THE EFFECT OF ACTIDIONE ON MITOSIS IN THE SLIME MOLD *PHYSARUM POLYCEPHALUM*

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ABSTRACT

Actidione, reportedly a specific inhibitor of protein synthesis, was found to reduce the incorporation of labeled amino acids into proteins of the slime mold *Physarum polycephalum* without drastically inhibiting the incorporation of nucleic acid precursors into RNA. This inhibitor was found to completely block the ensuing mitosis if it was added at any time between telophase and late prophase. Plasmodia given Actidione in late prophase (about the time of nucleolar dissolution) went on through telophase to reconstruction even though nuclear amino acid incorporation was drastically reduced during that period.

INTRODUCTION

The involvement of RNA and protein synthesis in the initiation and the completion of mitosis is one of the more challenging problems in cell biology. Previous experiments by members of this group (Mittermayer, Braun, and Rusch, 1965) showed that actinomycin, an inhibitor of messenger RNA synthesis, inhibited mitosis in *Physarum polycephalum*, but this inhibition was not observed immediately upon addition of the drug. At least one complete nuclear division cycle was completed after addition of actinomycin even though RNA synthesis was inhibited during that period. The involvement, on the other hand, of newly synthesized protein in mitosis remained unclear primarily because the most effective inhibitor of protein synthesis, puromycin, was ineffective in *P. polycephalum* (Mohberg, unpublished experiments). Recently, however, Actidione, an inhibitor of protein synthesis (Bennett, Smithers, and Ward, 1964; Ennis and Lubin, 1964; Siegel and Sisler, 1964) which interferes with the function of transfer RNA, was employed (Sussman, 1965) as a specific inhibitor of protein synthesis in the cellular slime mold, *Dictyostelium discoideum*. This drug prevented normal morphogenesis and inhibited the formation of an enzyme without having an immediate effect on RNA synthesis. Sussman's results (1965) prompted us to attempt to establish the requirement for newly synthesized protein in the mitotic process of the plasmodial slime mold. The objects of the experiments in this communication were, first, to determine the effect of Actidione on protein and RNA synthesis and, second, to attempt to establish the requirement for protein synthesis in the promotion and the completion of mitosis in *P. polycephalum*. This organism is well suited for studies of this type because quantities of highly synchronous plasmodial material are readily available (Guttes, Guttes, and Rusch, 1961).

MATERIALS AND METHODS

CULTURE OF P. POLYCEPHALUM: Previously published methods were used for the culture of this organism and for the observation of mitosis (Mittermayer, Braun, and Rusch, 1965; Guttes, Guttes, and Rusch, 1961). For isotope incubations the medium to

TABLE I

The Effect of Actidione on Ribonucleic Acid and Protein Synthesis in Physarum

Precursor	Protein					
	10 min.		30 min.		60 min.	
	Actidione	Control	Actidione	Control	Actidione	Control
	dpm/mg					
Hypoxanthine	0	37	168	141	342	512
Uridine	11,500	14,500	39,300	36,700	100,000	96,000
Leucine	150	346	198	590	220	1,450
Lysine	14	72	18	605	62	1,320

For these experiments cultures, approximately 2 hours past mitosis, were incubated for the times indicated in glucose-salts medium containing 10 μg of Actidione per cc. The H^3-uridine medium was 3.33 μc/cc, H^3-hypoxanthine was 1.66 μc/cc, C^{14}-leucine was 0.166 μc/cc, and C^{14}-lysine was 0.33 μc/cc.

Proteins were determined by the biuret (Gornall, Brodawill, and David, 1949) method.

which tracer was added consisted of citric acid·2 H_2O, 3.6 gm; $FeCl_2 \cdot H_2O$, 0.54 gm; $MgSO_4 \cdot 7\ H_2O$, 0.54 gm; $CaCl_2 \cdot 2\ H_2O$, 0.54 gm; $MnCl_2 \cdot 4\ H_2O$, 0.076 gm; $ZnSO_4 \cdot 7\ H_2O$, 0.03 gm; glucose, 10.0 gm; water to 100 ml; pH adjusted to 4.6 with KOH.

