

## ACTINOMYCIN D IN *TETRAHYMENA*

### *Non-Specific Inhibition of RNA Synthesis and Primary and Secondary Effects on Protein Synthesis*

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#### SUMMARY

The effects of actinomycin D on RNA and protein synthesis have been investigated in *Tetrahymena pyriformis*. The extent of inhibition of total RNA synthesis and the time before inhibition occurs are both dependent on the concentration of the drug over the range 0.5–10  $\mu\text{g/ml}$ . In contrast to reports on mammalian systems, actinomycin D at low doses is not a selective inhibitor of rRNA synthesis in *Tetrahymena*; at all doses tested, poly(A+) RNA and poly(A-) RNA syntheses were reduced to similar extents.

Finally, the action of actinomycin D was studied in conjunction with  $\gamma$ -radiation which alone causes a transient disaggregation of the polysomes. Actinomycin D partially inhibits the reassociation of polysomes in irradiated cells, but after recovery, the polysome content of the irradiated and drug-treated cells is greater than that of cells treated only with actinomycin D. The results support both a direct action of actinomycin D on translation and secondary effects resulting from inhibition of transcription.

The effect of actinomycin D on protein synthesis was determined by two independent methods.

1. Investigation of drug-induced alterations in polysome content showed that as the dose increases (a) the lag period before observable polysome dissociation decreases; (b) the rate of polysome dissociation increases; and (c) the final extent of polysome loss increases.

2. By measuring the relative rates of incorporation of [<sup>35</sup>S]methionine into N-terminal and internal positions of nascent peptides, it was determined that actinomycin D inhibits initiation and elongation to similar extents.

Actinomycin D is widely used as a selective inhibitor of RNA synthesis. It is known to bind to the DNA of many cells [1, 2], including *Tetrahymena* [3], and thereby to make the DNA template unavailable for transcription by RNA polymerase [4, 5]. In mammalian cells, low concentrations of actinomycin D (0.01–0.08  $\mu\text{g/ml}$ ) interfere primarily with the synthesis of nucleolar

(ribosomal precursor) [6–8] RNA. At higher drug concentrations ( $\sim 5 \mu\text{g/ml}$ ), all types of RNA synthesis are inhibited, polysomes dissociate, and mRNA is degraded (e.g., [9–11]). Initially, it was thought that the observed polysome loss in the presence of actinomycin D resulted from normal mRNA turnover [8, 12–14]. More recent investigations have shown, however, that the polysomes of mammalian cells may be affected by the drug in other ways, resulting in an inhibition of the initiation of protein synthesis [15, 16], and that the half-life of mRNA cal-

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culated from polysome loss in the presence of actinomycin D is considerably shorter than that determined by other means [17, 18]. However, no direct mode of action for the inhibition of protein synthesis by actinomycin D has been described.

RNA synthesis in the ciliate protozoan, *Tetrahymena*, is also susceptible to inhibition by actinomycin D [19–22]. Growth and division of exponential cultures are impeded by the drug [21, 23], and division of heat shock-synchronized cells is delayed if the drug is given before a critical time in the mitotic cycle [19, 22–25]. In cells that have been synchronized by repeated heat shocks and starved in an inorganic medium, a condition in which no rRNA synthesis occurs, 25–50  $\mu\text{g/ml}$  of actinomycin D inhibits the synthesis of DNA-like (presumably messenger) RNA and tRNA [22]. A lower dose of drug (5  $\mu\text{g/ml}$ ) interferes with rRNA and tRNA synthesis to a similar extent [26]. Prolonged treatment (5–24 h) with 10  $\mu\text{g/ml}$  actinomycin D leads to nucleolar aggregation and loss of granular elements of the nucleoli [27], and actinomycin D at 50  $\mu\text{g/ml}$  inhibits transport of previously synthesized rRNA into the cytoplasm [28]. Nothing has appeared concerning the specificity of low concentrations of the drug in exponentially growing cells of *Tetrahymena*.

When cultures of *Tetrahymena* are starved, the polysomes become dissociated [29]. Upon transfer of the cells to nutrient medium, the polysomes reform and protein synthesis resumes. If RNA synthesis is blocked by 25  $\mu\text{g/ml}$  of actinomycin D during refeeding, the reassociation of polysomes still occurs [29], suggesting that actinomycin D has no direct effect on the initiation of protein synthesis in *Tetrahymena*. In contrast, 10  $\mu\text{g/ml}$  is able to partially prevent the reformation of polysomes which have been dissociated by  $\gamma$ -radiation [30].

The purpose of the present study was two-fold: to determine (1) whether or not, in *Tetrahymena* as in mammalian cells, actinomycin D at low levels acts as a preferential inhibitor of rRNA synthesis while not affecting mRNA synthesis; (2) whether or not there is any direct effect of the drug on polysome-associated protein synthesis.

## MATERIALS AND METHODS

### *Culture conditions*

*Tetrahymena pyriformis* (amicronucleate strain GL-C) was grown in PPL medium, which consists of 1% (w/v) proteose-peptone (Difco), 0.1% liver extract (Nutritional Biochemicals Corp.), and 0.13%  $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ . Cultures were maintained at 28°C either in 250 ml flasks containing 50 ml of medium or in 3-1 Fernbach flasks containing 500 ml of medium. Under these conditions, the cell number doubled every 3 h during the exponential phase of growth. Cultures were used for experiments in early- to mid-log phase at a cell density of  $2\text{--}4 \times 10^4/\text{ml}$ . Further details have been described previously [31, 32].

