INVESTIGATIONS OF THE MECHANISM OF CELL KILLING INDUCED BY ACTINOMYCIN D

Thesis by

Mark James Miller

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To Gina

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ABSTRACT

At high concentration, actinomycin D kills the protozoan Chlamydomonas reinhardi with exponential kinetics. The rate of killing is dependent upon the temperature of incubation. This dependence is partially a function of the increased extent of binding of the drug at the higher temperature (33°C), but sensitivity of the cell must also be stimulated by the higher temperature. While actinomycin D probably kills cells in a reaction which requires binding to DNA, there is no correlation between the lethal event and the inhibition of macromolecular synthesis or the breakdown of macromolecules.

I have developed a simple model to explain the difference in sensitivity of various species of RNA to actinomycin D inhibition. This model predicts that frequently transcribed genes will be much more sensitive to the drug than infrequently transcribed genes.

Mutants of Chlamydomonas reinhardi have also been isolated which are both temperature sensitive in growth and resistant to killing by actinomycin D. These mutants, unlike other actinomycin D-resistant cell lines, are neither impermeable to the drug nor do they excrete it at an accelerated rate. The mutants are partially temperature sensitive in their ability to synthesize RNA. In the presence of actinomycin D, however, RNA synthesis is partially protected at the nonpermissive temperature (and, in some cases, at the permissive temperature also) when compared to the inhibition of wild type cells.

Extraction and examination of RNA from these mutants reveals that actinomycin D inhibits different species of RNA to different extents.

I propose that the mutants have an altered chromosomal constituent, which impedes the binding to the genome. At the nonpermissive temperature the alteration is postulated to partially interfere with transcription.

In the course of these experiments it became necessary to determine the maturation pathways of the ribosomal RNA species of Chlamydomonas reinhardi. Cytoplasmic rRNAs of C. reinhardi are cleaved from a single precursor of molecular weights $2.4 \cdot 10^6$ to a mature rRNA ($0.69 \cdot 10^6$mol.wt) and a $1.4 \cdot 10^6$-mol. wt precursor of the mature $1.3 \cdot 10^6$-mol. wt rRNA. The kinetics of incorporation of radioactive label into the rRNAs suggest that the $0.69 \cdot 10^6$-mol. wt rRNA gene is located closer to the promotor than is the gene for the $1.4 \cdot 10^6$-mol. wt rRNA. The synthesis of cytoplasma rRNAs is extremely sensitive to camptothecin, an inhibitor of nuclear rRNA synthesis, but synthesis of chloroplast rRNA is quite resistant to the inhibitor. This has allowed us to demonstrate that chloroplast rRNAs are processed from precursors which resemble those of blue-green algae. A $1.14 \cdot 10^6$-mol. wt precursor is processed to the $1.07 \cdot 10^6$-mol. wt mature chloroplast rRNA, and a $0.64 \cdot 10^6$-mol. wt precursor is cleaved to a $0.56 \cdot 10^6$-mol wt species and then to the mature $0.54 \cdot 10^6$-mol. wt rRNA. This study demonstrates two new ways in which the function of the chloroplast genome resembles those of prokaryotes more than those of the nucleus.

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GENERAL INTRODUCTION

General Introduction

Actinomycin D (AMD) is a widely used inhibitor of DNA-dependent RNA synthesis. The drug acts by intercalating between guanosine-cytosine base pairs of DNA and blocking the progress of RNA polymerase (3,7,9).

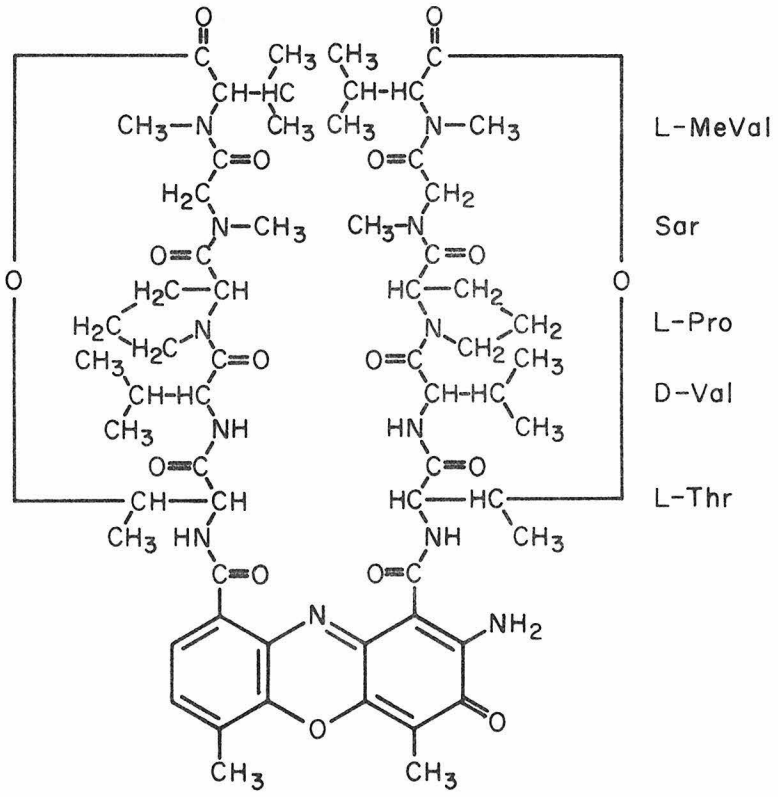
The specificity of AMD's action has been called into question by the broad range of effects the drug has on cellular processes. In addition to RNA, the drug inhibits the synthesis of DNA (6) and protein (4,5). It can also cause the "superinduction" of several inducible proteins such as ovalbumine and tyrosine aminotransferase (13, 20,24). AMD has also been reported to induce the degradation of RNA (19) and to inhibit RNA transport and processing (2,8). Finally, AMD is very toxic (18), but the toxicity does not correlate well with the degree of inhibition of RNA synthesis among different cell types (16) nor does the drug have the same specificity of tumor killing as do other inhibitors of RNA synthesis (15).

I have used the toxicity of AMD to isolate a series of AMD-resistant, temperature-sensitive mutants (ART mutants) of the alga Chlamydomonas reinhardi. These mutants are unique in that, unlike other resistant cell lines, they are permeable to the drug (1,14) and do not excrete it at an accelerated rate (17,21). Because I found that the synthesis of RNA in the ART mutants was partially protected from AMD inhibition and was also partially temperature-sensitive in the absence of the drug, it became of interest to study the synthesis

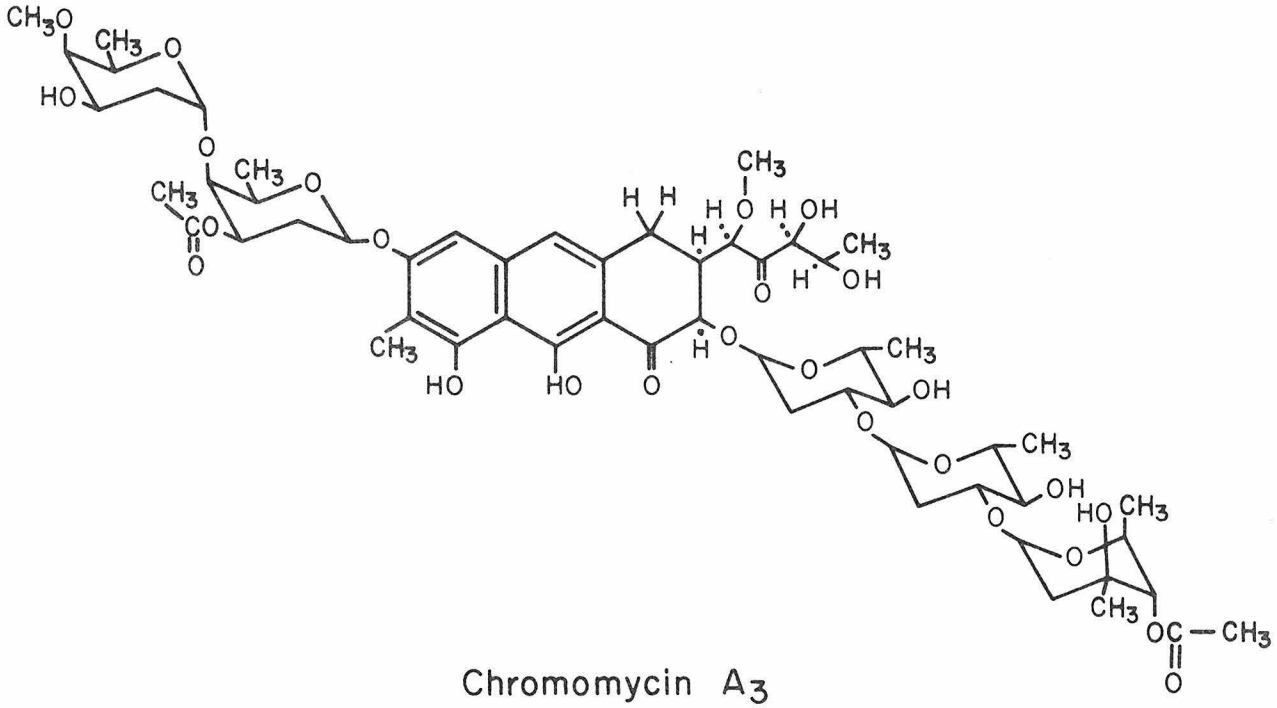
of RNA in wild type and mutant strains of C. reinhardi. As a result of these investigations, I determined the maturation pathways of both cytoplasmic and chloroplast ribosomal RNA in wild type C. reinhardi (11). The effects of AMD on RNA synthesis in mutant and wild type cells were also studied (10), as were the general effects of the drug on the synthetic processes of wild type cells (12). Finally, I have compared the toxicity of AMD to the toxicity of chromomycin A_3, a drug which, like AMD, inhibits RNA synthesis by binding to guanosine-cytosine-rich regions of DNA (22) but which does not bind by intercalation (23), and whose chemical structure differs greatly from that of AMD (fig. 1). These studies indicate that AMD kills primarily by binding to cellular DNA, although there is no simple relationship between its inhibition of nucleic acid synthesis and its killing.

Figure 1

Actimomycin D and Chromomycin A_3 (7)



Actinomycin D



Chromomycin A_3

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CHAPTER I:

SYNTHESIS AND MATURATION OF CHLOROPLAST AND CYTOPLASMIC RIBOSOMAL RNA IN *CHLAMDOMONAS REINHARDI*.

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SYNTHESIS AND MATURATION OF CHLOROPLAST AND CYTOPLASMIC RIBOSOMAL RNA IN *CHLAMYDOMONAS REINHARDI*

MARK J. MILLER and DANIEL McMAHON

Division of Biology, California Institute of Technology, Pasadena, Calif. 91109 (U.S.A.)

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Summary

The work of a large number of people has shown that the ribosomes of the chloroplast differ from those of the cytoplasm in the spectrum of their sensitivity to inhibitors of protein synthesis, the size of their rRNAs, and in the sensitivity of chloroplast rRNA synthesis to rifamycin SV. The genes for chloroplast rRNAs reside on the chloroplast DNA while those for cytoplasmic rRNAs are found on the nuclear DNA. We have characterized the synthesis and processing of cytoplasmic and chloroplast rRNAs of the alga *Chlamydomonas reinhardi* in order to further elucidate the difference between chloroplast and nuclear genetic systems.

Cytoplasmic rRNAs of *C. reinhardi* are cleaved from a single precursor of molecular weights $2.4 \cdot 10^6$ to a mature rRNA ($0.69 \cdot 10^6$ mol. wt) and a $1.4 \cdot 10^6$-mol. wt precursor of the mature $1.3 \cdot 10^6$-mol. wt rRNA. The kinetics of incorporation of radioactive label into the rRNAs suggest that the $0.69 \cdot 10^6$-mol. wt rRNA gene is located closer to the promotor than is the gene for the $1.4 \cdot 10^6$-mol. wt rRNA. The synthesis of cytoplasmic rRNAs is extremely sensitive to camptothecin, an inhibitor of nuclear rRNA synthesis, but synthesis of chloroplast rRNA is quite resistant to the inhibitor. This has allowed us to demonstrate that chloroplast rRNAs are processed from precursors which resemble those of blue-green algae. A $1.14 \cdot 10^6$-mol. wt precursor is processed to the $1.07 \cdot 10^6$-mol. wt mature chloroplast rRNA, and a $0.64 \cdot 10^6$-mol. wt precursor is cleaved to a $0.56 \cdot 10^6$-mol. wt species and then to the mature $0.54 \cdot 10^6$-mol. wt rRNA. This study demonstrates two new ways in which the function of the chloroplast genome resemble those of prokaryotes more than those of the nucleus.

Introduction

Chlamydomonas reinhardi, and other plant species, contain two major classes of ribosomes. Those found in the cytoplasm resemble the cytoplasmic

ribosomes of eukaryotes in general while those in the chloroplast resemble the ribosomes of prokaryotes [1—4]. Although *C. reinhardi* contain mitochondria, mitochondrial ribosomes form an insignificant fraction of cellular ribosomes. Examination of thin sections shows that the frequency of ribosomes within mitochondria is less than 1% of the frequency of those within the chloroplast and purified mitochondria are quite poor in ribosomes (Goodenough, U., personal communication). *C. reinhardi* is an organism, therefore, where the synthesis and processing of cytoplasmic and chloroplast RNAs can be compared without serious interference from mitochondrial rRNA syntheses.

The rRNAs of chloroplast and cytoplasm differ in a number of respects including their size [1,2,5] and the location of the genes which encode their primary structure [7—9]. The synthesis of chloroplast but not nuclear RNAs is sensitive to rifamycin SV [10], again resembling RNA synthesis in prokaryotes. Rifamycin SV also seems to be an agent which specifically disrupts the chloroplast genetic system as opposed to that of the mitochondrion. When *C. reinhardi* is treated with rifamycin SV, the structure of the chloroplast becomes disrupted and its content of ribosomes becomes severely depleted while the structure and ribosomal content of the mitochondria is unaffected [11] (Goodenough, U., personal communication).

We have examined the synthesis and processing of chloroplast and cytoplasmic rRNAs in *C. reinhardi* for several reasons. First, to re-investigate a report that its cytoplasmic rRNAs were not made from a single high molecular weight precursor [12]. If this were true it would be unique among the eukaryotes. In addition we wished to determine whether camptothecin, an agent which inhibits RNA synthesis in eukaryotes, but not in prokaryotes [13,14] was a more potent inhibitor of nuclear than chloroplast rRNA synthesis. This antibiotic then allowed us to demonstrate that precursors of chloroplast rRNA which resemble those of blue-green algae are made in *C. reinhardi*. Finally, McMahon and Langstroth [15] have demonstrated total RNA synthesis of *C. reinhardi* is under "stringent" control but that this control is regulated in a manner which differs substantially from prokaryotes. In order to investigate the precise locus of this control it is important to determine the pathways of RNA synthesis.

Materials and Methods

Chemicals

Radioactive chemicals were purchased from Schwarz/Mann. Diethyl oxydiformate was the product of Eastman Kodak Co., Rochester, N.Y. Sodium-*p*-aminosalicylate was from K and K laboratories, Hollywood, Calif. Sodium dodecylsulfate was purchased from Alcolac Chemical Co., Baltimore, Md. Rifamycin SV was purchased from Calbiochem, San Diego, Calif. and ribonuclease-free deoxyribonuclease was purchased from Worthington Biochemical Corp., Freehold, N.J. Both phenol and *m*-cresol were obtained from Mallinckrodt Chemical Works, St. Louis, Mo. The phenol was distilled over powdered zinc before use. The *m*-cresol was repeatedly distilled over zinc in vacuo until it was colorless and then stored under N_2 at $-20^{\circ}C$. Actinomycin D and camptothecin were the gifts of Dr Walter Gall (Merck and Co., Rahway, N.J.) and Dr Harry Wood (National Cancer Institute, Bethesda, Md.), respectively.