MATERIALS: A list of the chemicals used and their sources are as follows: Actidione, Upjohn Co., Kalamazoo, Michigan; H^3-uridine, 1.3 c/mmole, H^3-hypoxanthine, 0.83 c/mmole, and C^{14}-l-lysine, 240 mc/mmole, Schwarz Bioresearch Labs. Inc., Mount Vernon, New York; C^{14}-dl-leucine, 36.4 mc/mmole, Nuclear-Chicago Corporation, Des Plaines, Illinois; S^{35}-l-methionine, 20.0 mc/mmole at the time of its use, Schwarz Bioresearch Labs. Inc.

PROCEDURES: Nuclear isolations were done according to Mohberg and Rusch (1964). Protein determinations were made by either the biuret (Gornall, Brodawill, and David, 1949) or Folin (Lowry *et al.*, 1951) method after the plasmodia or isolated nuclei were first washed in 5 per cent w/v trichloroacetic acid in 50 per cent aqueous acetone, washed again in 0.25 M perchloric acid, and the resultant pellet was then dissolved in alkali. Radioactivity determinations were by liquid scintillation counting of the dissolved pellets.

RESULTS

Preliminary experiments showed that continuous treatment of surface-grown plasmodia with Actidione at concentrations of 5 μg and up per cc completely inhibited the growth of this slime mold. Treatment with Actidione at concentrations greater than 20 μg per cc led to the breakdown of the plasmodia within a few hours.

The effect of Actidione on the incorporation of the nucleic acid precursors uridine and hypoxanthine and the effect on the incorporation of the amino acids leucine and lysine are shown in Table I. This experiment showed that Actidione was a powerful inhibitor of amino acid incorporation and that the drug had relatively less effect on nucleic acid synthesis.

TABLE II

The Effect of Actidione on Incorporation of Methionine at Mitosis

Stage of mitosis at the beginning of incubation	Time after addition of actidione	Protein	Per cent inhibition relative to the control
	min.	*dpm/mg*	
Prometaphase (control)	—	11,700	0.0
Prometaphase	0–10	57	99.5
Metaphase-anaphase	20–30	170	98.5
Early reconstruction	25–35	106	99.0
Reconstruction	40–50	84	99.3
Reconstruction	50–60	89	99.2

These counts are the average of duplicate determinations of the S^{35}-methionine incorporated in a 10 minute pulse. Actidione at a level of 50 μg per cc of culture fluid was added to all cultures excepting the control at the time mitosis was observed and remained in contact throughout the experiment. Incubation was in the glucose-salts medium containing 50 μg per cc of Actidione and 3.2 μc per cc of S^{35}-methionine. Protein was determined by the biuret (Gornall, Brodawill, and David, 1949) method.

Table II shows the effect of Actidione on the rate of S^{35}-methionine incorporation into the proteins of intact plasmodia. At the time these plasmodia

entered prometaphase, Actidione treatment was initiated. This experiment shows that methionine incorporation was inhibited and that during mitosis and nuclear reconstruction there was no period of insensitivity to the inhibitor.

Results of detailed observations concerning the effect of Actidione on the course of the mitotic cycle are summarized schematically in Fig. 1. Although mitosis in *P. polycephalum* has been described in detail (Guttes, Guttes, and Rusch, 1961), a short description is given here. About 1 hour before mitosis (the intermitotic time is approximately 10 hours under the conditions of these experiments) the nucleolus begins to swell and condensed chromatin begins to pull away from the nuclear membrane. At the beginning of prophase, as defined by Guttes, Guttes, and Rusch (1961), the nucleolus moves to the periphery of the nuclear membrane, and within a few minutes the nucleolus begins to dissolve. In the period up to the time that the nucleolus begins to dissolve, Actidione treatment produces a permanent blockage. Actidione treatment, at approximately the time of nucleolar dissolution or shortly thereafter in late prophase, does not produce a permanent blockage but permits the progress of mitosis. In the untreated cultures the time from prophase to telophase is approximately 15 minutes, and of this time less than 5 minutes are available for exactly timing the critical period of blockage. Its precise timing has, as yet, proved elusive.