### *Labeling with [ $^3\text{H}$ ]uridine*

For experiments in which the kinetics of RNA synthesis were to be measured, it was necessary to have a means to correct for inhomogeneities in sample size, since it is difficult to obtain a reproducible cell count in successive samples from a suspension of swimming *Tetrahymena*. To this end, the DNA was uniformly labeled before beginning an experiment by incubating the cells overnight with [ $^{14}\text{C}$ ]TdR (0.1  $\mu\text{Ci/ml}$ ; 57 mCi/mmol; New England Nuclear Corp.). Experiments were initiated by the addition of [ $^3\text{H}$ ]uridine (10  $\mu\text{Ci/ml}$ ; 35 Ci/mmol; New England Nuclear Corp.) to a 10 ml culture in a 125 ml flask. After 15 min, actinomycin D (Cosmegen, Merck and Co., Rahway, N.J.), freshly dissolved in  $\text{H}_2\text{O}$  to 500  $\mu\text{g/ml}$ , was added to the appropriate cultures to reach the different preselected concentrations (0.05–50  $\mu\text{g/ml}$ ). At various times during the labeling with [ $^3\text{H}$ ]uridine, triplicate samples of 75  $\mu\text{l}$  were pipetted onto 2 cm squares of Whatman 3MM filter paper, which were washed in ice-cold 10% trichloroacetic acid (TCA), 5% TCA, and ethanol:ether (3:1), and counted for radioactivity as previously described [32, 33].

For experiments in which the RNA was to be extracted and analyzed, the DNA was not prelabeled. Cultures of 50 ml were treated with actinomycin D for the times and at the concentrations indicated in the figures. At the appropriate time, [ $^3\text{H}$ ]uridine (5  $\mu\text{Ci/ml}$ ) was added. After a 10 min labeling period, cultures were harvested by pouring them over frozen crushed medium. Total cellular RNA was extracted with phenol- $\text{CHCl}_3$ -SDS essentially according to the method of Perry et al. [34] with slight modifications [35].

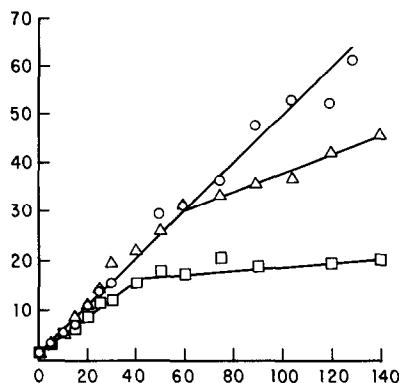


Fig. 1. Abscissa: time (min); ordinate:  $^3\text{H}$  dpm/ $^{14}\text{C}$  dpm.

Kinetics of inhibition of RNA synthesis in *Tetrahymena* by actinomycin D. Log-phase cells were pre-labeled overnight with [ $^{14}\text{C}$ ]TdR (0.1  $\mu\text{Ci/ml}$ ). At zero time, [ $^3\text{H}$ ]uridine (10  $\mu\text{Ci/ml}$ ) was added to 10 ml aliquots of the culture. After 15 min some of the aliquots received actinomycin D ( $\Delta$ , 5  $\mu\text{g/ml}$ ;  $\square$ , 10  $\mu\text{g/ml}$ ). One aliquot received an equal volume of  $\text{H}_2\text{O}$  as a control ( $\circ$ ). Triplicate samples were taken at the indicated times and processed for acid-precipitable radioactivity as described in Materials and Methods.

### Analysis of RNA

Aliquots of the extracted RNA were analyzed by electrophoresis on 2.4% acrylamide-1% agarose gels (6 $\times$ 95 mm) in the buffer system of Loening [36], containing 0.2% sodium dodecyl sulfate (SDS). Electrophoresis was carried out for 150 min at 50 V [35]. The gels were scanned for absorbance at 260 nm in a Gilford recording spectrophotometer equipped with a linear transport apparatus to determine the positions of the mature 25S and 17S ribosomal RNAs and to insure that these RNAs were not degraded. The gels were then sliced and counted for radioactivity.

In some experiments, RNA containing poly(A) [poly(A $^+$ ) RNA] was separated from the remaining RNAs [poly(A $^-$ ) RNA] by chromatography on 50 mg columns (7 mm i.d.) of oligo(dT) cellulose by a modification [35] of the method of Aviv & Leder [37]. All poly(A $^-$ ) RNA, including all rRNA, was eluted with 4 ml of application buffer (10 mM Tris-HCl, pH 7.5; 500 mM KCl); poly(A $^+$ ) RNA was then eluted with 4 ml of elution buffer (10 mM Tris-HCl, pH 7.5). Aliquots of the separated RNAs were taken for the determination of RNA concentration, radioactivity, and size distribution by SDS-polyacrylamide gel electrophoresis.

### Analysis of polysome content

Culture aliquots (25 ml) in 125 ml flasks were exposed to actinomycin D or gamma-radiation at 28°C at the doses indicated in the figure legends. Cells were harvested by pouring the culture over frozen crushed medium containing 5  $\mu\text{g/ml}$  cycloheximide. The cells

were washed and lysed, and polysomes were prepared and analyzed on sucrose density gradients, as previously described [31]. The polysome content, i.e., the percentage of the total ribosomes in polysomes, was determined by cutting out and weighing photocopies of the appropriate areas under the recorder tracings of the 260 nm absorption profile of each gradient. In some cases, these data are presented as "% of control", which relates the polysome content for a treated culture to that for an untreated control culture for the same experiment. Polysome content of control cultures is routinely 80–90%.