Cells and culture conditions

Two strains of *C. reinhardi* were used: *arg* 2, a mutant-lacking argino-succinate synthetase [16] was obtained from Dr W. Ebersold, Department of Biology, University of California, Los Angeles; CW-15, a mutant which lacks a normal cell wall [17] was obtained from Dr D. Roy Davies, John Innes Institute, Norwich, England. All cells were grown in HSM medium [18] containing in addition 24 mM sodium acetate. For *arg* 2 the medium was supplemented with 0.57 mM arginine. When cells were labeled with $^{32}PO_4^{3-}$ the medium was prepared with only 1% of its normal content of phosphate (0.133 mM PO_4^{3-}). In this case the medium was buffered with 20 mM Tris—HCl (pH 7.2). Cells grew at normal rates for more than 24 h in this medium. Cells were always grown at 22°C and a light intensity of 1500 lux to a concentration of $1 \cdot 10^6 - 2 \cdot 10^6$/ml.

Extraction of RNA

Method A: 25 ml of CW-15 cells were poured over 6—7 g of ice and then centrifuged at 1000 × *g* for 2 min. This and all subsequent steps were performed at 0—4°C. The lysis buffer was prepared just prior to centrifugation by adding 0.6 g of sodium-*p*-aminosalicylate, 0.2 ml of diethyl oxydiformate and 1 ml of 20% sodium dodecylsulfate to 10 ml of Buffer I of Brown and Haselkorn [19]. It was poured onto the cell pellet and the cells were disrupted by Vortex mixing. 10 ml of phenol—*m*-cresol—8-hydroxyquinoline (90:10:0.1, v/v/w) solution saturated with Buffer I were added and the mixture was shaken intermittently for 15 min. It was then centrifuged for 20 min at 900 × *g*. The lower, organic layer was removed and the aqueous layer re-extracted with phenol—*m*-cresol—8-hydroxyquinoline and centrifuged. After removal of the phenol—*m*-cresol—8-hydroxyquinoline solution, the aqueous layer was extracted with a solution of 4% iso-amyl alcohol in chloroform. Finally, 25 ml of ethanol were added to the aqueous layer and nucleic acids were allowed to precipitate for several hours at −25°C. The pellet was collected by centrifugation (11 000 × *g* for 20 min) and treated with deoxyribonuclease by the method of Brown and Haselkorn [19], with the exception that the final extraction was done twice with two volumes of a 1:1 mixture of phenol—*m*-cresol—8-hydroxyquinoline and chloroform—iso-amyl alcohol.

Method B: Cells with normal walls were harvested as above and the pellet was resuspended in 10 ml of Buffer I. The cells were disrupted using a French pressure cell operated at a pressure of less than 1600 lb/inch^2 and collected into a tube containing 0.2 ml diethyl oxydiformate. Sodium dodecylsulfate and sodium-*p*-aminosalicylate were added to the same concentrations as in Method A and the lysate extracted with phenol—*m*-cresol—hydroxyquinoline and chloroform—iso-amyl alcohol as in Method A.

Gel electrophoresis

Polyacrylamide-agarose gels were made by the method of Simmons and Strauss [20]. Electrophoresis was performed at 0—4°C using a buffer consisting of 0.04 M Tris, 0.02 M sodium acetate, 0.001 M EDTA (pH 7.8) and containing 0.1% sodium dodecylsulfate. After electrophoresis (2.2% acrylamide, 0.4% agarose) they were sliced on a Mickle gel slicer into 1.0-mm thick slices. The RNA

in the slices was hydrolyzed for at least 2 h at 70°C in 0.5 ml concentrated NH_4OH. The samples were cooled and, after evaporation of the ammonia, dioxane-based scintillation fluid was added and the samples were counted. The molecular weights of the species of RNA detected on the gel were assigned on the basis of the known proportionality between distance migrated and log molecular weight [21,22] using the molecular weights of the internal markers of *C. reinhardi* rRNAs as standards. These were assumed to be $1.30 \cdot 10^6$, $0.69 \cdot 10^6$, $1.07 \cdot 10^6$ and $0.54 \cdot 10^6$ molecular weights for the cytoplasmic 25-S and 18-S rRNAs and the chloroplast 23-S and 16-S rRNAs, respectively [23].

Results

Isolation of RNA

The rRNAs and their precursors of *C. reinhardi* are extremely labile. Several techniques for the isolation of RNA [1,19,23,24] failed to extract precursors; even the mature rRNAs were often degraded. A wide variety of other techniques and buffers also failed to extract a possible precursor to rRNAs. The extreme difficulty we encountered in developing an isolation procedure probably explains the failure of Wilson and Chiang [12] to isolate a precursor of the cytoplasmic rRNAs. Our initial success occurred using a mutant of *C. reinhardi* which lacks a cell wall, CW-15. In contrast to wild-type cells, these cells lysed instantaneously in the detergent, and polyacrylamide gel electrophoresis revealed potential precursors of the rRNAs. The presence of the nuclease inhibitor, diethyl oxydiformate, in the isolation buffer is critical for success, as is the requirement for rapid cooling before centrifugation. Omission of sodium-*p*-aminosalicylate from the lysis buffer reduces the yield of precursor. Precursors could also be isolated from wild-type cells by disrupting the wall in the French pressure cell (Method B) demonstrating that the presence of precursor is indeed a characteristic of normal cells and not a secondary mutation of CW-15.

Synthesis and processing of cytoplasmic rRNAs

Fig. 1 illustrates the pattern of incorporation of label into RNA as a function of the time of labeling. A molecule of molecular weight $2.4 \cdot 10^6$ ($\pm 0.1 \cdot 10^6$) is the first discrete species to be labeled by $^{32}PO_4^{3-}$. Incorporation of $^{32}PO_4^{3-}$ into a $1.4 \cdot 10^6$-mol. wt species occurs almost as rapidly. Label accumulates in a $2.2 \cdot 10^6$-mol. wt ($\pm 0.1 \cdot 10^6$) molecule (difficult to resolve from the $2.4 \cdot 10^6$-mol. wt molecule) and in molecules close to the molecular weight of the mature rRNAs as the period of labeling continues. A minor peak of molecular weight $1.14 \cdot 10^6$ ($\pm 0.05 \cdot 10^6$) is also labeled. This will be shown later to be a precursor of the $1.07 \cdot 10^6$-mol. wt chloroplast rRNA.

The pattern of incorporation of label into RNAs is very similar to that found in several other plant species [25—28]. The molecular weights of the molecules and their order of appearance are in accord with the scheme for synthesis of cytoplasmic rRNA presented in Fig. 2. The position occupied by the $2.2 \cdot 10^6$-mol. wt molecule is ambiguous and will be discussed later.

The scheme in Fig. 2 is supported by the experiments illustrated in Fig. 3

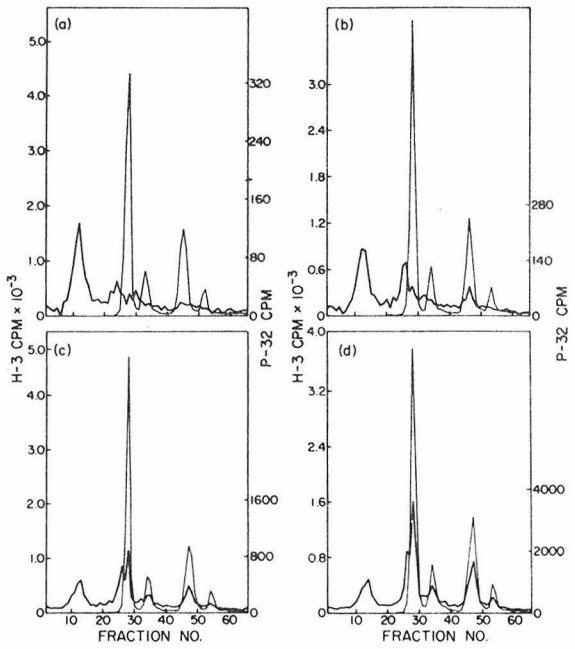

Fig. 1. Synthesis and processing of rRNAs. CW-15 cells were incubated in 0.1 μCi/ml [^{3}H]adenine (22 Ci/mM) for 3 h to label mature rRNAs. They were washed twice and resuspended in 0.133 mM phosphate–20 mM Tris–HCl–HSM (pH 7.2) medium and then divided into four equal cultures. The cultures were pulse labeled with $^{32}PO_4^{3-}$ (20 μCi/ml) for: a, 5 min; b, 10 min; c, 20 min; d, 30 min. The pattern of incorporation of [^{3}H]adenine is drawn with thin lines, the $^{32}PO_4^{3-}$ with thick ones. The ^{3}H cpm scale is omitted in subsequent figures, but is of similar magnitude.

in which actinomycin D was used to stop incorporation of $^{32}PO_4^{3-}$ into RNA and the flow of precursor RNAs into mature rRNA observed. In this and similar experiments the ^{32}P does not appear to chase from the $2.4 \cdot 10^6$-mol. wt species through the $2.2 \cdot 10^6$-mol. wt species of RNA but instead appears to directly enter the pools of $0.69 \cdot 10^6$ and $1.4 \cdot 10^6$-mol. wt RNAs.

Actinomycin D does not inhibit chloroplast rRNA synthesis very effectively. This may be the result of a permeability barrier for actinomycin D at the chloroplast membrane, or it may reflect a lower affinity of chloroplast DNA for actinomycin D because of its low content of guanine plus cytosine [29].

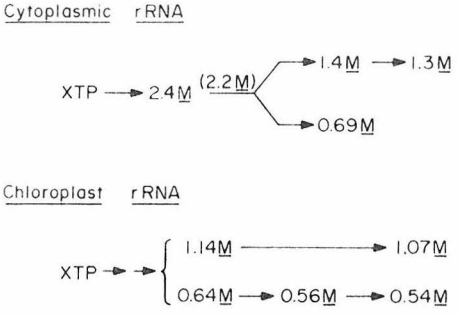

Fig. 2. Maturation pathway of cytoplasmic and chloroplast ribosomal RNA.

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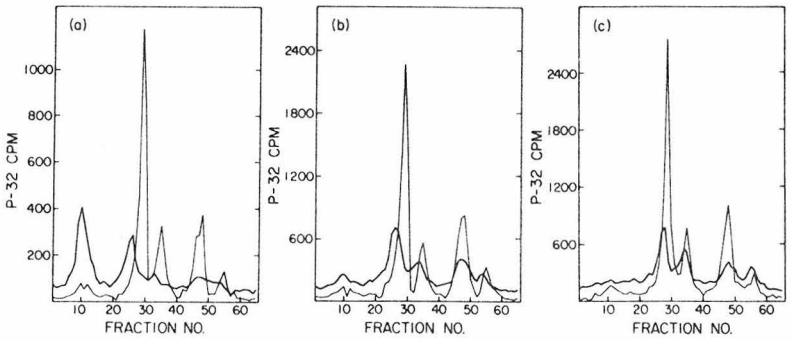

Fig. 3. Processing of rRNA precursors in the presence of actinomycin D. Cells of CW-15 were labeled for 2 h with 0.5 μCi/ml [^{3}H]adenine (27 Ci/mM). Their RNA was extracted and mixed with RNA extracted from cells which had been labeled for 10 min with $^{32}PO_4^{3-}$ (40 μCi/ml) and chased for varying lengths of time using actinomycin D (40 μg/ml). a, 10 min $^{32}PO_4^{3-}$ pulse; b, pulse followed by a 10-min actinomycin D chase; c, 20-min chase. The $^{32}PO_4^{3-}$ cpm are indicated by the thick lines.

Rifamycin SV, an inhibitor of chloroplast rRNA polymerase [10] reduces incorporation of $^{32}PO_4^{3-}$ into the chloroplast rRNA by 80—90%. It has no effect on the synthesis of the $2.4 \cdot 10^6$, $1.4 \cdot 10^6$, $1.3 \cdot 10^6$ or $0.69 \cdot 10^6$-mol. wt rRNAs (Fig. 4). Similarly, the absence of these molecules in cells labeled in the presence of camptothecin (Fig. 5) also supports their nuclear origin.

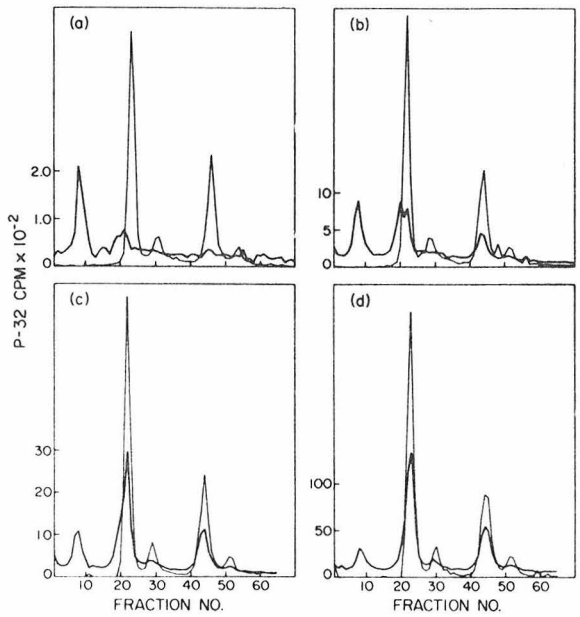

Fig. 4. The effect of rifamycin SV upon the pattern of incorporation of $^{32}PO_4^{3-}$ into RNA. CW-15 cells were labeled overnight in 0.5 μCi/ml [^{3}H]adenine (0.84 Ci/mM) then washed and resuspended in 0.133 mM phosphate—20 mM Tris–HCl—HSM (pH 7.2) medium + 500 μg/ml rifamycin SV and divided into four equal aliquots. $^{32}PO_4^{3-}$ (30 μCi/ml) was added after 1 h and the cells were incubated for: a, 5 min; b, 15 min; c, 30 min; d 45 min. The $^{32}PO_4^{3-}$ cpm are indicated by the thick lines.

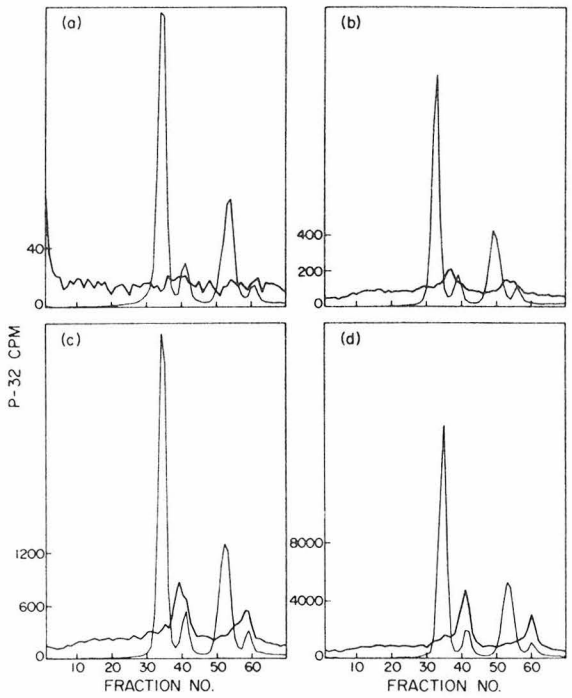

Fig. 5. Incorporation of $^{32}PO_4^{3-}$ into RNA in the presence of camptothecin. CW-15 cells were labeled overnight in 0.5 μCi/ml [^{3}H]adenine (0.84 Ci/mM). They were then washed and resuspended in 0.133 mM phosphate–20 mM Tris—HCl—HSM (pH 7.2) medium and camptothecin (8 μg/ml). The culture was divided into four aliquots and pulse labeled with 40 μCi/ml $^{32}PO_4^{3-}$. Lengths of $^{32}PO_4^{3-}$ incubation: a, 8 min; b, 15 min; c, 30 min; d, 60 min. The $^{32}PO_4^{3-}$ cpm are indicated by the thick lines.