Immediately after telophase, nucleolar materials begin to appear as tiny granules, and within 2 hours (in the uninhibited mitosis) these granules fuse to form a single nucleolus. In cultures treated with Actidione in late prophase, the time from late prophase to telophase varied from that of the control by as much as 1 hour; reconstruction and the formation of a single nucleolus varied in time from that of the control by as much as 3 hours. In cultures treated in late prophase with Actidione at concentrations from 5 μg per cc to 50 μg per cc, there were no striking differences in the times required for either mitosis or reconstruction. In one case, however, treatment of a culture with the higher Actidione concentration (50 μg per cc) led to a high frequency of binucleolate reconstructions. Nevertheless, the majority of plasmodia treated with Actidione at late prophase did not vary from the controls either in the duration of mitosis and reconstruction or in their topology.

From the preceding observations we suggest that the observed variation in the speed of mitosis probably results from the timing of the treatment. The treatment may have been made either before or after the appearance of a factor which regulates the duration of mitosis and nucleolar reconstruction. A nuclear event of this type would be very difficult to resolve because short time-periods are involved and because the time of appearance of such a factor could vary significantly from one plasmodial preparation to another.

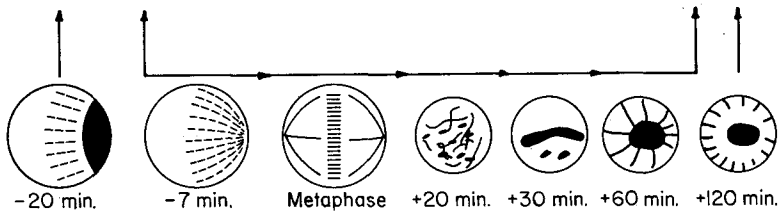

FIGURE 1. A schematic representation of the course of mitosis in *P. polycephalum*. Arrows show the effect of Actidione, vertical arrows indicate complete blockage at that stage of mitosis, horizontal arrows show that the nuclei may progress through the given stages in the presence of this inhibitor.

It is worth reiterating that, even though the time course of mitosis and reconstruction was, in a few cases, affected by Actidione treatment, nucleolar reformation was observed in every plasmodium which was examined.

Actidione treatment in late prophase permitted mitosis and reconstruction, as we have just described, but in every case the following mitosis was completely inhibited provided that Actidione treatment was continuous. Continuous Actidione treatment beginning at any time between telophase and late prophase eliminated the ensuing mitosis.

Our methionine incorporation experiments (Table II) show that plasmodial protein synthesis was inhibited by Actidione during the period of mitosis. It can, however, be argued that mitosis and nuclear reconstruction depend upon the syn-

thesis of an Actidione-resistant protein. This argument has some apparent validity in our observation (Tables I, II) that there is a small but significant amount of radioactivity present in the acid-insoluble fraction of the Actidione-treated plasmodium. To investigate this proposition, the following experiment was designed. Whole plasmodia at definite stages in or near mitosis were labeled with S^{35}-methionine in the presence or in the absence of Actidione. Nuclei were isolated from these plasmodia, and the specific activity of the nuclear proteins was determined. The results of this experiment are shown in Fig. 2, which shows that Actidione treatment significantly inhibited the formation of sulfur-containing acid-insoluble material. The traces of residual radioactivity in the acid-insoluble material of the treated cultures may be owing to the presence of a trace of Actidione-resistant protein synthesis, or, on the other hand, to the formation of a non-protein sulfur-containing polymer. Even if this material were protein, it is unlikely that the observed incorporation contributed a great deal to the structural reformation of the nucleus because in this experiment the time required for reconstruction was approximately the same for both treated and control cultures.

These observations and data provide substantial evidence that the proteins necessary for mitosis are synthesized just prior to mitosis and that the proteins required for reconstruction are, for the most part, also formed prior to mitosis.