### [ $^{35}\text{S}$ ]Methionine incorporation

Cells were concentrated to 1–2 $\times$ 10 $^9$ /ml in their growth medium and 1 ml aliquots were incubated at 28°C in tubes in which the medium was never more than 1 cm deep to assure adequate oxygenation. Actinomycin D was added to some of the aliquots, and [ $^{35}\text{S}$ ]methionine (New England Nuclear Corp., 200–250 Ci/mmol) was added to all of the cultures to 10  $\mu\text{Ci/ml}$ . Samples of 75  $\mu\text{l}$  were removed at 5–10 min intervals and each applied to a 2 cm square of Whatman 3MM paper. All the papers from an experiment were washed with hot 10% and cold 5% TCA, ethanol-ether, and ether, and the precipitated peptides and proteins were subjected to a modified Edman degradation on the filter paper as described previously [38]. Incorporation into *N*-terminal positions results from the initiation of new peptide chains, while incorporation into internal positions is due to the elongation of peptides.

### Irradiation

A US Nuclear Corp. [ $^{60}\text{Co}$ ] irradiator, model GR-9, provided  $\gamma$ -radiation at a dose-rate of 3.5–4.3 krad/min as determined with LiF thermoluminescent dosimeters. Samples were always kept in water baths at 28°C during exposure.

### Determination of radioactivity

Gel slices were digested in 0.5 ml Soluene-100 (Packard) per slice. All samples were counted in 10 ml of scintillation fluid (Omnifluor, 8 g/l, New England Nuclear Corp., in toluene: Triton X-100, 2:1). After temperature equilibration in the cold, samples were counted in a Packard Tri-Carb or an Intertechnique liquid scintillation spectrometer. Correction for counting efficiency was made with an automatic external standard.

## RESULTS

### Actinomycin D and RNA synthesis

In the first series of experiments, the extent and kinetics of inhibition of total RNA synthesis were determined for different doses of actinomycin D. Representative curves for 5 and 10  $\mu\text{g/ml}$  of drug are displayed in

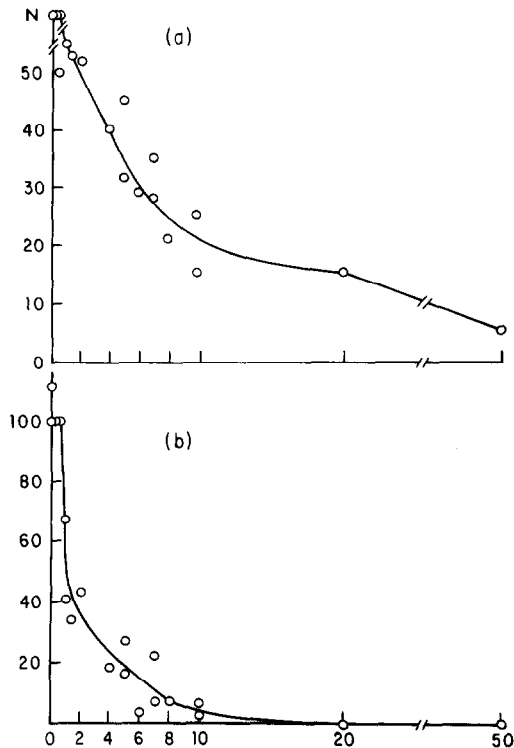


Fig. 2. Abscissa: actinomycin D conc. ( $\mu\text{g}/\text{ml}$ ); ordinate: (a) time to inhibit (min); (b) % of control rate.

The effect of concentration of actinomycin D on the inhibition of RNA synthesis in *Tetrahymena*. The data for each point were obtained from experiments similar to those shown in fig. 1. (a) The time for the drug to become effective is the interval between the time of drug addition (at 15 min on the abscissa of fig. 1) and the time at which the maximum change in rate of uridine incorporation is first noted (the intersection of the line for the inhibited rate with the control line). *N*, no inhibition was observed during the course of the experiment; (b) the degree of inhibition is expressed as the % of control rate. The slope of the inhibited portion of the kinetic curve is compared to the slope of the control curve as in fig. 1.

fig. 1. It is apparent that neither concentration is immediately effective against [ $^3\text{H}$ ]uridine incorporation (cf [26]). However, the larger dose acts sooner and more extensively than the smaller dose. The treated cultures can be compared to the control culture both in terms of the time after addition of the drug when the change in rate of incorporation is first noted and in terms of the