Processing of chloroplast rRNA

Camptothecin inhibits the incorporation of precursors into cytoplasmic rRNA in *C. reinhardi* at lower concentrations than those which inhibit chloroplast rRNA synthesis (Table I). Therefore, we examined the pattern of $^{32}PO_4^{3-}$ incorporation into RNA in cells which had been inhibited with camptothecin. Fig. 5 shows that isotope initially appeared in species of RNA whose molecular weight was $1.14 \cdot 10^6$ ($\pm 0.05 \cdot 10^6$) and $0.64 \cdot 10^6$ ($\pm 0.02 \cdot 10^6$) and that a

TABLE I

INCORPORATION INTO CYTOPLASMIC AND CHLOROPLAST RIBOSOMAL RNA IN THE PRESENCE OF CAMPTOTHECIN

Camptothecin concentration (μg/ml)	Cytoplasmic rRNA		Chloroplast rRNA	
	cpm/μg RNA layered	Percent of control	cpm/μg RNA layered	Percent of control
0	5100	100	650	100
1	2000	44	490	75
5	0	0	110	17
20	0	0	68	10

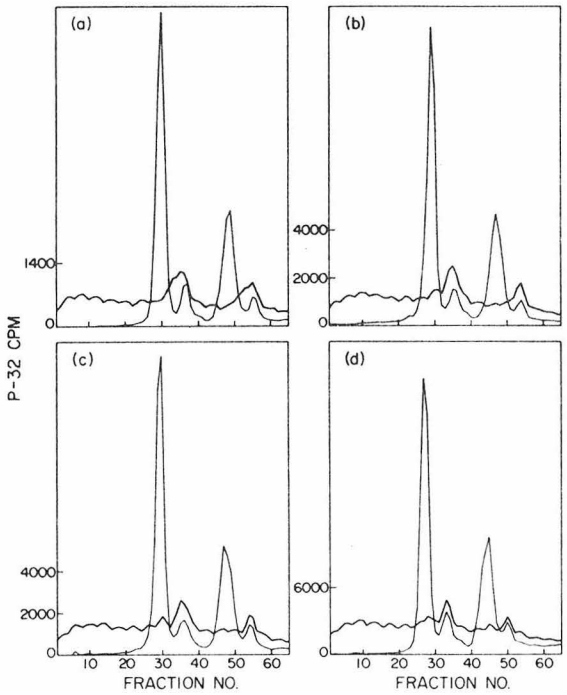

Fig. 6. Processing of chloroplast rRNA. CW-15 cells were prelabeled and resuspended in 0.133 mM phosphate—20 mM Tris—HCl—HSM(pH 7.2) medium + camptothecin as noted in Fig. 5. After a 45-min pulse with $^{32}PO_4^{3-}$ (40 μCi/ml), solid rifamycin SV was added to 500 μg/ml and the RNA was extracted at intervals. a, 45-min $^{32}PO_4^{3-}$ pulse; b, pulse followed by a 30-min rifamycin SV chase; c, 60-min chase; d, 120-min chase. The $^{32}PO_4^{3-}$ cpm are indicated by the thick lines.

species of molecular weight $0.56 \cdot 10^6$ appears with longer labeling. After 60 min the label had accumulated primarily in mature chloroplast rRNAs. Although the background here appears high, its absolute magnitude is about the same as that found in uninhibited cells. This can be illustrated by comparing the backgrounds in Figs 1 and 5, noting that the camptothecin-treated cells were labeled with phosphate of twice the specific activity of that used to label the uninhibited cells. The molecular weights and order of appearance of RNAs suggested the processing scheme which is illustrated in Fig. 2.

Since actinomycin D is not an efficient inhibitor of chloroplast rRNA synthesis we have used rifamycin SV to determine whether label incorporated in the $1.14 \cdot 10^6$-, $0.64 \cdot 10^6$- and the $0.56 \cdot 10^6$-mol. wt molecules would accumulate in chloroplast rRNAs in the absence of further synthesis of RNA. This is the case as Fig. 6 illustrates. Therefore these molecules appear to be precursors of chloroplast rRNAs. Definitive proof of this will require hybridization competition experiments, however.

Discussion

The processing of *C. reinhardi* rRNA does not show any fundamental differences from that of other eukaryotes [25—28], although, compared to

some, it is made from a smaller primary precursor. As we noted earlier, there are two discrete species of very high molecular weight RNA ($2.4 \cdot 10^6$ and $2.2 \cdot 10^6$), the larger of which is labeled first. Our results indicate that the peak of molecular weight $2.2 \cdot 10^6$ is not a normal intermediate between the $2.4 \cdot 10^6$-mol. wt precursor and the mature rRNAs since the material in the $2.4 \cdot 10^6$-mol. wt peak is not chased into the $2.2 \cdot 10^6$-mol. wt peak (Fig. 3), and because this species does not appear in any significant quantity until after the mature rRNAs have appeared. The $2.2 \cdot 10^6$-mol. wt species may be another primary product of transcription which is more stable and which is transcribed at a slower rate. Alternatively, it may be a product of incorrect processing of the $2.4 \cdot 10^6$-mol. wt molecule which is not processed into mature rRNA but which is eventually degraded. The existence of such defects in processing can be inferred from the results of Wellauer and Dawid [30] in HeLa cells.

Assuming that there is no preferential extraction of precursors, the order of nucleolar rRNA genes within the unit of transcription can be determined by examining the relative rates of appearance of labeled $1.4 \cdot 10^6$- and $0.69 \cdot 10^6$-mol. wt RNAs. Since the first RNAs released from the transcriptional complex following the addition of label will be labeled predominantly at their 3′ end, our data suggests that the rapidly labeled $1.4 \cdot 10^6$-mol. wt RNA (precursor of 25-S rRNA) is located nearer the 3′ end than is the $0.69 \cdot 10^6$-mol. wt (18 S) rRNA. The gene order then would be: promotor, 18 S, 25 S. This is directly opposite the order of genes in HeLa [30] and rat [31] cells but agrees with the order of genes in *Euglena gracilis* [19]. It would be of interest to know at what point in the evolution of the eukaryote this inversion in gene order occurred. Moreover, since the rRNA genes of *C. reinhardi* and of a variety of prokaryotes and eukaryotes are multiply re-iterated [9,23,32,33] the question of how such an inversion was accomplished is intriguing. This inversion of gene order appears to demand that an intermediate stage exists in the life cycle or existed in evolution where the number of replicated rRNA transcriptional units is one. There is also the alternative that inversion of gene order has resulted from inversion of the structural gene order with regard to a promotor, but this intermediate state would, of course, produce rRNAs which were the complements of the original rRNAs. Finally this might be the result of unequal crossing over or of a polyphyletic origin of the eukaryotes.

Chloroplast rRNAs are synthesized from discrete precursors which resemble those of blue-green algae [34,35]. Although no RNA large enough to be a precursor for both rRNAs had been detected this does not exclude the possibility that such a common precursor does exist but has not been detected because of the speed of its processing, etc. Recently, reports have appeared describing potential precursors of chloroplast rRNA in *E. gracilis* [36], and in spinach leaves [37,38]. In neither case was the possibility of mitochondrial rRNA contamination considered. A precursor—product relationship was not adequately demonstrated with pulse-chase experiments. The case of *E. gracilis* [36] was especially puzzling since label appeared in the mature 23-S chloroplast rRNA before becoming apparent in its presumed precursor.

The chloroplast resembles prokaryotes in its relative resistance to camptothecin [13,14] and in the size of the precursors of its rRNAs. Thus, in two more ways the properties of the genetic system of the chloroplast resemble those of prokaryotes more than those of the rest of the cell.

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Acknowledgements

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CHAPTER II:

THE EFFECTS OF ACTINOMYCIN D ON *CHLAMYDOMONAS REINHARDI* I. GENERAL EFFECTS.

THE EFFECTS OF ACTINOMYCIN D ON *CHLAMYDOMONAS REINHARDI*

I. GENERAL EFFECTS

Mark J. Miller and Daniel McMahon

California Institute of Technology

Division of Biology

Pasadena, California 91125

Correspondence and Proofs should be addressed to:

ABSTRACT

At high concentration, actinomycin D kills the alga Chlamydomonas reinhardi with exponential kinetics. The rate of killing is dependent upon the temperature of incubation. This dependence is partially a function of the increased extent of binding of the drug at the higher temperature (33°C), but sensitivity of the cell must also be stimulated by the higher temperature. While actinomycin D probably kills cells in a reaction which requires binding to DNA there is no correlation between the lethal event and the inhibition of macromolecular synthesis or the breakdown of macromolecules.

We have developed a simple model to explain the difference in sensitivity of various species of RNA to actinomycin D inhibition. This model predicts that frequently transcribed genes will be much more sensitive to the drug than infrequently transcribed genes.

INTRODUCTION

Actinomycin D (AMD), an inhibitor of RNA transcription, acts by intercalating into double strand DNA and retarding the progress of RNA polymerase (11, 13). The efficiency with which the drug inhibits transcription varies greatly between different species of RNA (34). Ribosomal RNA (rRNA) is particularly sensitive to the drug, being inhibited at concentrations 100-fold lower than are necessary to cause equivalent inhibition of other species of RNA (29, 34). This greater sensitivity of rRNA synthesis has been attributed to the higher GC content of ribosomal DNA (9), or to the possibility that a single RNA polymerase may read through several rRNA cistrons (33, 34).

AMD is a valuable cancer chemotherapeutic agent for the treatment of Wilm's tumor (7), gestational choriocarcinomas (10) and mixed metastatic embryonal carcinomas of the testes (23). Its use in therapy is restricted, however, by its failure to affect more prevalent human tumors, and by its high cytotoxicity for normal cells (40). The mechanism of this cytotoxicity is unknown. An understanding of the mechanism of cytotoxicity could conceivably facilitate the design of more effective drugs for cancer chemotherapy.

Several observations suggest that the lethal effects of AMD are not due exclusively to its ability to inhibit RNA transcription. Reich (35) noted that daunorubicium, mithramycin, and AMD, all of which inhibit RNA synthesis,have different spectra of tumor killing. Schwartz et al. (40) reported that the ability of the drug to kill

cells correlates more easily to the amount of drug those cells contain, than to the relative inhibition of RNA synthesis caused by the drug. Sawicke and Godman (37) have shown that, under conditions where there is equivalent inhibition of transcription, L cells and Vero cells are much more resistant to the toxic effects of AMD than are HeLa cells.

In order to investigate the mechanisms of cytotoxicity of AMD, we have examined its effects on macromolecular synthesis and on cellular viability of the alga Chlamydomonas reinhardi. AMD is not toxic to cells at concentrations which result in the greatest increase in the relative inhibition of macromolecular synthesis. We also have developed a simple model which described AMD's ability to inhibit different species of RNA with greatly different efficiencies.

MATERIALS AND METHODS

Chemicals

^{3}H-actinomycin D was purchased from Schwarz/Mann, while non-radioactive actinomycin D was the gift of Dr. Walter Gall, Merck and Co., Rahway, NJ. Hyamine hydroxide was purchased from Nuclear Chicago, and the GF/A and GF/C filter papers were from Whatmann. 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron) was a gift from E. I. DuPont de Nemours & Co., Wilmington, DE. RNase A and RNase T_1

were purchased from Worthington Biochemical Co., Freehold, NJ., and pancreatic DNase I was from Sigma Chemical Co., St. Louis, MO. Sources of other chemicals have been described previously (26).

Culture Conditions

Culture conditions have been described previously (26). Two strains of *Chlamydomonas reinhardi* have been used. One, Arg 2, is an arginine requiring auxotroph (16). The other is CW-15, a mutant without a cell wall, was isolated by Dr. D. Roy Davies (5). Experiments in white light were done under General Electric Red Fluorescent lights at an intensity of 500 lux. Incubations at 33°C were carried out in a Scherer Environmental Chamber.

Actinomycin D Binding Assay

AMD binding to cells was measured as ethanol precipitable counts in a modification of the method of Pederson and Robbins (32). Labeled cells were harvested by centrifugation (1 min at 1000xg), resuspended in 0.5 ml cold H_2O, and quickly precipitated in 10 ml cold (-20°C) 100% ethanol. After at least 30 min in an ice bath, the precipitate was washed onto GF/C filter papers with at least 100 ml cold 100% ethanol. After drying, the samples were dissolved in 0.5 ml hyamine hydroxide by incubating them for 2 h at 70°C, cooled, and counted in 10 ml toluene-based scintillation fluor. The efficiency of counting was 23%.

Incorporation of Labeled Precursors into Nucleic Acids and into Protein

Incorporation into nucleic acids was measured with $^{32}PO_4^{-3}$. Cells were washed into low phosphate medium (26) and labeled with 0.5 μCi/ml $^{32}PO_4^{-3}$. At intervals, two 1.0 ml aliquots (or 4 aliquots, if a DNA determination was also to be done) were added to 1.0 ml of cold 20% TCA, 0.1 M H_3PO_4. After at least 30 min in the cold, samples were washed onto GF/A filters (presoaked in 0.1 M H_3PO_4) with three 10 ml washes of 10% TCA in 0.01 M H_3PO_4 followed by five 4 ml washes with methanol:chloroform:water (12:5:3) (8). After drying at 70°C, the samples were cooled and if DNA determinations were to be done, the RNA was hydrolyzed in 0.5 M NaOH as described by McMahon and Blaschko (25) except that after the TCA washes, the samples were washed with chloroform, methanol and water as above. Incorporation into protein was measured with $^{35}SO_4$ as described by Cross and McMahon (4). Cells were washed and resuspended in HSMA containing 0.2 the normal concentration of sulfate (normal = 81 μM) and labeled with 0.5 μCi/ml of $H_2{}^{35}SO_4$ (New England Nuclear). At intervals 1.0 ml samples were added to 1.0 ml of 10% (w/v) trichloroacetic acid (TCA) + 10 mM Na_2SO_4 + 1 mM l-cysteine, mixed and kept at 0°C for at least 30 min. An additional 1.0 ml of 10% TCA was added, the tubes were heated to 90°C for 30 min and then chilled to 4°C. Samples were filtered onto GF/A filters (presoaked in 10 mM Na_2SO_4), washed three times with 10% TCA, 10 mM Na_2SO_4, and five times with 4 ml methanol:chloroform:water (12:5:3) (8). After drying, the samples were counted in 10 ml toluene based scintillation fluor. The efficiency of counting was 95%.

Quantitative Plating of Cells

After appropriate dilution, 0.20 ml Arg 2 cells were pipetted into petri plates containing HSMA + Arg, in 1.5% agar and spread with a glass rod. The efficiency of plating of this technique was 90-100%, as determined by counting the cells to be plated on a Coulter Counter. The CW-15 mutants, which lack a cell wall, lyse when spread on agar plates with a glass rod. Quantitative plating was done by pipetting 0.20 ml of a cell suspension into 2.0 ml of 0.6% agar in HSMA supplemented with 0.3% yeast extract (Difco), and maintained at 46°C. The solution was immediately poured onto HSMA plates containing 0.8% agar and distributed evenly by swirling. Cells could be maintained for 2 min at 46°C with no detectable loss of viability. The plating efficiency of this technique was about 50% as determined by counting the cells to be plated on a Coulter Counter.

Autoradiography of Cells

CW-15 cells were washed and resuspended in HSMA containing 90 μCi/ml, 40 μgm/ml ^{3}H-AMD at a concentration of 3 x 10^{6} cells/ml. After 10 h at 22°C, the cells were washed five times in HSMA (0-4°C) containing carrier AMD (40 μg/ml) and fixed according to Karnovsky (20). This procedure does not increase AMD binding to cells (Table I). Afterwards, cells were post-fixed in OsO_4, stained with Uranyl acetate, dehydrated through a series of ethanol steps, and embedded in epon-araldite (28). Thick sections were cut using a Ladd diamond knife, placed on glass slides and dipped in Kodak NTB-2 emulsion. After 30

days exposure the slides were developed, stained for 1 min in 5% Giemsa, washed 3 times in water, dried, and examined under a Zeiss picto-microscope using phase contrast microscopy.