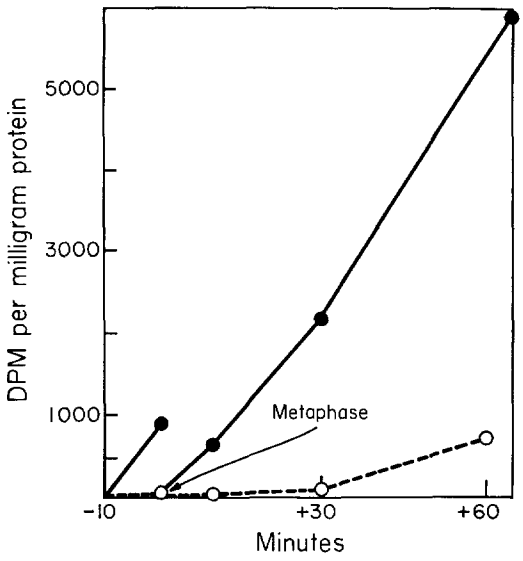

FIGURE 2 Incorporation of S^{35}-methionine into nuclei labeled *in vivo*. For these determinations cultures at a definite mitotic stage were incubated in glucose-salts medium containing 1 μc per cc S^{35}-methionine. The incubation mixture contained 20 μg per cc of Actidione. Five cultures were used for each determination. Protein was analysed by a modified Folin procedure (Lowry *et al.*, 1951). Treated cultures are designated by the broken lines and open circles, control cultures by solid lines and closed circles.

DISCUSSION

With an efficient inhibitor it should be possible to establish the "last" time at which a specific component required for the completion of a given biological process is formed. Such an approach yields little information about the quantitative contribution of the last component. It is, however, obvious that all other critical components of the process must have been completed prior to the blockage. Earlier experiments (Mittermayer, Braun, and Rusch, 1965) with *P. polycephalum* suggested that the last RNA message supplying information for a protein required for mitosis was synthesized at least one full nuclear mitotic cycle prior to the completion of a mitotic event. The present experiments show that the last protein is synthesized shortly before mitosis. This behavior resembles that of the fertilized sea urchin egg (Gross, Malkin, and Moves 1964) in which proteins necessary for mitosis appear to be translated from stable RNA templates. We have, unfortunately, little information concerning the quantitative contribution of the last protein to the mitotic process, nor have we an answer to the question whether or not these proteins are synthesized continuously or discontinuously. Discontinuous protein synthesis from a stable RNA template would be a process of great theoretical interest.

Two observations suggest that the nucleolus may play a more than passive part in the mitotic process: (*a*) mitosis became relatively resistant to Actidione treatment at some time shortly after the beginning of nucleolar dissolution, and (*b*) nucleolar reconstruction proceeded (albeit at times at a reduced rate) even though the synthesis of nuclear proteins was inhibited. A relationship between the nucleolus and mitosis has frequently been suggested from earlier experiments based on direct observation (Robinow, 1963), the behavior of plant micronuclei (La Cour, 1953; McLeish, 1954; Darlington and Hague, 1955), genetic ex-

periments (Philp and Huskins, 1931; McLeish, 1955; and McClintock, 1934), and ultraviolet microbeam irradiation experiments (Gaulden and Perry, 1955). On the other hand, embryos of frog (Elsdale, Fischberg, and Smith, 1958) and insect (von Borstel and Rekemeyer, 1958) went through several cleavage divisions when nucleolar organizing regions of the chromosomes were absent. It was, however, difficult to determine how much "nucleolar" material of maternal origin had been stored in the egg. Even though the latter reports (Elsdale, Fischberg, and Smith, 1958; von Borstel, 1958) are somewhat discordant observations on the general theme of nucleolar function in mitosis, it is probable that in many organisms the nucleolus plays an important part in the initiation of mitosis and that this function is probably separate from the well known nucleolar production of ribosomes (Edström, Grampp, and Schar, 1961) and from its function in the synthesis and methylation of transfer RNA (Sirlin, 1963).

It would be tempting to propose that, in those organisms where the nucleolus participates in the formation of the mitotic apparatus, the nucleolus is, in part, reconstructed from elements of the mitotic apparatus. Along this line of speculation our data suggest that proteins necessary for nucleolar reconstruction are conserved through mitosis, and, since both light (Guttes, Guttes, and Rusch, 1961) and electron (Kessler, 1964) microscopic studies of mitosis in *P. polycephalum* show that the nuclear membrane retains its integrity throughout mitosis, it is probable that these nucleolar proteins are retained within the nuclear envelope. Any attempt to carry this speculation further and to propose a relationship between the proteins of mitosis and nucleolar proteins would, based on these experiments, be premature. At any rate, the inhibitor Actidione should prove a useful tool in future experiments which will attempt to unravel some of the complexities of the mitotic process in *P. polycephalum*.

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