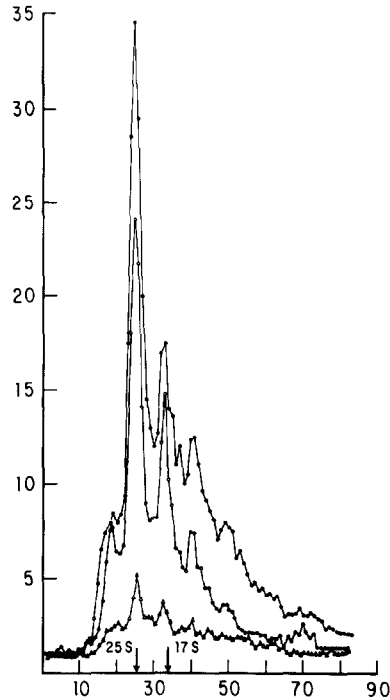


Fig. 3. Abscissa: slice no.; ordinate:  $^3\text{H}$  dpm  $\times 10^{-2}$ . Electrophoretic analysis of the [ $^3\text{H}$ ]uridine-labeled RNA synthesized in actinomycin D-treated *Tetrahymena*. Cultures of *Tetrahymena* were treated with 0, 1.0, or 10  $\mu\text{g}/\text{ml}$  actinomycin D. After a suitable time interval, determined from the data of figs 1 and 2, [ $^3\text{H}$ ]uridine was added to give 5  $\mu\text{Ci}/\text{ml}$ . Ten min later, the cells were harvested, and total RNA was isolated and separated by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Direction of migration is from left to right. After scanning at 260 nm to determine the positions of the mature 25S and 17S ribosomal RNAs (arrows), the gels were sliced and counted for radioactivity. ●, Control, 0.80  $A_{260}$  units of RNA applied to gel; ○, labeled from 85–95 min after the addition of 1  $\mu\text{g}/\text{ml}$  of drug, 0.54  $A_{260}$  units of RNA applied to gel, data normalized to 0.80  $A_{260}$  units; △, labeled from 10–20 min after 10  $\mu\text{g}/\text{ml}$  of drug, 0.60  $A_{260}$  units of RNA applied to gel, data normalized to 0.80  $A_{260}$  units.

extent of inhibition. These two parameters have been determined in this and similar experiments for concentrations of actinomycin D between 0.05 and 50  $\mu\text{g}/\text{ml}$  (fig. 2). No inhibition is observed with doses of actinomycin D below 0.5  $\mu\text{g}/\text{ml}$ . [ $^3\text{H}$ ]Uridine incorporation becomes progressively inhibited (up to 60–70%) as the dose is raised

Table 1. Comparison of the effect of actinomycin D on the synthesis of total RNA, Poly(A-) RNA, and Poly(A+) RNA in *Tetrahymena*

Actinomycin D			Spec. act. (% of control)	
Conc. ( $\mu\text{g/ml}$ )	Time of pulse (min)	Total RNA	Poly(A-) RNA	Poly(A+) RNA
1	85-95	57.4	50.7	77.0
2	75-85	34.1	30.9	46.0
5	15-25	22.8	28.7	32.2
5	35-45	8.2	7.9	9.4
10	10-20	8.0	7.2	1.2
10	35-45	1.4	1.7	0.3

Exponential cultures of *Tetrahymena* were treated with various concentrations of actinomycin D. At the indicated times after drug addition, the cultures were pulsed for 10 min with [ $^3\text{H}$ ]uridine ( $5 \mu\text{Ci/ml}$ ). RNA was extracted and fractionated on oligo (dT) cellulose columns. The specific activity was calculated from the [ $^3\text{H}$ ] dpm and  $A_{260}$  units for each fraction as well as from the total unfractionated RNA. In each case, the results are compared to a control from a non-drug treated culture labeled in parallel with the treated culture.

from 0.5 to  $2 \mu\text{g/ml}$  and is completely eliminated by  $10 \mu\text{g/ml}$  (fig. 2b). Further increases in the actinomycin D concentration allow a more rapid attainment of complete inhibition (fig. 2a).

The types of RNAs synthesized in actinomycin D-treated *Tetrahymena* were investigated by labeling the cells for 10 min with [ $^3\text{H}$ ]uridine at a time when the drug had become effective (as determined from fig. 2a). RNA synthesis and processing are very rapid in *Tetrahymena*, and mature labeled 25S and 17S rRNA are apparent by 5-7 min after addition of the radioactive precursor [28, 35, 39]. The RNA was extracted and electrophoresed on polyacrylamide-SDS gels (fig. 3). Increasing levels of actinomycin D ( $1.0$  and  $10 \mu\text{g/ml}$ ) lead to progressively reduced incorporation of [ $^3\text{H}$ ]uridine into positions corresponding to the mature 25S and 17S rRNAs (fig. 3, arrows) as well as into the 34-35S precursor rRNA. From these data, a pronounced decrease in rRNA synthesis in actinomycin D-treated cells can be readily discerned. However, it is not clear whether other RNAs are affected similarly.

To determine the extent to which the syn-

thesis of poly(A+) RNAs is depressed, RNA was again labeled by a 10 min pulse with [ $^3\text{H}$ ]uridine after treatment of the cells with various concentrations of actinomycin D. Total RNA was extracted and then fractionated into poly(A+) RNA and poly(A-) RNA on columns of oligo(dT) cellulose (table 1). Actinomycin D-induced altera-

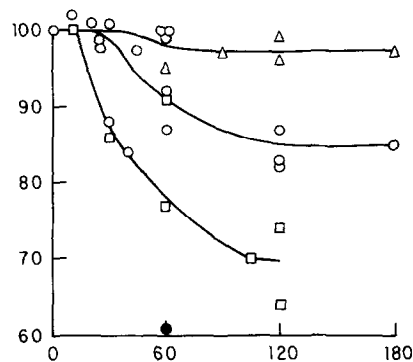


Fig. 4. Abscissa: time (min); ordinate: % of control polysomes.

The effect of time of exposure to actinomycin D on the polysome content of *Tetrahymena*. Exponential cultures of *Tetrahymena* (25 ml) received actinomycin D. After 0.5-180 min, the cells were harvested and the size distribution of polysomes determined on sucrose density gradients. In the untreated controls, the percentage of ribosomes in polysomes ( $\geq$  dimers) was between 80 and 90% in various experiments. The results of a series of experiments are reported in the figure.  $\Delta$ , 1;  $\circ$ , 5;  $\square$ , 10;  $\bullet$ , 50  $\mu\text{g/ml}$ .