Mutagenesis Using Actinomycin D

Arg 2 cells (25 ml at 2 x 10^6 cells/ml) were treated with 0, 3, or 40 μgm/ml of AMD for 8 h at 22°C. Ninety percent of the cells treated with 40 μg/ml of AMD are killed. The other treatments do not affect cellular viability. The drug was washed away, and the cells were diluted ten-fold and allowed to grow for 7 days to stationary phase. They were then counted and plated onto petri plates containing nutrient agar + 10 μgm/ml cycloheximide.

RESULTS

Toxic Effects of Actinomycin D

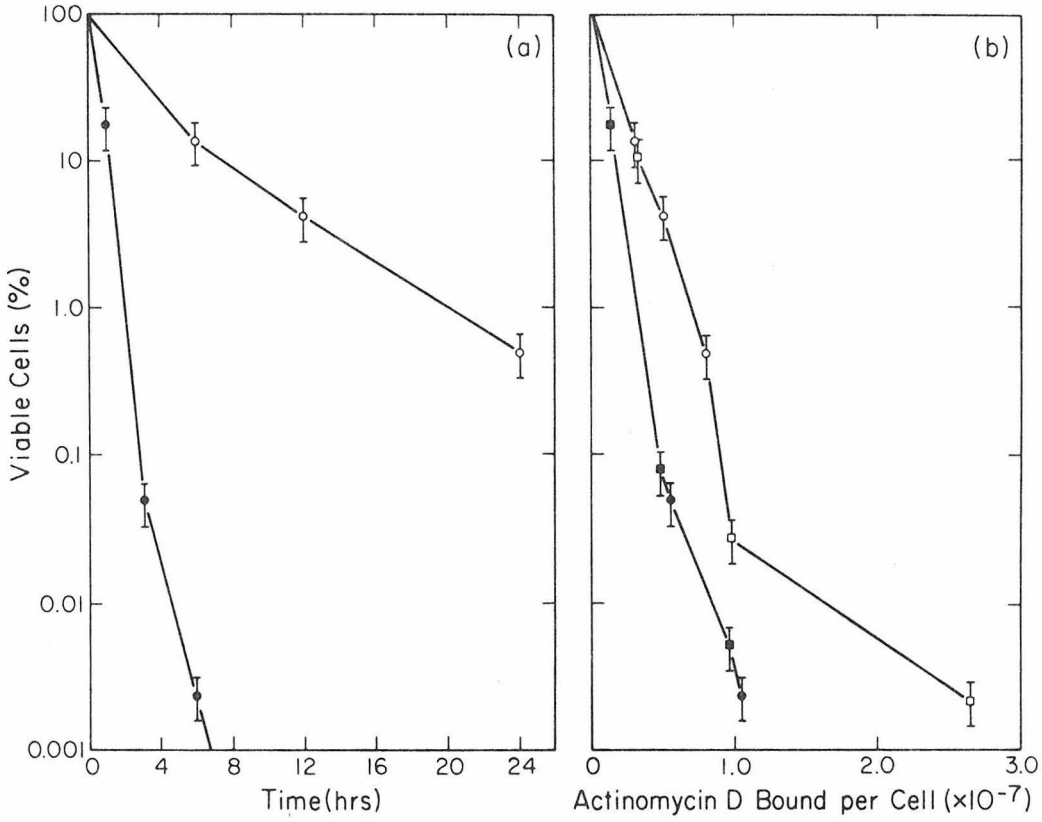
Fig. 1a shows the exponential kinetics with which 40 μgm/ml of AMD kills C. reinhardi. It also shows that there is a considerable dependence of the rate upon the temperature of incubation. In four such experiments incubated at 22°C the average half life of survival of CW-15 was 2.2 h. In two experiments at 33°C the average half life was 0.35 h. These experiments and others show that few, if any, cells are resistant to AMD killing. If there is a subpopulation of cells resistant to AMD as has been reported in mammalian systems (1) it comprises less than 0.01% of the total population.

Figure 1

Cellular survival in the presence of actinomycin D. CW-15 cells were suspended in HSMA (2×10^6 cells/ml) and divided into two aliquots, one being placed at 22°C and the other at 33°C. After 1 h, ^{3}H-AMD was added to each culture (2 µCi/ml, 40 µgm/ml) and at intervals binding of AMD was measured. The cells were washed free of AMD and plated as described in Methods. The relative cellular viability was determined by dividing the number of colonies per plate by the number of cells plated (as measured on a Coulter counter) and correcting for plating efficiency as determined from controls which were not treated with AMD.

a) Percent survival vs. time. 22°C: -O- ; 33°C -O-.

b) Percent survival vs. molecules of AMD bound per cell. Figure 1b presents data from two experiments. The circles indicate data from the experiment in Fig. 1a. The squares indicate the other experiment, 22°C: -O-, -□- ; 33°C: -●-, -■- .


100
10
1.0
0.1
0.01
0.001
Viable Cells (%)
(a)
0
4
8
12
16
20
24
Time(hrs)
(b)
0
1.0
2.0
3.0
Actinomycin D Bound per Cell (×10⁻⁷)

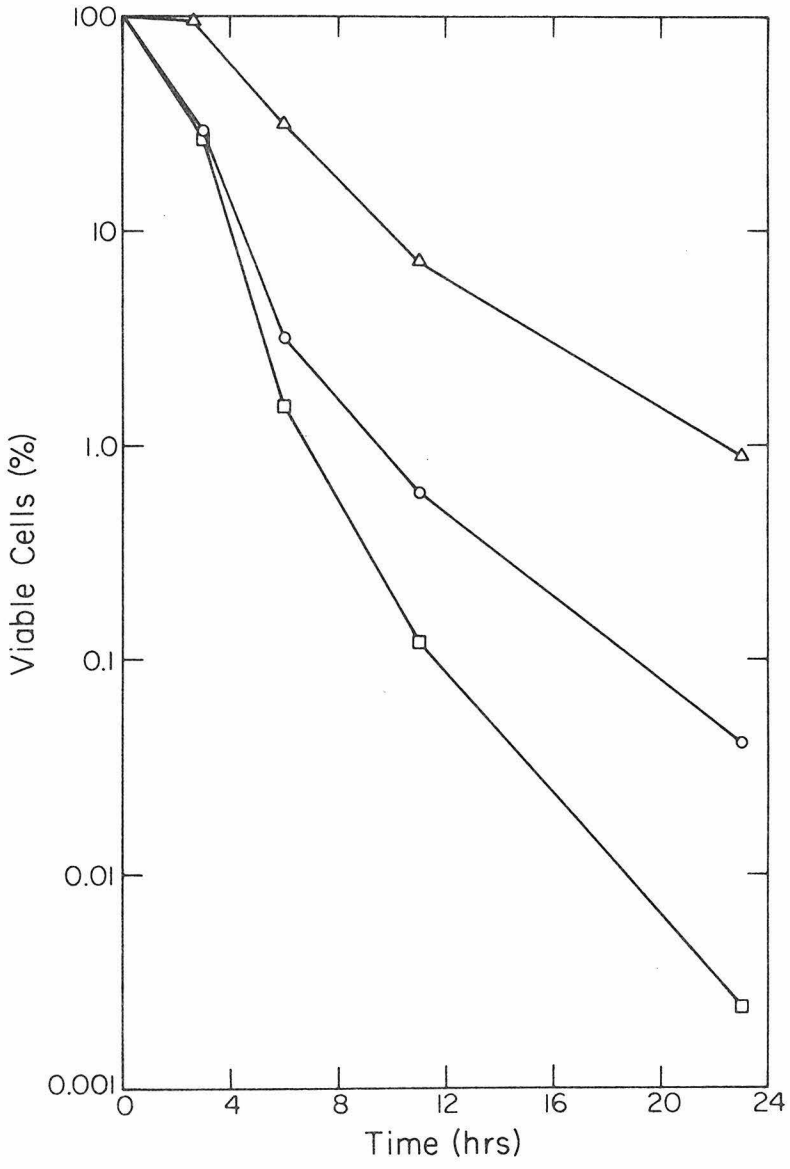
Since AMD absorbs visible light and since the survival experiments were done in the light, we investigated whether killing might depend on some photochemical reaction mediated by AMD (e.g., cleavage of DNA). Experiments of several sorts indicate that this possibility is extremely unlikely. Fig. 2 compares the survival of cells incubated in AMD in white light, red light, and in the dark. There is an initial lag period of about 3 h in the dark after which cells are killed at rates which are essentially identical to those of cells incubated in white light. We were curious whether the lag did in fact represent some transient effect of light on AMD or on the cells (e.g., a slowing of growth or of some other temporary adaptation of the cells to the dark.) Fig. 2 shows that the latter possibility is more likely. Cells were illuminated under red fluorescent lamps whose spectral distribution (Iscotables, [1974], Instrument Specialties Co., Lincoln, Nebraska, p. 45) does not overlap the absorption spectrum of AMD but does overlap the absorption spectrum of chlorophyll and so stimulates photosynthesis. Although the intensity of the red light was only one-third that of white, C. reinhardi dies with no apparent lag period and at a greater rate than do cells grown under white light. Furthermore, when cells were treated with AMD in white light in the presence of 10^{-5} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea, a photosynthetic inhibitor (12), they were substantially protected from killing (previously unpublished observations).

Binding of Actinomycin D

More than 50% of the AMD in the cell can be removed by washing in ethanol (Table 1, Experiment 1). Fig. 3 shows autoradiographs of cells

Figure 2

Cell survival under different conditions of illumination. A culture of Arg 2 was washed into fresh HSMA + Arg and adjusted to 5×10^5 cells/ml. Aliquots of the culture were incubated under white light (1500 lux), red light (500 lux, λ_{max} = 650 mμ) or in the dark for 1 h prior to adding AMD (40 μgm/ml), and throughout the course of the experiment. At intervals relative cellular viability was determined as described in Fig. 1. Incubation in white light (O); in red light (□), in the dark (Δ).


100
10
1.0
0.1
0.01
0.001
Viable Cells (%)
0
4
8
12
16
20
24
Time (hrs)

which were incubated in ^{3}H-AMD in order to identify the sites at which the bound (ethanol-insoluble) AMD is localized. Several things are obvious. There is little or no absorption to the cell wall or plasma membrane. The silver grains are often found scattered throughout the cell but many cells show a single region of intense localization slightly displaced from their center (large arrows). From the relative size and position of the binding site, and from results to be presented below, we assume that the localized silver grains represent nuclear binding (36, and our unpublished results). We were curious whether the diffuse binding resulted from nonspecific fixation of AMD to cellular material by the glutaraldehyde-formaldehyde fixation. Experiment 1 of Table I shows that this fixation decreases rather than increases the measured AMD binding.

The binding of AMD to a site which makes it insoluble in ethanol is not directly proportional to the external concentration of the drug but arises as the 0.86 power (Fig. 4). The equation which describes this binding is:

$$A_i = 1.5 \times 10^5 A_e^{0.86}$$

where A_i and A_e are the internal and external concentrations of AMD.

In Fig. 5 we have plotted the time dependence of incorporation of AMD at 22°C and 33°C. This experiment shows that, after a slight lag, the rate of incorporation is stimulated by higher temperatures. This suggests that the effects of temperature on killing may result from stimulation of the rate of binding of AMD. Fig. 1b shows that, in fact, when survival of cells is plotted as a function of the amount of bound

Figure 3

Autoradiographs of CW-15 cells labeled with ^{3}H-actinomycin D for 10 h at 22°C. The large arrows indicate regions of intense localization of silver grains, P indicates the pyrenoid. (2620X). The bar represents 5μ.

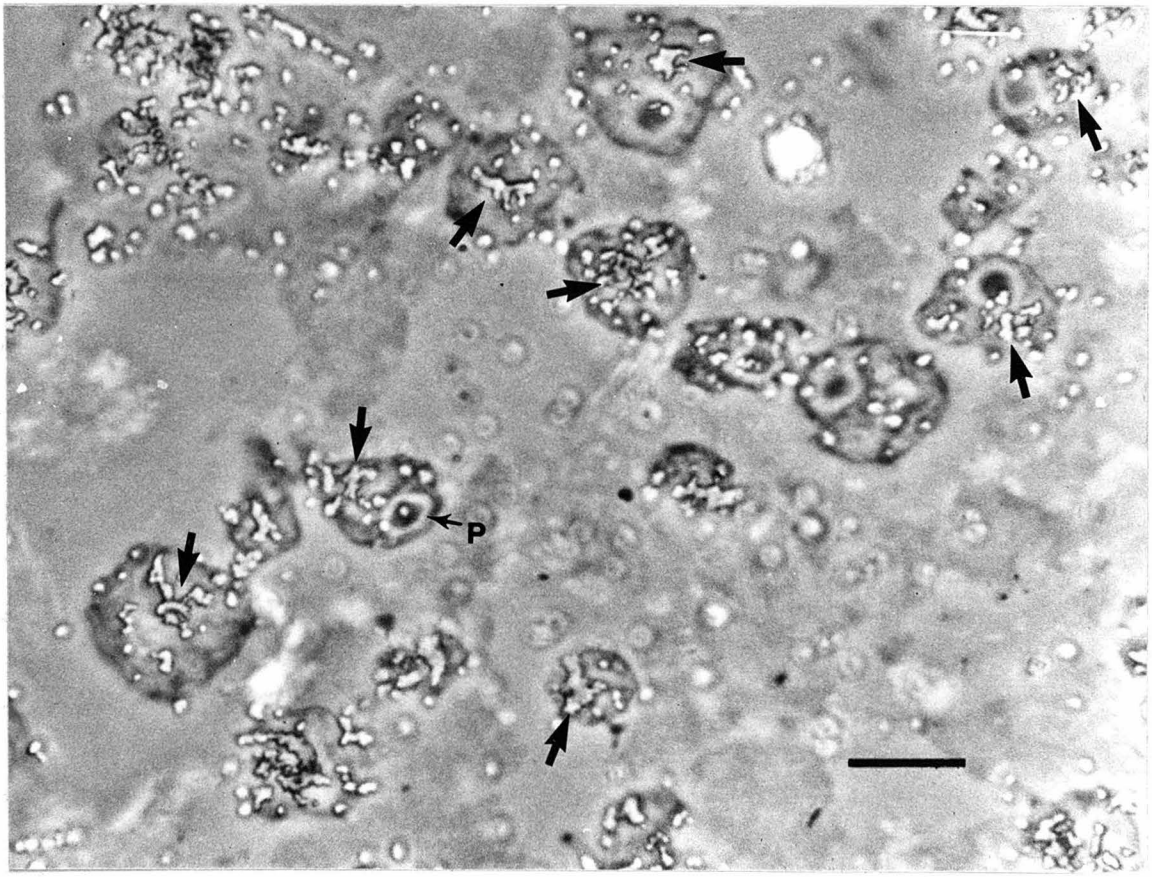

P

Figure 4

External concentration of actinomycin D vs the amount of actinomycin D bound per cell. Four and one-half ml of CW-15 (4×10^6/ml) cells in HSMA were transferred to 4.5 ml HSMA containing 18 µCi ^{3}H-AMD (2.6 Ci/mM) and enough added carrier to make the final concentration of the drug given on the abscissa. Since 2µ Ci/ml of pure ^{3}H-AMD has a concentration of 1.0 µgm/ml, smaller quantities of label were used for external concentration below 1.0 µgm/ml. A background determination at zero time was made for each culture. After 8 h incubation at 22°C the cell concentration was measured and AMD binding was determined as described in the Methods.

