autoradiogram of section like that in Fig. 4, at higher magnification. Cells exposed to H^3-uracil (3 μc/ml) for 11 hours, beginning 44 hours after the start of an experiment. See text for discussion. Focus on the emulsion; silver grains represent incorporation of uracil into acid-insoluble material. Fig. 7. Autoradiogram of section from experimental root tip, to parallel that in Fig 6; isotope data as above, but actinomycin was also present from the start of the experiment. Focus on emulsion. A high degree of tissue disorganization is evident from spaces between cells. Grain count does not differ from background.

bers of pycnotic nuclei represent death of cells in some other mitotic stage. Thus in the vast majority of mitotic cells in these roots the current divisions were completed. Entry into another mitosis was then blocked. It is possible that the long delay in response is in part a result of slow penetration of the drug into the cells. But elongation and prevascular differentiation stop long before the mitotic index drops, so that there is a period of at least 24 hours during which actinomycin suppresses differentiation without holding up the progress of cells already in mitosis. Whether the interphase block is during the period or periods of DNA synthesis or in one of the two gaps (G_1, G_2) will have to be determined by a different sort of experiment.

The recovery of growth after removal of actinomycin is an argument against a nonspecific injury effect, unrelated to macromolecule synthesis; but the recovery is in itself somewhat surprising, in view of the strong binding of antinomycin D to DNA (*2*). A similar recovery has, however, been observed by Goldstein and coworkers (*12*), who studied actinomycin effects on HeLa cells.

That some messenger RNA and some new protein must be made for each mitosis in root meristem cells (*13*) is supported by our data. Once prophase is underway, RNA synthesis probably stops normally; presumably protein synthesis can stop without affecting a division already past prophase. Three classes of "division" protein are of importance in cells of this type, possibly requiring renewal in each cycle: the structural proteins of the mitotic spindle, enzymes required for mitotic movements and for breakdown of the nuclear membrane, and the histones of reconstituting nuclei. The observed failure of tissue to become differentiated, presumably soon after the addition of actinomycin, may reflect dependence upon messengers whose half-lives are shorter than one cell cycle (*14*).

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Eye Fixation Aspect of Attention to Visual Stimuli in Infant Chimpanzees

Abstract. *Infant chimpanzees look at a visual stimulus for a regularly decreasing proportion of the time after presentation of the stimulus. Individual differences and presentation of a novel object affect the general level of fixation but do not significantly influence the slope of the curve showing the decline of fixation with time.*

Classical theories of attention (*1*) and more recent studies of the orienting reflex (*2*) have been concerned with moment-to-moment adjustments made by an organism for the efficient reception of information. An especially significant aspect of orientation to a visual stimulus involves the turning of the eyes toward potentially important stimuli.

While autonomic and electroencephalographic changes are manifestations of a general mobilization of the organism to receive stimuli, it is the direction of gaze that can often indicate which stimulus the organism is responding to at any moment. Thus, patterns of eye movement are used to analyze visual search of a complex stimulus field (*3*) and to assess which aspects of such a field are discriminated (*4*) and preferred (*5*).

By considering the shift of eye movements to a new stimulus as one component of the orienting reflex, as Sokolov (*2*) has done, it is possible to regard a change in eye fixation as a phasic psychophysiological response to the onset of a simple stimulus. The purpose of the studies we report was to consider eye fixations in this way, to examine the course of eye fixations after presentation of a visual stimulus, and to analyze certain factors that might affect the resulting curve.

Five infant chimpanzees (aged 7 to 22 months) were tested in two experiments. Testing took place in a 31-by-18-by-29-inch box from which light was excluded. The animal was strapped into a chair inside the box facing a 20¼-by-16¾-inch panel, 14½ inches away. In the panel, at the subject's eye level, were two 3¼-by-3-inch windows, 10¾ inches apart. Each window could be illuminated by a 7-watt bulb masked by frosted Plexiglas. There was a 5/16-inch peephole halfway between the two windows through which the subject could be observed from outside the box.

The general procedure involved turning on one of the lights and observing the animal's eye fixations toward that light during a number of successive 5-second intervals. An eye fixation toward the stimulus was counted if the stimulus light was reflected in the center of the pupil, and an event key connected to a pen writer was depressed for the duration of each fixation. Scoring the records involved determination of the duration of eye fixation within each 5-second interval. The median correlation between observers on this measure was .84 (range, .66 to .94).

In a first experiment it was found that duration of eye fixations toward the light was maximum immediately after the light was turned on and that there was a regular decrease in fixation of the stimulus in the following 25 seconds. There was typically at least one fixation during the first 5 seconds. It was followed by looking away and then by repeated fixations later in the trial.

In the second experiment, three conditions were presented to determine whether a retinal adaptation process alone could account for this short-term decrement in eye fixation. In this experiment a 3¾-by-3¾-by-3¼-inch lighted white box into which the subject could see was placed behind each window. A hinged door on the top of each box permitted the experimenter to insert objects into it. A guillotine door between the window and the box