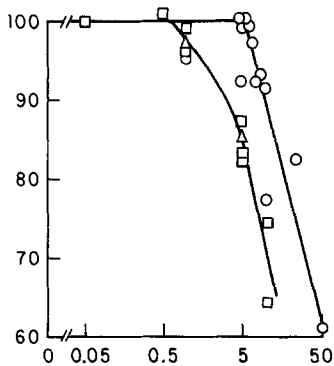


Fig. 5. Abscissa: actinomycin D conc. ( $\mu\text{g/ml}$ ); ordinate: % of control polysomes.

The dependence of the polysome content of actinomycin D-treated *Tetrahymena* on drug concentration and exposure time. Cultures of *Tetrahymena* were treated as in fig. 4, and part of these data are from the same series of experiments as presented in fig. 4. ○, 1; □, 2; △, 3 h-exposure. Note the logarithmic scale on the abscissa.

tions in [ $^3\text{H}$ ]uridine incorporation into total RNA, poly(A $-$ ) RNA and poly(A $+$ ) RNA were determined by comparing the specific radioactivities of RNAs from control and irradiated cells. Throughout the range of actinomycin D concentrations, 1–10  $\mu\text{g/ml}$ , the specific radioactivities of the two fractions were reduced to an approximately similar extent as each other and as the total RNA, showing clearly that rRNA synthesis is not preferentially inhibited even at the lowest effective concentrations (table 1). The differences observed at 1 and 2  $\mu\text{g/ml}$  are not great enough to warrant a conclusion of selectivity in view of the 10–200-fold differences observed in mouse L cells [7]. Similarly, at 10  $\mu\text{g/ml}$  both poly(A $+$ ) RNA and poly(A $-$ ) RNA are inhibited to less than 10% of the normal synthesis rate so that we do not feel that the differences are significant.

#### Actinomycin D and protein synthesis

Actinomycin D concentrations of greater than 1  $\mu\text{g/ml}$  lead to the dissociation of polysomes after a delay period (fig. 4). In

the experiments, the drug was continuously present. As the concentration is raised from 1 to 10  $\mu\text{g/ml}$  the lag period before dissociation decreases, the rate of polysome dissociation increases, and the extent of polysome loss increases. These data and others have been replotted in fig. 5 to show the concentration dependence of actinomycin D-induced polysome dissociation. Doses above 5  $\mu\text{g/ml}$  can elicit the dissociation of polysomes in a one-hour incubation. Two- to 4-fold lower concentrations are effective when the incubation period is 2 or 3 h (fig. 5).

The mechanism of the actinomycin D-induced dissociation of polysomes was investigated by measuring the relative rates of incorporation of [ $^{35}\text{S}$ ]methionine into *N*-terminal and internal positions of nascent peptides, which is a measure of the relative rates of the initiation and elongation phases of protein synthesis [38]. When 1  $\mu\text{g/ml}$  actinomycin D is added to cultures of *Tetrahymena*, incorporation into both *N*-terminal and internal positions continues at the control rate for some 30–40 min, after which the rate becomes reduced (fig. 6*b*). This reduction in rate is observed if the [ $^{35}\text{S}$ ]methionine is added immediately after the drug (at zero min) or 40 min later. Higher concentrations (5 or 10  $\mu\text{g/ml}$ ) elicit an earlier response, but the magnitude of change is similar to that of 1  $\mu\text{g/ml}$  actinomycin D, on the order of a 50% inhibition (fig. 6*c, d*). The time lag before any one of the drug concentrations elicits inhibition of methionine incorporation is similar to the time lag before polysome dissociation begins in the presence of the same concentration (fig. 4).

We previously showed that recovery of polysomes from  $\gamma$ -radiation-induced dissociation was partially prevented by 10  $\mu\text{g/ml}$  actinomycin D [30]. The experiment displayed in fig. 7 was designed to analyze

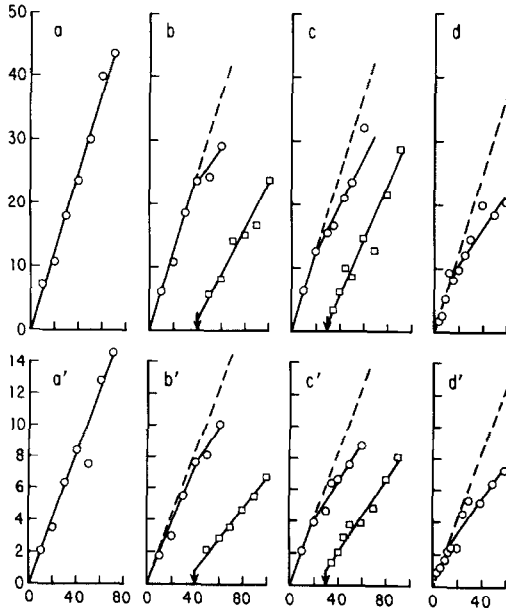


Fig. 6. Abscissa: time (min); ordinate:  $^{35}\text{S}$  dpm  $\times 10^{-3}$ .