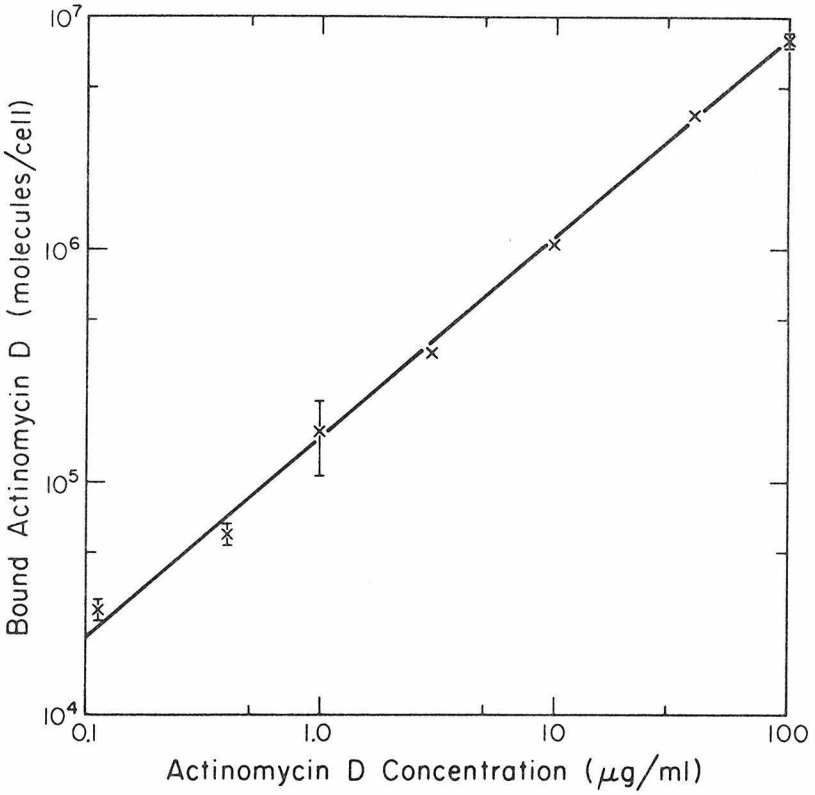
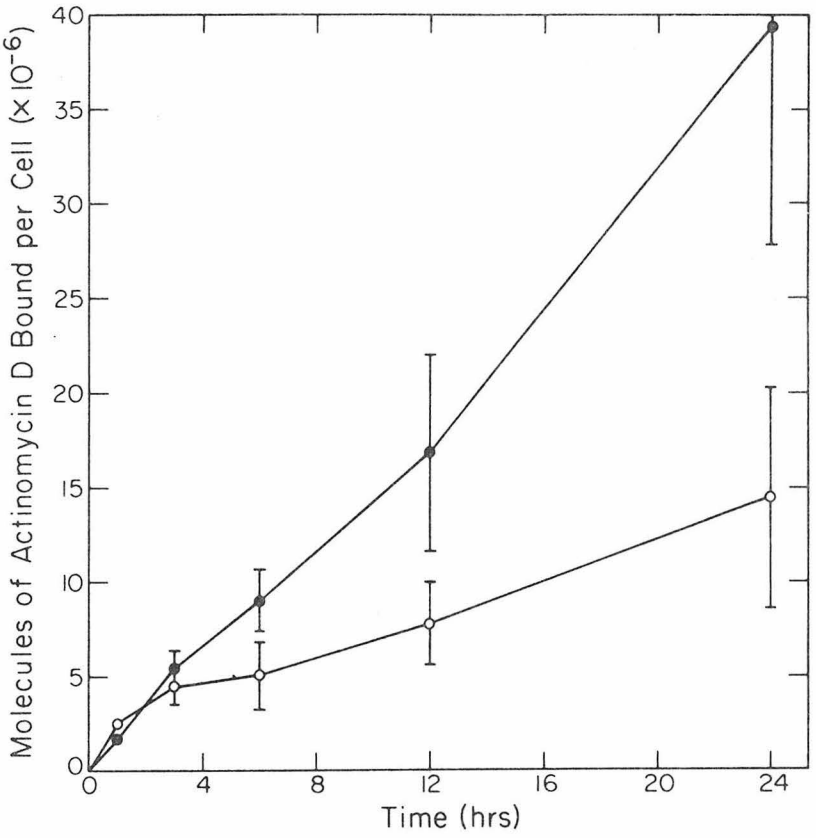

10^7
10^6
10^5
10^4
Bound Actinomycin D (molecules/cell)
0.1
1.0
10
100
Actinomycin D Concentration (μg/ml)

Figure 5

Rate of binding of actinomycin D to CW-15 at 22°C and 33°C. Cells were resuspended in HSMA (2×10^6 cells/ml) and preincubated at 22°C or 33°C for 2 h. ^{3}H-AMD was added at time 0 (2 µCi/ml, 40 µgm/ml) and binding was measured at the indicated times. The error bars represent 95% confidence limits. 22°C: (O); 33°C (●).


Molecules of Actinomycin D Bound per Cell (X 10⁻⁶)
0
5
10
15
20
25
30
35
40
0
4
8
12
16
20
24
Time (hrs)

AMD a considerable amount of the temperature dependence of killing is eliminated. This correlation might have resulted because dead cells bind much more AMD than living ones, but this possibility is eliminated by the fact that mutants, which are not killed by AMD, show a similar temperature sensitive enhancement of incorporation (see accompanying paper). Finally, it is important to note even when killing is corrected for the differences in binding of AMD there is still a substantial stimulation of the rate of killing by higher temperature.

We examined the chemical nature of the binding sites for AMD in the cell to verify that AMD was binding to cellular DNA. Our initial attempts to release the ethanol-insoluble AMD with DNase were unsuccessful. To determine whether the intracellular contents of the cells precipitated with ethanol were accessible to enzymes, RNase was added to them and was unable to degrade radioactively labeled RNA (Table I, Experiment 2). It appeared the fixed cells were very impermeable even to enzymes as small as pancreatic RNase A and T_1. In Experiment 3 we examined the effects of detergents on the release of the bound AMD. Triton X-100 (1%) released a small amount (12%) of the bound AMD. SDS (1%) increased this release by only 8%, indicating that relatively little of the AMD is non-covalently bound to lipids or proteins. Incubation with nuclease in the presence of Triton X-100 was much more effective. DNase released 40-50% of both the bound AMD and radioactively-labeled DNA (Table I, Experiments 4 and 5). Our results differed from those of Pederson and Robbins (32) who obtained 94% release of ethanol precipitated AMD with 100 μgm/ml DNase in RSB buffer. Unfortunately, they did not measure the extent of degradation of DNA in their experiments. In

TABLE I. Effects of Various Treatments on the Binding of Actinomycin D*.

Experiment No.	Material Measured	Treatment		CPM	% Released
1	a) AMD	i)	Untreated whole cells	2682	
		ii)	Ethanol washed	1146	57
		iii)	Formaldehyde-glutaraldehyde fixed, ethanol washed	750	71
2	a) AMD	i)	Control	630	
		ii)	+ DNase	638	-
	b) RNA	i)	Control	16952	
		ii)	+ RNase	15449	9
3	a) AMD	i)	Control	2737	
		ii)	+ Triton X-100	2396	12
		iii)	+ Triton X-100 + SDS	2197	20
4	a) AMD	i)	Control (+ Triton X-100)	2396	
		ii)	+ DNase	1214	49
	b) DNA	i)	Control (+ Triton X-100)	95792	
		ii)	+ DNase	47028	51
	c) AMD	i)	Control (+ Triton X-100 + NaCl)	1899	
		ii)	+ DNase	1213	36
	d) DNA	i)	Control (+ Triton X-100 + NaCl)	102168	
		ii)	+ DNase	65488	36
5	a) AMD	i)	Control (+ Triton X-100)	633	
		ii)	+ DNase	374	41
		iii)	+ RNase	653	-
	b) DNA	i)	Control (+ Triton X-100)	36844	
		ii)	+ DNase	24014	35
	c) RNA	i)	Control (+ Triton X-100)	262528	
		ii)	+ RNase	120832	54

(Continued)

TABLE I (continued)

*Cells (CW-15) were labeled for 10 h in AMD (2-4 μCi/ml, 20 μgm/ml) at 22°C. A separate culture was labeled for 14 h with ^{3}H-adenine (.5 μCi/ml, 27 Ci/mM), and for an additional 10 h in the presence of unlabeled AMD (20 μgm/ml). Cells (unless otherwise indicated) were washed free of label and precipitated in ethanol. The precipitate was collected by centrifugation at 10,000 rpm (16,300 xg) for 10 min in a Sorvall HB-4 centrifuge rotor, washed once with 80% ethanol and dried in vacuo. The pellet was resuspended in 1.0 ml of RSB buffer (32) by gently sonicating until no large clumps were visible. The treatments in experiments 3-5 were done in the presence of 5 μgm/ml AMD. Reagent concentrations, when added, were: Triton X-100, 1%; SDS, 1%; NaCl, 1.5 M; DNase, 250 μgm/ml; and RNase A + RNase T_1, 100 units/ml and 20 units/ml, respectively. Enzymatic digestions were done for 90 min at 37°C. Following digestion, the ^{3}H-adenine labeled cells were hydrolyzed with NaOH to determine DNA content (25). In Experiment 1 the untreated AMD counts were determined by pipetting washed cells onto GF/C filters and washing them with 30 ml cold water.

retrospect their result was surprising, since chromosomal proteins have been reported to protect 50% of the chromosomal DNA from digestion by endonuclease (3). For this reason, we also treated cells with DNase in the presence of high salt hoping to expose all of the DNA to digestion by decreasing the amount of bound chromosomal proteins (Experiment 4c and d). However, release of AMD and degradation of DNA were somewhat less than found in the absence of the salt, although both were released to the same extent. RNase did not release any AMD, although it did solubilize more than half of the endogenous RNA (Experiment 5c).

Relative Sensitivity of Macromolecular Synthesis and Cellular Survival to Actinomycin D

We examined the relationship between the inhibition of nucleic acid synthesis and the survival of cells. Figure 6 represents a comparison of the extent of inhibition of total nucleic acid synthesis and cellular viability under various conditions of temperature and AMD concentration. In three experiments which are not included in this figure, concentrations of AMD which inhibited 6-58% of nucleic acid synthesis (1-10 μg/ml) did not kill cells. There is only a very crude correlation, at best, between the survival of the cells and the extent of inhibition of nucleic acid synthesis by AMD.

In order to examine further whether there was a correlation between the extent of killing and the inhibition of the synthesis of macromolecules, we examined the relative effects of different doses of AMD. Figure 7 shows that cells are completely resistant for at least 8 h to doses of AMD of 10 μgm/ml or less. In contrast, the extent of macromolecular

Figure 6

Cellular viability vs relative inhibition of nucleic acid synthesis. CW-15 or Arg 2 cells were resuspended in 0.133 mM phosphate -20 mM Tris-HCl-HSMA (pH 7.2) media (26) and preincubated for at least 1 h at 22°C or 33°C. AMD was added to various concentrations (1-80 µgm/ml) along with 0.5 µCi/ml $^{32}PO_4^{-3}$. After 2 h the incorporation into nucleic acids was determined and compared to controls containing no AMD. The relative inhibition of incorporation is plotted against the half lives of viability of cells treated at the same concentration of AMD and temperature. The half lives were determined over the first 12 h of incubation. The error bars represent the standard errors of the mean half life where more than one determination was made. The numbers in parentheses indicate the number of determinations when more than 1. CW-15: 22°C, (□); CW-15: 33°C, (■), Arg 2: 22°C, (O).

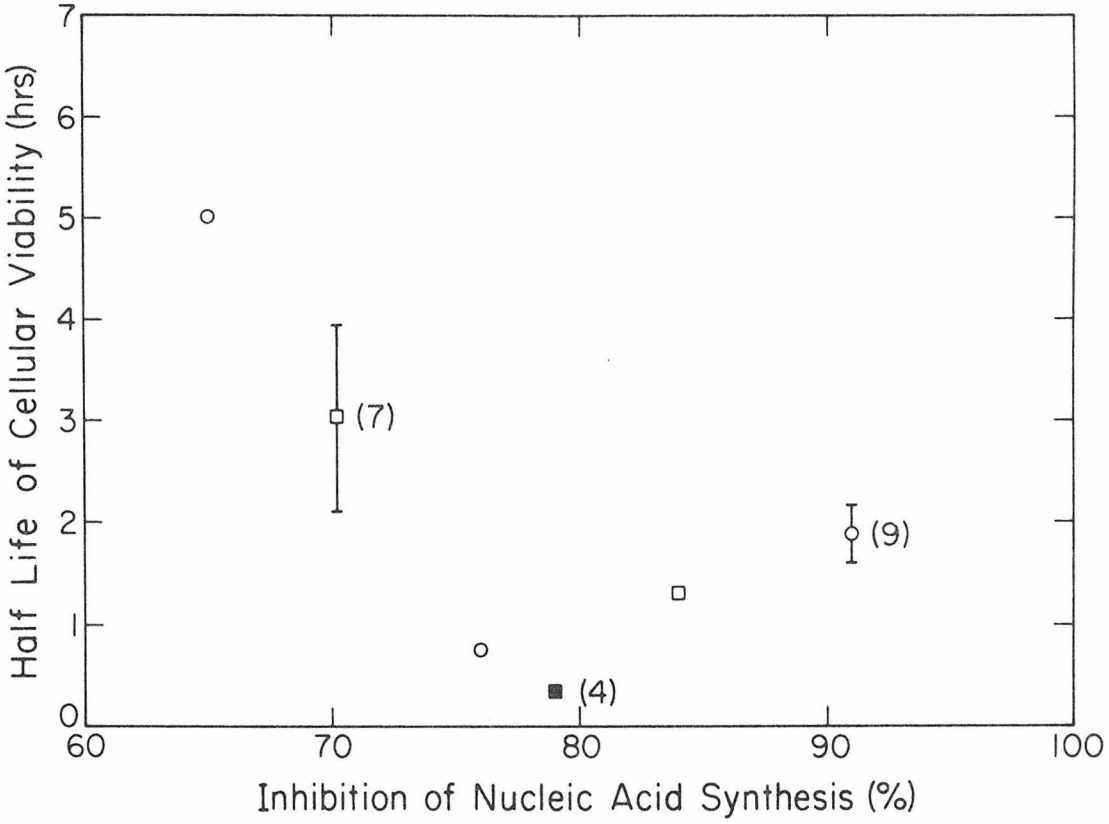
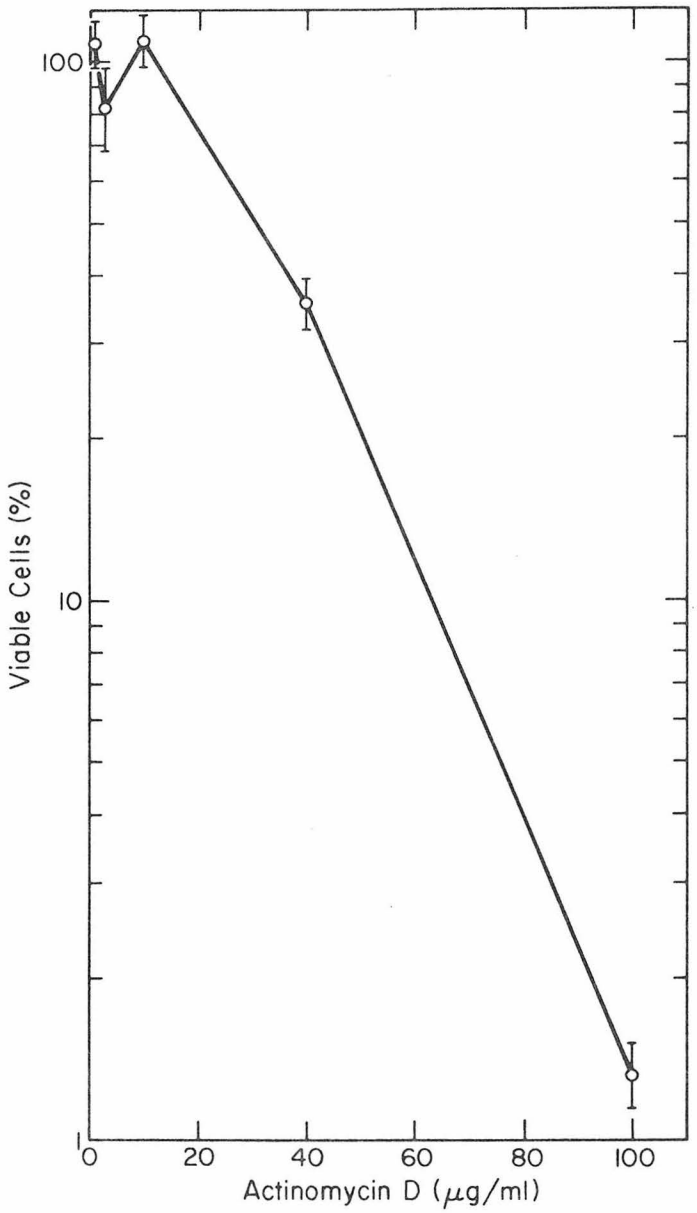

Half Life of Cellular Viability (hrs)
0
1
2
3
4
5
6
7
60
70
80
90
100
Inhibition of Nucleic Acid Synthesis (%)
(7)
(4)
(9)

Figure 7

Relative cellular viability after 8 h in various concentrations of actinomycin D. Cells (CW-15) were incubated in various concentrations of AMD as described in Fig. 9. After 8 h they were washed free of the drug, counted on a Coulter counter, and plated as described in Methods. The data in this figure were collected in the same experiment illustrated in Figs. 8 and 9.