The incorporation of [ $^{35}\text{S}$ ]methionine into *N*-terminal and internal positions of nascent peptides in actinomycin D-treated *Tetrahymena*. An exponentially-growing culture was concentrated 5-fold in growth medium, and 1 ml aliquots were incubated in 30 ml tubes. Control cells (a) were given 10  $\mu\text{Ci}$  [ $^{35}\text{S}$ ]methionine at zero min, and the culture was sampled (75  $\mu\text{l}$ ) at 10-min intervals. The resultant kinetic graphs (a, a') are reproduced in subsequent panels for comparison (---). Other cells (b, c, d) were treated with actinomycin D (b, 1; c, 5; d, 10  $\mu\text{g}/\text{ml}$ ) at zero min. [ $^{35}\text{S}$ ]Methionine (10  $\mu\text{Ci}$ ) was added either at zero min immediately after actinomycin D (O) or 30 or 40 min later (arrow,  $\square$ ). Seventyfive  $\mu\text{l}$  samples were removed at the indicated times and processed as described in Materials and Methods to determine the incorporation into *N*-terminal (a'-d') and internal (a-d) positions of nascent and completed peptides.

whether this actinomycin D effect resulted from inhibition of the synthesis of an RNA necessary for recovery or from a direct interference with the polysomes. Actinomycin D at 5 or 10  $\mu\text{g}/\text{ml}$  was added to cells at a time such that by the end of irradiation, it would be effective in inhibiting protein and RNA synthesis (cf figs 2, 4 and 6). In non-drug-treated cells, 40 krad results in the loss of  $\sim 30\%$  of the polysomes, and the polysome level returns to the control value

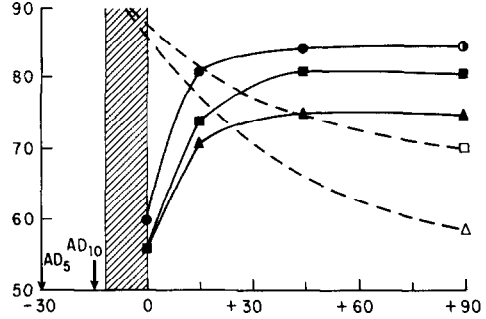


Fig. 7. Abscissa: time rel. to end of irradiation (min); ordinate: % ribosomes in polysomes.

Kinetics of dissociation and reassociation of polysomes in actinomycin D-treated and irradiated *Tetrahymena*. An exponential culture of *Tetrahymena* was divided into 25 ml aliquots. Some of these were irradiated with 40 krad (solid symbols) while others were not (open symbols). The interval in which the radiation was delivered is represented by the shaded area on the abscissa. The end of this interval is considered zero min. Some of the cultures received 5  $\mu\text{g}/\text{ml}$  actinomycin D at -30 min ( $\square$ ,  $\blacksquare$ ), some received 10  $\mu\text{g}/\text{ml}$  of drug at -15 min ( $\Delta$ ,  $\blacktriangle$ ), while others received no drug (O,  $\bullet$ ). The cells were harvested at the indicated times and the polysome content determined as described in Materials and Methods. The dashed lines are redrawn from fig. 4 and represent the loss of polysomes in drug-treated, unirradiated cells.

during the next 45-90 min (cf [31]). When actinomycin D is present, the shape of the recovery curve is the same as in the non-drug-treated cells, but the final extent of recovery is reduced. Actinomycin D alone, i.e., in unirradiated cells, causes a progressive loss in polysomes (cf fig. 4). By the time the polysomes of the irradiated, drug-treated cells have recovered to the full extent (90 min on the abscissa of fig. 7), the polysome content of the cells receiving only the drug is less than that of the cells receiving both drug and radiation.

## DISCUSSION

### *Actinomycin D and RNA synthesis*

The data presented here show clearly that actinomycin D is not a selective inhibitor of rRNA synthesis in *Tetrahymena*. Even at

the lowest effective concentrations, there is only a slightly greater inhibition of poly(A<sup>-</sup>) RNA than of poly(A<sup>+</sup>) RNA (fig. 3; table 1). This finding was initially unexpected for the following reasons: (a) Very low concentrations of actinomycin D, lower than those which are inhibitory to *Tetrahymena*, are 10–200 times more effective against rRNA synthesis in mammalian cells [6–8]; (b) actinomycin D binds to DNA through GC base pairs [2]. Therefore, GC-rich DNA is better able to bind the drug, and actinomycin D is a better inhibitor of transcription on GC-rich DNA [3]. In *Tetrahymena*, as in mammalian cells, the ribosomal genes have a greater content of GC base pairs than the bulk DNA and should, therefore, bind more actinomycin D than non-ribosomal RNA genes [39, 40]. (c) Actinomycin D produces striking morphological changes in the nucleoli (the sites of rRNA synthesis) after prolonged treatments of *Tetrahymena* [27] and other cells [e.g., 41]. These apparently contradictory observations could be resolved if the accessibility of the extrachromosomal rDNA of *Tetrahymena* were equal to or less than that of the chromosomal DNA and/or if the eventual inhibition of rRNA synthesis were secondary to an inhibition of the synthesis of a controlling element or ribosomal protein(s) which is encoded in a chromosomal gene.

In support of the first model, it should be noted that unlike the case in mammalian cells, the rRNA genes exist in *Tetrahymena* as single extrachromosomal molecules of DNA [42, 43]. Approx. 10000 rDNA gene copies are found per cell in exponentially growing cultures in 500–1000 nucleoli located around the periphery of the macronucleus [44, 45]. Perhaps the extrachromosomal location of these genes or the extent to which they are covered by nascent RNA

or protein allows them to assume a morphology which is less susceptible to direct attack by actinomycin D. A report by Mathis & Gorovsky [46] showing that chromatin containing the rDNA in *Tetrahymena* is apparently packaged in the same subunit structure as the bulk of the chromatin in *Tetrahymena* and other eukaryotes makes this possibility less likely. However, this point cannot be completely resolved until the binding of actinomycin D to nucleolar and main nuclear DNA are compared, since it is known that transcriptional changes which alter chromosome morphology affect the amount of bound actinomycin D [47, 48]. It has also been shown that the time of ribosomal gene replication is distinct in the cell cycle from the replication of the other nuclear DNA [49] and that the intracellular concentration of actinomycin D in *Tetrahymena* varies as a function of the cell cycle [50].

Perry & Kelley [7] have proposed that the greater sensitivity of 45S rRNA synthesis to actinomycin D in mouse L cells may be the result of the transcription of stretches of contiguous repetitive rRNA genes with RNA polymerase beginning transcription only at initiator regions which occur once in several gene repeats; binding of the drug near an entry site for the polymerase may cause the shut-down of a whole set of repeated genes. In a negative sense, the results in *Tetrahymena* may provide some support for their proposal. The rDNA copies in *Tetrahymena* are on single molecules of  $12.6 \times 10^6$  D. Because these molecules are palindromes, there are two copies of the rRNA cistrons on each molecule [40]. The binding of actinomycin D to any one molecule could inhibit RNA synthesis on at most two gene copies. Hence, if the model of Perry & Kelley [7] were correct, one would predict that where long stretches of

Table 2. The inhibition of RNA and protein synthesis by actinomycin D in *Tetrahymena*

Actinomycin D conc. ( $\mu\text{g/ml}$ )	RNA synthesis (fig. 2)		Polysome content (fig. 4)		Methionine incorporation (fig. 6)	
	Lag time (min)	Inhibition (%)	Lag time (min)	Inhibition <sup>a</sup> (%)	Lag time (min)	Inhibition (%)
1	60	30-60	40-60	5	40	27-56
5	30-45	70-85	20-30	15	20	25-36
10	15-25	95	10-20	30	10-20	42-50

This table is a summary of the data from all experiments such as those in figs 2, 4, and 6. The range of observed lag times and range of inhibitions are recorded.

<sup>a</sup> Average value of plateau.

tandem rDNA repeats do not occur, actinomycin D would not be more inhibitory to rRNA synthesis.

The second model, that of an indirect action of actinomycin D on rRNA synthesis and nucleolar morphology, has a precedent in the indirect action of a variety of adenosine analogs on the production of "nucleolar necklaces" in cultured chick embryo fibroblasts [51, 52]. Exposure of these cells to actinomycin D leads to unraveling of the nucleoli into formations having the appearance of beaded strands, and Granick [51, 52] has proposed that these effects are secondary to inhibition of the synthesis of mRNA for ribosomal protein(s). Similar alterations in nucleolar ultrastructure are produced by many drugs, including some cancer chemotherapeutic agents other than actinomycin D, such as daunomycin, toyocamycin, and bleomycin [53].

#### *Actinomycin D and protein synthesis*

Actinomycin D inhibits methionine incorporation (fig. 6) and leads to the dissociation of polysomes (fig. 4). The lag time for these actions at the three concentrations tested is slightly less than the lag time for inhibition of total RNA synthesis (fig. 2, table 2). The more rapid inhibition of protein synthesis is consistent with a direct

action of actinomycin D on translation; however, further analysis of these data suggests that some of the effects on translation are probably secondary to effects on transcription.

Actinomycin D causes a similar inhibition of methionine incorporation into *N*-terminal and internal positions of nascent chains (fig. 6), which we interpret as an inhibition of elongation alone or of both initiation and elongation. The assay employed measures the incorporation of methionine into acid-precipitable material, eliminating the smallest nascent peptides. As we showed previously with other agents [38], specific inhibitors of initiation reduce incorporation into *N*-terminal methionine either earlier or more extensively than incorporation into internal positions; agents which are specific for elongation block incorporation into both *N*-terminal and internal positions; and those which inhibit elongation and, to a similar or lesser extent, initiation, will appear as inhibitors of elongation. The fact that the polysome content of these cells also decreases in the presence of actinomycin D (fig. 4) is consistent with a concomitant inhibition of initiation.

There is a drug concentration-dependence for the effects of some, but not all, of the parameters of protein synthesis; in-

creasing the concentration of actinomycin D allows a more rapid attainment of inhibition of polysome content and methionine incorporation and a greater net loss of polysomes, but all three doses tested result in a similar (approx. 40–50%) inhibition of methionine incorporation. The present data do not allow a complete description of the mechanism of these responses, but we offer two hypotheses: (1) actinomycin D within the concentration range studied interferes with the function of a factor which couples ribosome movement to peptide-bond synthesis. Hence, peptide bond synthesis may be inhibited by a constant factor (40%) at all concentrations tested, occurring more rapidly at the higher drug levels. An acceleration in ribosome movement (translocation) may depend upon the concentration-dependent removal of this putative factor from the ribosome, resulting in a decreased polysome level once a new steady-state is reached; or (2) actinomycin D becomes concentrated either on the ribosome or on a requisite factor for protein synthesis, reducing the rate of elongation by 40%. In this case, the loss of polysomes may be a secondary effect of a concentration-dependent decrease in the synthesis of mRNA or of a regulatory RNA, such as that proposed by Goldstein & Penman [16].

We previously showed that when exponential cultures of *Tetrahymena* are exposed to 40 krad of  $\gamma$ -radiation, the polysome content initially is decreased and then recovers to control levels over the next 45–90 min [31, 54; cf fig. 7]. In addition, rRNA synthesis in the irradiated cells is preferentially inhibited and then recovers in approx. 90 min, while poly(A+) RNA (mRNA) is synthesized at a supranormal rate during the entire 2 h post-irradiation period [35]. Actinomycin D-treated *Tetrahymena*, on the other hand, show an almost

parallel decrease in the synthesis of both rRNA and poly(A+) RNA (figs 3 and 4). In cells exposed to both  $\gamma$ -radiation and actinomycin D, the polysome content is initially depressed by the radiation but subsequently recovers at a rate comparable to that of the cells receiving only radiation. This suggests that there is no direct effect of the drug on initiation of polysome formation. The final level of recovered polysomes in radiation-plus-drug-treated cells is less than in the untreated control, but greater than in the corresponding drug-treated unirradiated cells (fig. 7). In other words, irradiation provides an apparent protection of the polysomes against actinomycin D-induced dissociation. This may result from the increased amounts of mRNA synthesized in the irradiated cells before the end of the irradiation at which time the drug should have become effective in inhibiting RNA synthesis.