100
10
1
Viable Cells (%)
0
20
40
60
80
100
Actinomycin D (μg/ml)

synthesis is very sensitive to inhibition by those concentrations of AMD. Ten μgm/ml of AMD inhibit protein synthesis by 40% (Fig. 8), DNA synthesis by 25%, and RNA synthesis by two thirds (Fig. 9). Concentrations of AMD greater than 10 μgm/ml kill the cells rapidly but are proportionately less effective than lower concentrations in inhibiting macromolecular synthesis. As might be expected, RNA synthesis is more sensitive to AMD inhibition than DNA synthesis during the first 4 h of incubation. However, during the second 4 h the increase in the inhibition of RNA synthesis is approximately the same as in the first 4 h. The relative effect of AMD on protein and RNA synthesis is very dependent upon the dose of AMD. Low concentrations of AMD inhibit RNA synthesis more effectively than protein synthesis. The opposite is true of high concentrations of AMD.

Effect of Actinomycin D on the Stability of Macromolecules

Actinomycin D has been reported to cause RNA degradation (22, 44) and chromosome breaks (31). We were therefore interested to see what effect lethal (40 μgm/ml) and sublethal (3 μgm/ml) doses of AMD would have on the stability of prelabeled macromolecules. Fig. 10 shows that neither concentration of AMD had a significant effect on the stability of protein or DNA. The lethal, but not the sublethal, dose of AMD stimulates the breakdown of prelabeled RNA. The RNA is degraded with a half life of 28 h, far slower than the rate at which cells die.

Figure 8

Incorporation of $^{35}SO_4^{-2}$ into protein during 8 h incubation in various concentrations of AMD. CW-15 cells were washed and resuspended in .016 mM SO_4^{-2} HSMA and allowed to incubate for 2 h at 22°C. Aliquots were then pipetted into an equal volume of medium containing 1.0 μCi/ml $^{35}SO_4^{-2}$ and twice the final concentration of AMD. Incorporation into protein as described in the Methods was determined after 0, 4 and 8 h of incubation, and was divided by the cell concentration. The data are plotted as percent inhibition compared with a control containing no AMD. 0-4 h (X——X); 0-8 h (O——O); 4-8 h: (Δ——Δ).

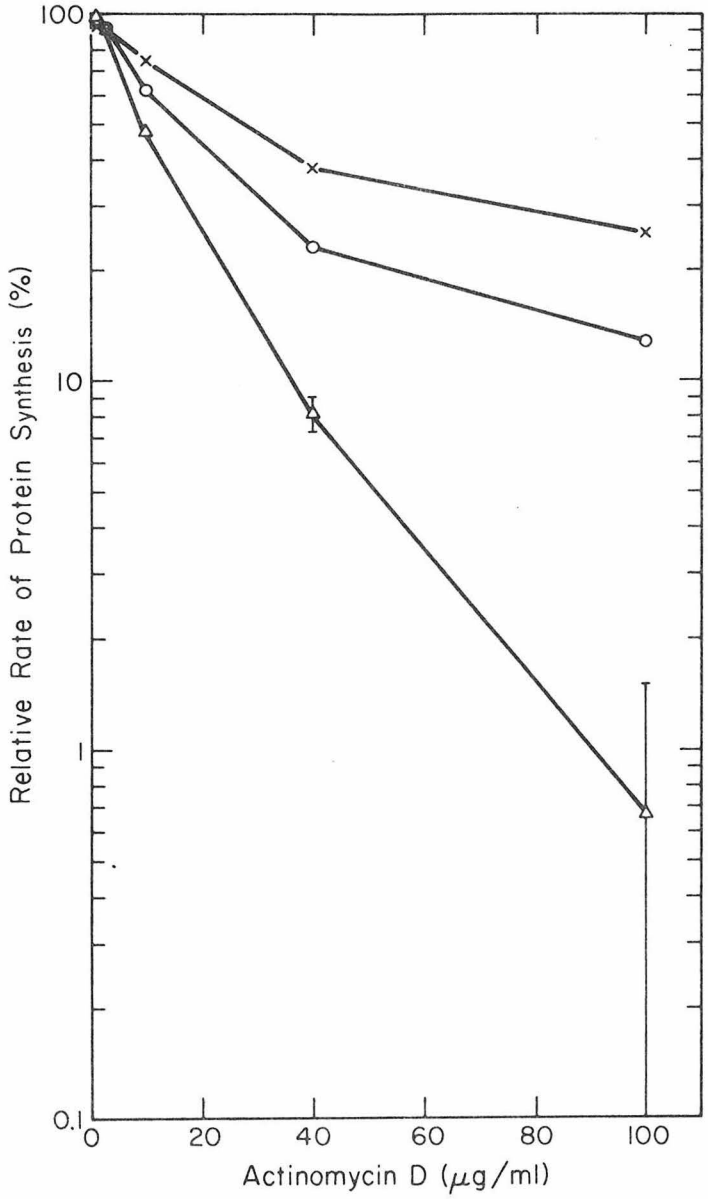

100
10
1
0.1
Relative Rate of Protein Synthesis (%)
0
20
40
60
80
100
Actinomycin D (μg/ml)

Figure 9

Incorporation of $^{32}PO_4^{-3}$ into DNA and RNA during 8 h incubation in various concentrations of AMD. CW-15 cells were washed and resuspended in 0.133 mM phosphate-20 mM Tris-HCl-HSMA (pH 7.2), and allowed to incubate for 2 h at 22°C. Aliquots were then pipetted into equal volumes of medium containing 1.0 μCi/ml $^{32}PO_4^{-3}$ and twice the final concentration of AMD. Incorporation into DNA and RNA were determined after 0, 4 and 8 h of incubation and was corrected for the cell concentration. The data are plotted as percent inhibition compared with a control containing no AMD. 0-4 h: (X——X); 0-9 h (O——O); 4-8 h (Δ——Δ).

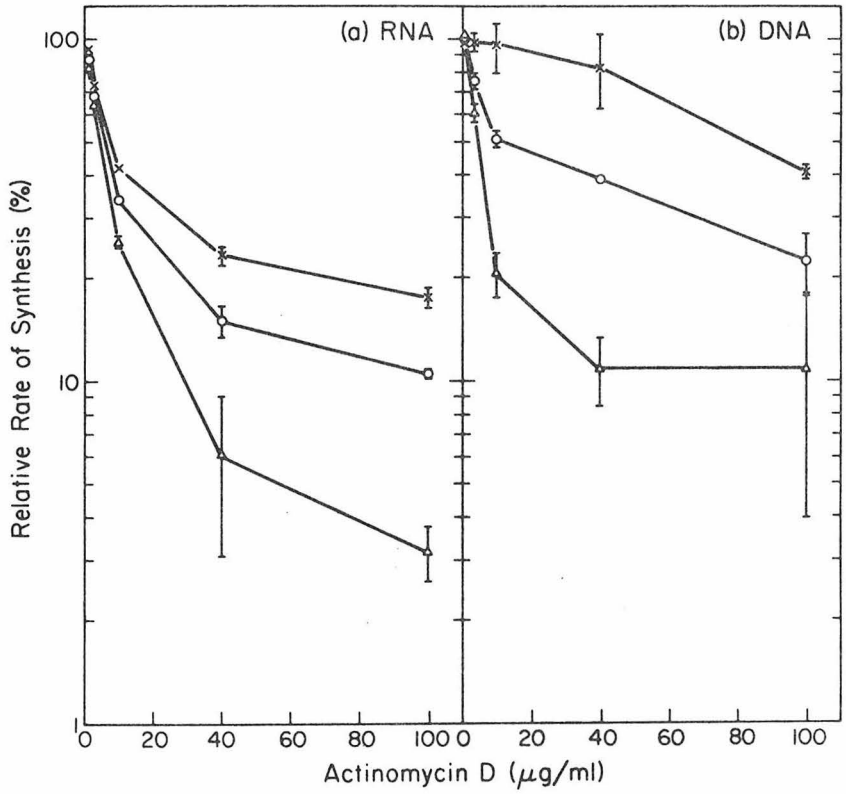
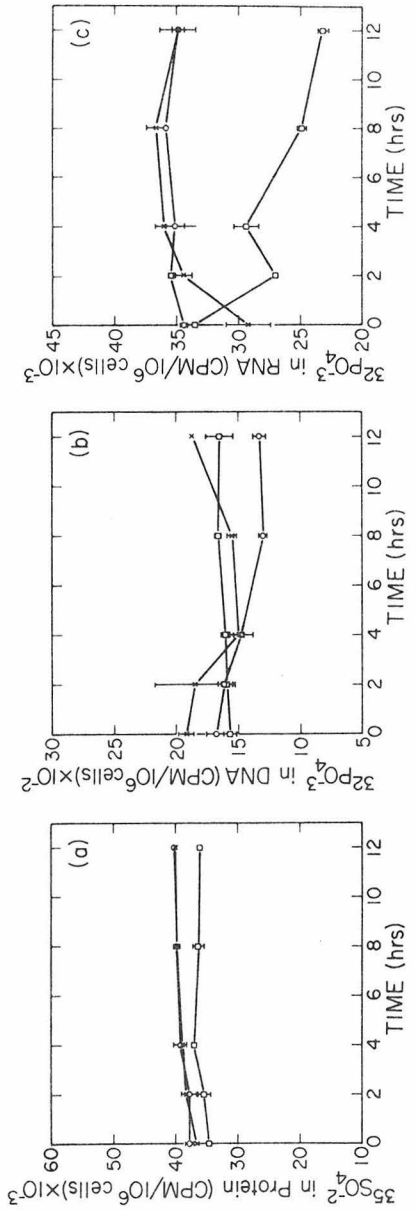

(a) RNA
(b) DNA
100
10
1
Relative Rate of Synthesis (%)
0
20
40
60
80
100
Actinomycin D (μg/ml)

Figure 10

Effect of actinomycin D on the stability of prelabeled RNA, DNA and protein. CW-15 cells (2.5×10^5 cells/ml) were incubated in either 0.5 µCi/ml $^{32}PO_4^{-3}$ (in 0.133 mM phosphate -20 mM Tris-HCl-HSMA (pH 7.2))(26), or in 0.5 µCi/ml $^{35}SO_4^{-2}$ (in HSMA) for 22 h at 22°C. The cultures were washed free of label, resuspended in HSMA, and allowed to incubate for 4 h at 22°C in order to let the intracellular pools equilibrate with unlabeled precursor. Each culture was counted, split into three aliquots and treated with 0 µgm/ml (X——X), 3 µgm/ml (O——O), or 40 µgm (□——□) of AMD. Label retained per cell was determined at the indicated times. a) $^{35}SO_4^{-2}$ in protein; b) $^{32}PO_4^{-3}$ in DNA; c) $^{32}PO_4^{-3}$ in RNA.


(a)
(b)
(c)
TIME (hrs)
TIME (hrs)
TIME (hrs)
$^{35}SO_4^{-2}$ in Protein (CPM/10^6 cells)$\times 10^{-3}$
$^{32}PO_4^{-3}$ in DNA (CPM/10^6cells)$\times 10^{-2}$
$^{32}PO_4^{-3}$ in RNA (CPM/10^6 cells)$\times 10^{-3}$

Is Actinomycin D a mutagen?

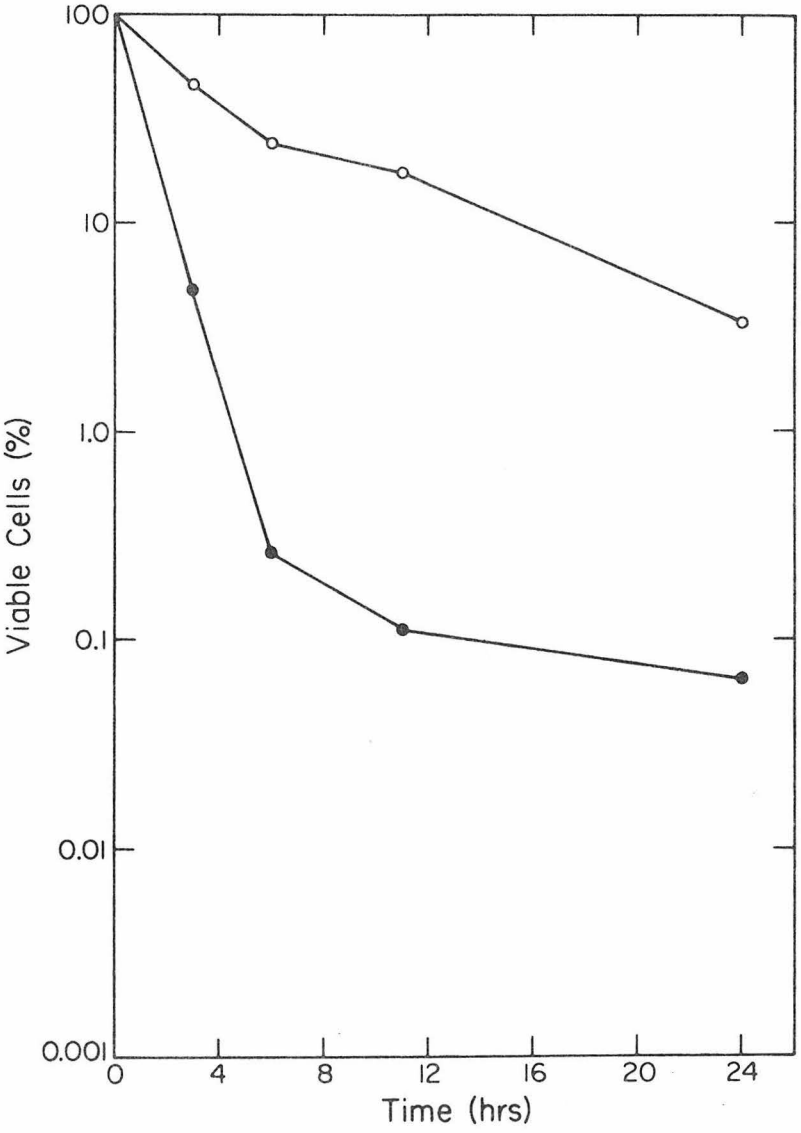
Cells were treated with AMD as described in the materials and methods and examined for cycloheximide resistant mutants (after a period of growth to allow phenotypic expression). No resistant strains were found among 3.5×10^7 cells which arose from the untreated culture, 9.4×10^7 cells from a culture treated with 3 μg/ml of AMD nor among 6.2×10^7 cells from a culture treated with 40 μg/ml of AMD. In contrast, cells mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (NMG) until 90% were killed (24) exhibited 660 resistant colonies from 5.8×10^6 cells plated (previously unpublished results). The frequency of mutants induced by NMG was 0.011% but 40 μg/ml AMD did not produce any mutants ($<2 \times 10^{-6}$%) although it killed cells to the same extent as NMG. Thus if AMD is a mutagen it is more than a thousand times less effective than NMG.

Lethality of Other Antibiotics which Inhibit RNA Synthesis

Chromomycin A_3, like AMD, binds to regions of DNA rich in guanine and cytosine containing bases, and inhibits the progress of RNA polymerase (45). In C. reinhardi it inhibits both RNA and DNA synthesis. For example 47 μg/ml inhibits RNA synthesis, measured with ^{3}H-adenine, by 70-80% after 4 h (previously unpublished experiments). Like AMD, chromomycin A_3 kills cells with exponential kinetics and with a temperature-sensitive stimulation of killing (Fig. 11). In other experiments, using Arg 2, cells were killed with exponential kinetics for up to

Figure 11

Cellular survival in the presence of chromomycin A_3. CW-15 cells were resuspended in HSMA (1×10^6 cells/ml) and divided into two aliquots, one placed at 22°C and the other at 33°C. After 2 h an equal volume of 94 µgm/ml chromomycin A_3 (preincubated at 22°C or 33°C) was added. At intervals the cells were washed free of chromomycin A_3, counted on a Coulter counter and plotted. 22°C: (O); 33°C: (●).


100
10
1.0
0.1
0.01
0.001
Viable Cells (%)
0
4
8
12
16
20
24
Time (hrs)

24 h. On the other hand, camptothecin, at a concentration which inhibits 90% of total RNA synthesis, measured with ^{3}H-adenine or 70% as measured with $^{32}PO_4$, causes very little cell death (Fig. 12), nor does it protect cells from AMD.

DISCUSSION

Effects of AMD on Cellular Viability

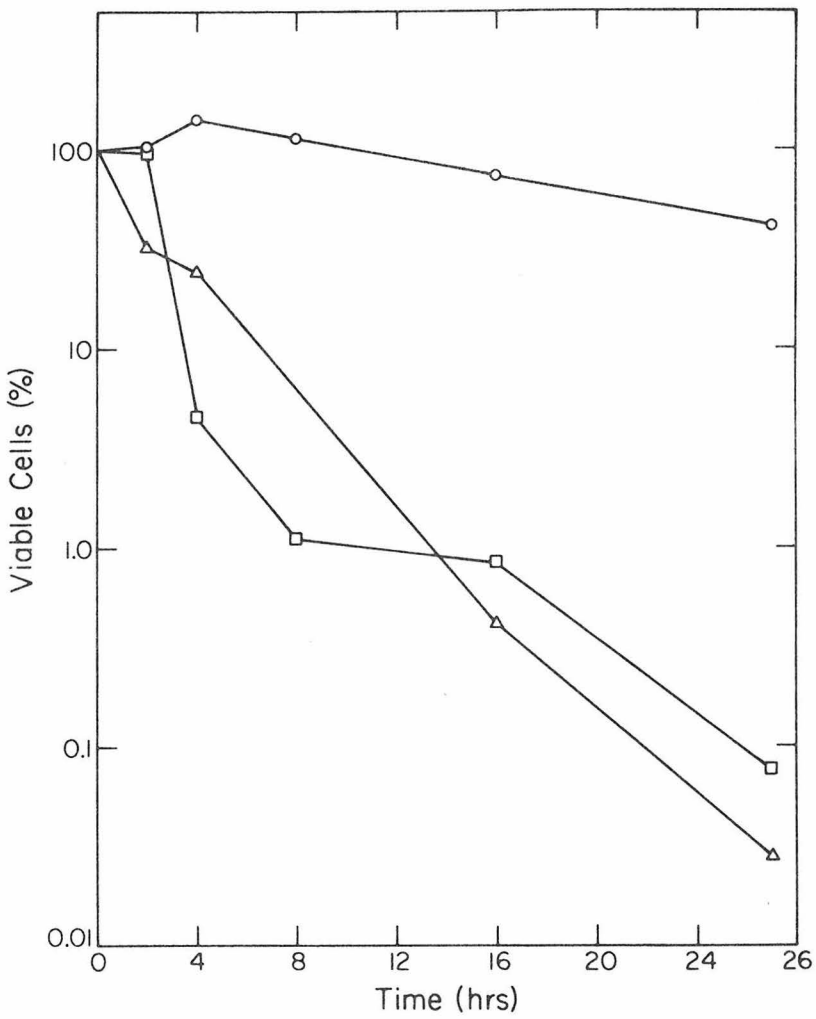
The toxic effects of AMD are not due to a reaction which is photochemically sensitized by AMD nor by photoconversion of the drug to some more active species. Three kinds of experiments indicate this. First, actinomycin D kills cells in the dark with a rate, after a lag period, which is equal to that in the light. Secondly, in red light, which is not absorbed by AMD, cells die without a lag and at a more rapid rate than in white light. Third, little or no degradation of AMD occurs under the conditions of these experiments (see accompanying paper).

Actinomycin D does not appear to kill cells via the general inhibition of either RNA, DNA or protein synthesis. This is indicated by the data in Fig. 6 and Fig. 12, and by a comparison of Figs. 7, 8 and 9. At concentrations up to 10 μgm/ml, cells are resistant to AMD killing for at least the first 8 h of incubation, but at these drug levels macromolecular synthesis is very sensitive to AMD inhibition. These data, of course, do not exclude the possibility that a subset of RNAs or proteins resistant to low doses of AMD are required for survival.

There is a strong correlation between the ethanol-insoluble binding of AMD and cell killing. Our data are consistent with the hypothesis that

Figure 12

Cellular survival in the presence of camptothecin. Arg 2 cells were resuspended in HSMA (5×10^5 cells/ml) and divided into three aliquots. After 1 h preincubation they were treated with 10 µgm/ml camptothecin (O), 40 µgm/ml AMD (Δ), or 10 µgm camptothecin + 40 µgm/ml AMD (□). At intervals, cells were washed free of drug, counted on a Coulter counter, and plated.


100
10
1.0
0.1
0.01
Viable Cells (%)
0
4
8
12
16
20
24
26
Time (hrs)

all of the ethanol-insoluble AMD is bound to DNA. Since some of the DNA is resistant to DNase we cannot obtain complete release. This agrees with the observation of others that about 50% of the nuclear DNA is protected from nucleases by proteins (3). However, the site of the ethanol-binding needs further investigation.

Autoradiography of bound ^{3}H-AMD also agrees with the interpretation that AMD is bound to DNA since the majority of radioactivity appears to be localized in the nucleus. The distribution of silver grains over the cytoplasm may result from binding of AMD to chloroplast DNA. This is consistent with the fact that AMD effectively inhibits the synthesis of chloroplast rRNA (26, and accompanying paper).

Chromomycin A_3, which like AMD binds strongly to the GC rich regions of DNA (45) kills cells rapidly and with temperature-dependent kinetics. There are important differences between the molecular nature of the binding of the two drugs to DNA, since chromomycin-A_3 does not appear to bind by intercalation (46). Since mutants which are resistant to AMD are also resistant to chromomycin A_3 (see accompanying paper) it is likely the two drugs kill in the same way. Therefore, it is unlikely that AMD kills cells by intercalating into DNA and altering its tertiary structure. On the other hand, campothecin, which inhibits RNA synthesis, but binds only weakly to DNA (43), is not very toxic to cells. It thus appears that the primary site of AMD's lethal interaction with cells is associated with its ability to bind to DNA.

While the killing of cells might result from extended binding of AMD this does not appear likely. The temperature sensitivity of killing, which persists even when corrected for differences in the rate of binding

of AMD, implies that the lethal event occurs while the cells are being incubated with AMD rather than after they have been plated.

The drug does not stimulate the breakdown of protein. Although a lethal, but not a sublethal, dose of AMD does cause breakdown of RNA, the extreme difference between the kinetics of the extent of killing and of RNA breakdown suggest that the degradation of RNA is not the cause of cell death but follows it.

AMD induces breakage of DNA in KB cells (31). Lethal doses of the drug do not induce degradation of Chlamydomonas DNA. These doses are also ineffective in stimulating the rate of mutagenesis. There is no detectable affect of the drug on the stability of DNA, but some breakage of DNA cannot be excluded in C. reinhardi. It should be noted, however, that camptothecin has also been reported to cause breaks in DNA (43), but is not toxic to C. reinhardi

Inhibition of Macromolecular Synthesis

Honikel and Santo have presented evidence derived in vitro that AMD inhibits DNA synthesis either at the complex forming step or at the initiation step of the DNA polymerase reaction (15). The inhibition of DNA synthesis might result because AMD inhibits the synthesis of RNA necessary for the initiation of DNA synthesis (42). Our data are consistent with this model. DNA replication takes approximately 4 h in Chlamydomonas (21). Since replication which has already begun will not be affected by AMD (15), we would expect to see little inhibition of DNA synthesis in the first four hours of incubation. During the second

4 h, however, the inhibition should be significant. In fact, it is very similar to the inhibition of RNA synthesis.

As expected, the synthesis of RNA is more sensitive to AMD at low doses than the synthesis of DNA or protein, but the specificity of the drug is not great. In these experiments the inhibition of total RNA synthesis reflects the sum of the inhibitions of a wide spectrum of RNAs, whose sensitivity to AMD will vary greatly. Previous investigators have suggested that the differences in sensitivity results from differences in the guanine + cytosine content of the transcribed gene (9) or are the result of polycistronic transcription (33, 34).

We were intrigued by the hyperbolic shape of the curve of inhibition of synthesis of RNA versus dose of AMD as shown in Fig. 9, and attempted to develop a model for the sensitivity of a gene or population of genes to AMD. Although initially developed for this rather crude measure of sensitivity, the model is successful in explaining the sensitivities of individual RNA species as measured by Perry and Kelley(34) in L cells and Bleyman and Woese in Bacillus subtilis (2). The reader should also examine the interesting discussion of Perry and Kelley concerning this subject (34).

Our model postulates only that AMD binds randomly to guanine and cytosine base pairs and leads to the conclusion that the frequency of transcription is an important determinant of a gene's sensitivity to AMD.

Consider a gene, i, in a population of genes exposed to AMD. Assuming that there is random binding to guanine-cytosine base pairs, AMD is distributed in the population of genes according to a Poisson distribution. The inhibition of transcription of a population of genes

is given by equation 1:

$$1) \qquad R_i = e^{-rn} \sum_{h=o}^{h=n/7} \frac{(rn)^h}{h!} S_i(h)$$

where:

R_i is the relative rate of synthesis of RNA of the population of genes i

r is the average frequency of AMD molecules bound per base pair

n is the number of base pairs in gene i

h is the number of AMD molecules bound to any particular copy of genes i

$S_i(h)$ is the rate of synthesis of RNA from a copy of gene i containing h molecules of AMD, divided by the rate of synthesis of the same gene in the absence of bound AMD.

The summation is limited to h = n/7 because of the steric inhibition of binding (18, 41) which limits the number of "strong" binding sites to n/7 (17). This model also contains the implicit assumption that the dissociation of AMD from the gene is slow compared to the time taken to transcribe the gene. This seems to be a reasonable assumption in light of the very long half-life of AMD excretion and in light of the time required for the recovery of RNA synthesis after the treatment of other cells (38, and accompanying paper).

Hyman and Davidson (17) have shown that AMD slows chain propagation by reducing the rate terms for the incorporation of GTP and CTP into RNA by factors of 390 and 980, respectively. Therefore, any RNA polymerase will be slowed down, on the average, approximately 700-fold as it

transcribes a guanine-cytosine pair containing AMD. Thus the transit time $\langle\tau\rangle$, of a gene containing h AMDs transcribed by a single RNA polymerase at velocity, v, will be

$$2) \qquad \langle\tau\rangle = \frac{(n-h) + 700\ h}{v} \approx \frac{n + 700\ h}{v}$$

The relative rate of transcription is

$$3) \qquad S_i(h) = \frac{n/v}{\langle\tau\rangle} = \frac{n}{n + 700\ h}$$

Combining 3) and 1)

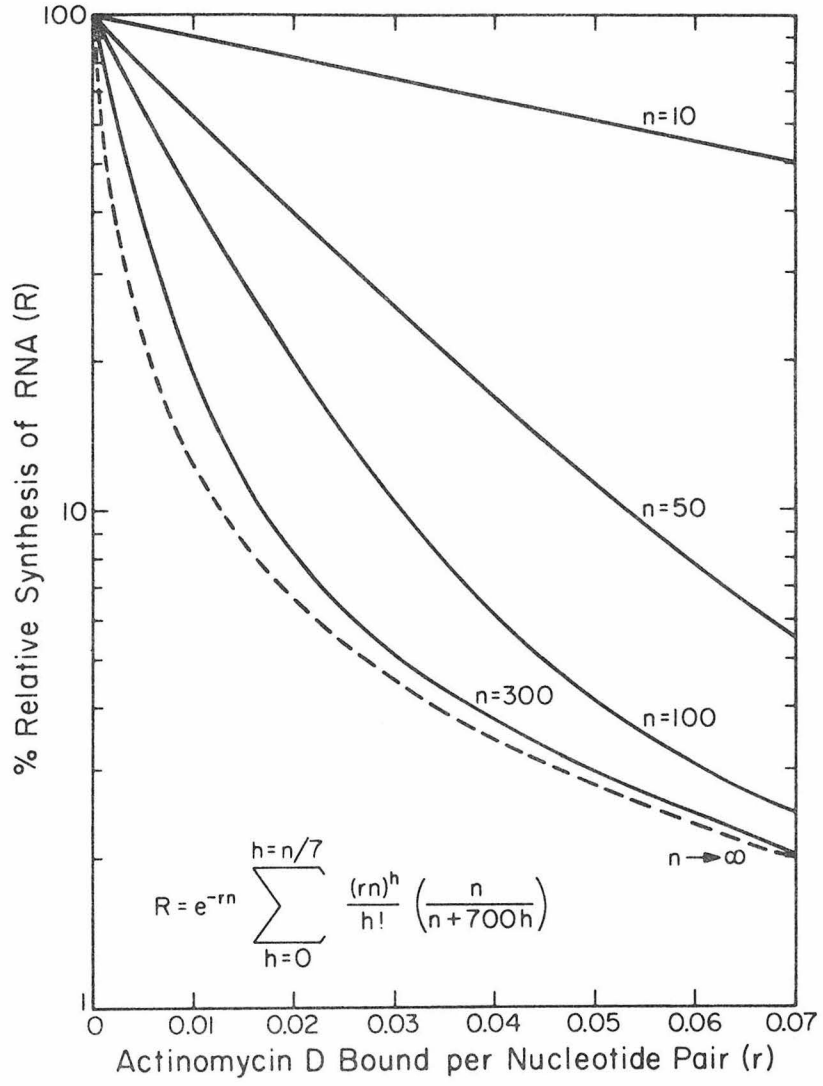
$$4) \qquad R_i = e^{-rn} \sum_{h=o}^{h=n/7} \frac{(rn)^h}{h!} \left(\frac{n}{n + 700\ h}\right)$$

Figure 13 illustrates a series of solutions of equation 4 for cases of n = 10 to n = 300. RNA synthesis is inhibited with essentially exponential sensitivity only for small r and/or n.

Equation 4 is true only for infrequently transcribed genes. The spacing of RNA polymerase molecules on different genes varies greatly (19, 30). Ribosomal RNA cistrons, for example, contain tightly packed polymerases (27). When AMD binds to a gene which contains polymerases spaced more closely than one per 700 base pairs the polymerases will tend to accumulate behind the first AMD block as automobiles do in a traffic jam. The speed with which the steady state is reached will depend on the initial packing and on the absolute rate of RNA transcription. In the case of tightly packed ribosomal RNA, the steady state should be reached very quickly since 45S ribosomal precursors

Figure 13

Plot of the relative synthesis of a species of RNA, R, of a gene, i, versus the average number of AMD molecules bound per base pair in that gene, r, according to equation 4. The value given by n indicates the size of the gene in base pairs. The dashed line represents the sensitivity to inhibition for a theoretical gene of the size of the whole genome.