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## REFERENCES

1. Reich, E & Goldberg, I, Progress in nucleic acid research and molecular biology (ed J Davidson & W Cohn), vol. 3, p. 183. Academic Press, New York (1964).
2. Sobell, H, Jain, S, Sakore, T & Nordman, C, Nature new biol 231 (1971) 200.
3. Goldberg, I, Rabinowitz, M & Reich, E, Proc natl acad sci US 48 (1962) 2094.
4. Goldberg, I & Rabinowitz, M, Science 136 (1962) 315.
5. Reich, E, Franklin, R, Shatkin, A & Tatum, E, Proc natl acad sci US 48 (1962) 1238.
6. Perry, R P, Proc natl acad sci US 48 (1962) 2179.
7. Perry, R & Kelley, D, J cell physiol 76 (1970) 127.
8. Cheevers, W & Sheinin, R, Biochim biophys acta 204 (1970) 449.
9. Stewart, G & Farber, E, J biol chem 243 (1968) 4479.
10. Rovera, G, Berman, S & Baserga, R, Proc natl acad sci US 65 (1970) 876.
11. Scholtissek, C, Eur j biochem 28 (1972) 70.

12. Penman, S, Scherrer, K, Becker, Y & Darnell, J, Proc natl acad sci US 49 (1963) 654.
13. Staehelin, T, Wettstein, F & Noll, H, Science 140 (1963) 180.
14. Craig, N, Kelley, D & Perry, R, Biochim biophys acta 246 (1971) 493.
15. Craig, N, J cell physiol 82 (1973) 133.
16. Goldstein, E & Penman, S, J mol biol 80 (1973) 243.
17. Singer, R & Penman, S, Nature 240 (1972) 100.
18. Murphy, W & Attardi, G, Proc natl acad sci US 70 (1973) 115.
19. Mita, T, Biochim biophys acta 103 (1965) 182.
20. Moner, J, Exp cell res 45 (1967) 618.
21. Satir, B, Exp cell res 48 (1967) 253.
22. Yuyama, S, Exp cell res 90 (1975) 381.
23. Frankel, J, J exp zool 159 (1965) 113.
24. Lazarus, L, Levy, M & Scherbaum, O, Exp cell res 36 (1964) 672.
25. Nachtwey, D & Dickinson, W, Exp cell res 47 (1967) 581.
26. Leick, V, Eur j biochem 8 (1969) 215.
27. Satir, B & Dirksen, E R, J cell biol 48 (1971) 143.
28. Eckert, W A, Franke, W W & Scheer, U, Exp cell res 94 (1975) 31.
29. Cameron, I, Griffin, E & Rudick, M, Exp cell res 65 (1971) 265.
30. Kuncio, G S, Rustad, R C & Oleinick, N L, Biochem biophys res comm 48 (1972) 457.
31. Oleinick, N L, Rustad, R C & Kuncio, G S, Biochim biophys acta 366 (1974) 215.
32. Ernst, S G, Rustad, R C & Oleinick, N L, Int j radiat biol 28 (1975) 67.
33. Byfield, J E & Scherbaum, O H, Anal biochem 17 (1966) 434.
34. Perry, R P, La Torre, J, Kelley, D E & Greenberg, J R, Biochim biophys acta 262 (1972) 220.
35. Ernst, S G, Oleinick, N L & Rustad, R C. Submitted for publication.
36. Loening, U E, Biochem j 102 (1967) 251.
37. Aviv, H & Leder, P, Proc natl acad sci US 69 (1972) 1408.
38. Oleinick, N L & Salengo, J J, Anal biochem 73 (1976) 27.
39. Leick, V, Eur j biochem 8 (1969) 221.
40. Karrer, K M & Gall, J G, J mol biol 104 (1976) 421.
41. Scheer, U, Trendelenburg, M & Franke, W, J cell biol 65 (1975) 163.
42. Engberg, J, Nilsson, J R, Pearlman, R E & Leick, V, Proc natl acad sci US 71 (1974) 894.
43. Gall, J G, Proc natl acad sci US 71 (1974) 3078.
44. Nilsson, J & Leick, V, Exp cell res 60 (1970) 361.
45. Yao, M., Kimmel, A & Gorovsky, M, Proc natl acad sci US 71 (1974) 3082.
46. Mathis, D J & Gorovsky, M A, J cell biol 67 (1975) 265a.
47. Brachet, J & Hulin, N, Exp cell res 59 (1970) 486.
48. Cionini, P G & Avanzi, S, Exp cell res 75 (1972) 154.
49. Andersen, H A & Engberg, J, Exp cell res 92 (1975) 159.
50. Cleffmann, G, Fehrendt, I & Behrendt, W, Exp cell res 87 (1974) 139.
51. Granick, D, J cell biol 65 (1975) 389.
52. — Ibid 65 (1975) 418.
53. Daskal, Y, Crooke, S, Smetana, K & Busch, H, Cancer res 35 (1975) 374.
54. Oleinick, N L & Rustad, R C, Radiat res 64 (1975) 509.

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