100
10
1
% Relative Synthesis of RNA (R)
n=10
n=50
n=100
n=300
n→∞
$R = e^{-rn} \sum_{h=0}^{h=n/7} \frac{(rn)^h}{h!} \left(\frac{n}{n+700h}\right)$
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
Actinomycin D Bound per Nucleotide Pair (r)

are transcribed in about 2.3 min (14). The model suggests that intercalation of AMD into a frequently transcribed gene would introduce gaps in the appearance of nascent RNA molecules which would shorten the apparent length of the gene. Scheer, Trendelenburg and Frank (39) have examined, in the electron microscope, rRNA cistrons of Triturus oocytes which have been treated with AMD. Their pictures illustrate such a situation, although they interpret this as resulting from premature release of the 45S rRNA precursor caused by AMD. Therefore a single AMD bound to a frequently transcribed gene will reduce transcription of the whole gene by up to 700-fold, hence,

5) $S_i\ (0) = 1$ and $S_i\ (\geq 1) \simeq 0$, so, from 1.)

6) $R_i = e^{-rn}$

Therefore the sensitivity of frequently transcribed molecules should be an exponential function of both the size of the gene and the amount of AMD bound per base pair. This will, however, be true only when the amount of AMD bound is relatively small. A moderate to large size gene with low transcriptional efficiency will be relatively more resistant to AMD and synthesis of its RNA will be inhibited hyperbolically.

Ribosomal RNA synthesis is inhibited very rapidly and as an exponential function of dose in both prokaryotes (2) and eukaryotes (34). Furthermore, equation 5 predicts that the level of AMD binding, when corrected for background, necessary to reduce RNA synthesis to 1/e will be the reciprocal of the length of the gene. The most accurate calculation of RNA size using the model would correct for differences in base content of different RNAs. This correction would probably be most

important to small RNAs. However, even calculations neglecting base composition are in rough agreement with the actual sizes of the molecules. The data of Perry and Kelley (34) demonstrate this proportionality for ribosomal precursors, 5S and tRNAs. Using their data (for total uptake of AMD into the cell) to estimate r and their value for the uptake of AMD necessary to reduce transcription of 45S RNA to 1/e, we estimate n to be 1.8×10^4 base pairs which is close to the actual value of 1.3×10^4. Their data for the sensitivity of 4S and 5S RNA yield a calculated size of 190-300 using our model. These numbers are 2-3 times too large. A very considerable difference between calculated and actual values results when their data for heterogeneous nuclear RNA are used in our model. hnRNA of molecular weight equal to 4.2×10^6 daltons gives a target size of only 309 bases by our model while 7×10^5 daltons hnRNA gives a size of only 68 bases. The considerable difference between the value measured by our model and the actual values of 13,000 and 2200 base pairs suggests that much of the genes for these two RNAs has very little affinity for AMD, possibly because of their base structure or because of the structure of their chromatin. Perry and Kelley (34) also showed that mRNA was less sensitive to AMD and was inhibited as a hyperbolic function of dose. In our model, this implies that the bulk of the mRNA genes are infrequently transcribed. This seems to be generally true (19, 30). However, it could reflect heterogeneity of the genes coding for the mRNAs. The inhibition of total RNA synthesis in C. reinhardi as a function of dose probably reflects the heterogeneous population of species being inhibited.

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CHAPTER III:

THE EFFECTS OF ACTINOMYCIN D ON CHLAMYDOMONAS REINHARDI II. ISOLATION AND CHARACTERIZATION OF MUTANTS RESISTANT TO KILLING BY ACTINOMYCIN D.

THE EFFECTS OF ACTINOMYCIN D ON *CHLAMYDOMONAS REINHARDI* II. ISOLATION AND CHARACTERIZATION OF MUTANTS RESISTANT TO KILLING BY ACTINOMYCIN D

Mark J. Miller, William Fry, and Daniel McMahon

California Institute of Technology

Division of Biology

Pasadena, California 91125

Correspondence and Proofs should be addressed to:

ABSTRACT

Mutants of Chlamydomonas reinhardi have been isolated which are both temperature sensitive in growth and resistant to killing by actinomycin D. These mutants, unlike other actinomycin D-resistant cell lines, are neither impermeable to the drug nor do they excrete it at an accelerated rate. The mutants are partially temperature sensitive in their ability to synthesize RNA. In the presence of actinomycin D, however, RNA synthesis is partially protected at the nonpermissive temperature (and, in some cases, at the permissive temperature also) when compared to the inhibition of wild type cells.

Extraction and examination of RNA from these mutants reveals that actinomycin D inhibits different species of RNA to different extents.

We propose that the mutants have an altered chromosomal constituent, which impedes the binding to the genome. At the nonpermissive temperature the alteration is postulated to partially interfere with transcription.

INTRODUCTION

Actinomycin D (AMD) is a widely used inhibitor of DNA-dependent RNA synthesis. It is also very toxic to cells and, in the previous paper, we have shown that the toxicity, while it is probably associated with the binding of AMD to DNA, results from a cause which is more subtle than the inhibition of general macromolecular synthesis. One way of studying the toxicity of AMD is to study mutants which are resistant to the drug. To date, all of the resistant strains studied by others have proven to be either impermeable to the drug (3, 13) or to excrete it more rapidly than do wild type cells (16, 22).

Since a mutation of the function which AMD inhibits might of itself be fatal to the cell, we sought to isolate, by suicide selection, conditionally resistant mutants. This method has previously been used for the isolation of mutants in protein synthesis of Chlamydomonas reinhardi (9, 10).

All of the mutants which have been found are more resistant to AMD at the nonpermissive temperature (33°C) than at the permissive temperature (22°C) although some of these are also resistant at the permissive temperature when compared to wild type cells.

MATERIALS AND METHODS

Chemical and Culture Condition

The chemicals and culture conditions not described here have been described in the preceding paper. Only CW-15 or mutants derived from it have been used here.

Mutagenesis and Selection of Mutants

Cells were mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine as described by McMahon (9). Following mutagenesis, the cells were grown for a week at 22°C (about 14 doublings) to allow phenotypic expression of the mutants. The cells were centrifuged and resuspended in 5 ml HSMA and then incubated for 1.5 h at 33°C. Five ml of growth medium containing 75 µgm/ml AMD (prewarmed to 33°C) was added and the cells incubated for 8 h at 33°C. Only 0.1 to 0.01% of wild type cells survive this treatment. Afterwards they were centrifuged, resuspended in fresh medium and allowed to grow for seven days before the selection procedure was repeated. After 4 days the cells were plated on HSMA agar, and after 4 days of growth the colonies were replica plated onto plates of HSMA agar which were incubated at 22°C and 33°C. Approximately 0.1% of the colonies were temperature sensitive. These were purified by streaking and retested for temperature sensitivity. Five mutants were examined in some detail. One mutant, ART-10A, was lost due to reversion after the experiments described here.

Actinomycin D Extraction and Chromatography

Cells which had been labeled for 8 h with ^{3}H-AMD were washed 5 times with cold water (0-4°C), resuspended in 2.0 ml cold water containing 20 µgm/ml carrier AMD, and extracted three times with $CHCl_3$ at 0-4°C. After each extraction the mixture was centrifuged for 5 min at 3000 x g. The organic layers were removed and combined. Five volumes of ethanol (-20°C) was added to the aqueous layer and the resultant precipitate was used to determine unextracted AMD counts. The $CHCl_3$

was evaporated in a stream of dry air and the residue was taken up in 0.25 ml acetone. An aliquot of the extract was spotted onto silica gel thin layer chromatograms (EM Laboratories, Elansford, N. Y.) and chromatographed in ethyl acetate:acetone (2:1) (7). The chromatography must be done as soon as possible to avoid degradation of the drug after extraction. After drying, the plates were cut into 1/4-inch strips, and the silica gel was scraped into scintillation vials. Counting was done in aquasol liquid scintillation solution (New England Nuclear, Boston, Mass.) after allowing the samples to elute overnight. Eighty-two percent of the total AMD taken up by the cell is extracted and the ethanol precipitable counts are reduced by 49%. Therefore, about 22% of the extracted counts are derived from ethanol precipitable AMD.

RNA Extraction

The methods of extraction and electrophoresis of RNA have been described previously (11). This procedure proved to be unsuitable for the isolation of RNA containing poly(A). The following procedure was developed for the extraction of this species. Cells were poured over cracked ice to chill them to 0-4°C and centrifuged for 1 min at 1000 x g. The supernatant was poured off and 10 ml of a solution containing 1.5% SDS, 2.0% diethyl pyrocarbonate (DEP), 5 mM EDTA, 25 mM Tris-HCl (pH 7.6, 0-4°C) was poured over the cells to lyse them (the SDS and DEP were added to the cold Tris-EDTA solution immediately before adding it to the cells). The lysate was extracted with phenol:chloroform:isoamyl alcohol (49:49:2, v:v:v) and centrifuged at 12,000 x g for 20 min. The upper, aqueous layer was transferred to a glass conical bottomed centrifuge tube, extracted again with phenol:chloroform:isoamyl alcohol, and

centrifuged for 20 min at 2000 rpm (1000 x g). The organic layer was drawn off and the aqueous layer was extracted once more with chloroform, isoamyl alcohol (96:4, v:v) and centrifuged. The aqueous layer was precipitated in 2.5 volumes of ethanol. The samples were treated with DNase (11) before being passed through poly(U) filters.

Measurement of poly(A) containing species of RNA

Poly(A) containing RNA was isolated on poly(U) filter as described by Sheldon et al. (18), and modified by Firtel (4, and personal communication). The RNA isolated above was dissolved in 1.0 ml buffer BP (0.15 M Na_2HPO_4, 0.1 mM EDTA, 0.5% SDS, 0.01 M Tris-HCl, pH 7.6) and its concentration measured by UV spectrophotometry assuming that a 1.0 mg/ml RNA solution has an A_{260} = 23. The poly(U)-filter (or blank, control filters containing no poly(U)) were placed in conical Bucherer funnels and washed with at least 50 ml distilled water, followed by a few ml of buffer BP. The filters were aspirated as dry as possible and the vacuum released. Between 0.1 and 0.2 ml of the RNA solution was applied to the filter, suction was resumed and the filter washed with at least 50 ml buffer BP and then with at least 50 ml 3 M ammonium acetate in 50% ethanol. The filter was dried at 70°C until no ammonia smell could be detected. It was then cooled and counted in dioxane based scintillation fluor. The efficiency of counting of ^{32}P was 100%. Filters without adsorbed poly(U) were run for each sample and retained less than 1% of the counts retained by the poly(U) filter. Predigestion of the RNA solution with RNase A (20 µgm/ml in 50 mM KCl, 1 mM Mg Cl_2, 50 mM Tris-HCl pH 7.6) for 1 h at 30°C reduces the RNA retained by the filter

by 97.5%. The RNA which was bound was hydrolyzed in 0.3 M KOM for 18 h at 37°C and the hydrolysate was absorbed on acid washed charcoal (21). It was eluted with 0.1 N NH_4OH in 60% acetone at 37°C for 1 h. The acetone was removed in a stream of dry air and the remaining sample was lyophylized. The dried extract was taken up in 0.5 ml H_2O and chromatographed on a PEI-cellulose plate (14) as described by Nazar et al. (12). Only 2' and 3' AMP residues were recovered indicating that the filters were binding poly(A) and that C. reinhardi does contain poly(A)-containing RNA sequences.

RESULTS

Resistance to Actinomycin D

Five actinomycin D-resistant temperature sensitive clones (ART clones) have been isolated from CW-15, all of which are resistant to AMD at 33°C (Table I). (Similar resistant clones were isolated in strain Arg 2 but these are not discussed here.) Three clones, ART-7, -8, and -10A are also partially resistant at 22°C. The clones are up to 10^4 times more resistant to AMD at 33°C (Table I). These clones were produced by a mutagen and have remained resistant to AMD when cultured in its absence for over a year. Stable revertants of these clones which lose their sensitivity to high temperature and their resistance to AMD coincidently have been produced by mutagenesis (Table II). So we shall refer to these clones as mutants although definitive proof of their nature is contingent upon mapping of their location on the chromosomes.

TABLE I

Survival and Binding of Actinomycin D in ART Mutants After Incubation for 12 h in Actinomycin D

Strain	AMD/cell ($X10^{-6}$)		% Survivors	
	22°C	33°C	22°C	33°C
CW-15	6.0	16.8	2.9	0.015
ART-7	5.4	10.7	11.5 (1.9)	45.2 (<.01)
ART-10A	5.2	10.2	52.0 (2.2)	62.0 (<.01)
ART-6A	6.8	10.2	4.6 (0.6)	88.1 (<.01)
ART-10	5.5	8.9	1.4 (1.7)	108.0 (<.01)
ART-8	4.7	7.5	61.6 (3.2)	122.0 (<.03)

Cells were washed and resuspended in fresh HSMA. After 1 h at 22°C, one aliquot of cells was placed at 33°C and the other left at 22°C. Two hours later AMD was added to a final concentration of 40 µgm/ml. For the binding assay, 2 µCi/ml ^{3}H-AMD was included. After 12 h incubation, binding of AMD and the relative number of survivors was determined as described in the preceeding paper. The numbers in parentheses indicate the expected percentage survival of wild type cells at the same temperature if the same amount of AMD were bound to them (derived from Fig. 1b of the accompanying paper).

TABLE II

Survival of ART-Revertants After 12 h Incubation in Actinomycin D

Strain	% Survivors	
	22°C	33°C
CW-15	4.0	.0051
Rev-6A	11.9	.21
Rev-10	2.7	.074
Rev-10A	11.7	.24

Cells were incubated in 40 µg/ml of AMD for 12 h at 22°C or 33°C as described in the legend of Table I.

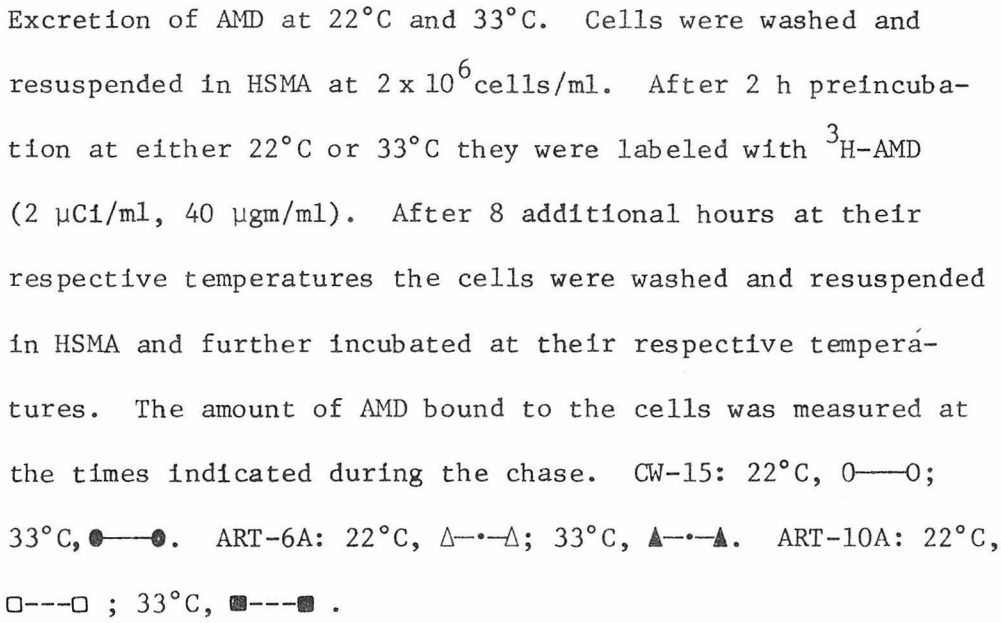
Table I also shows that the resistance of the clones is not due to a failure of the drug to enter the cell. At 22°C the mutants bind approximately as much AMD in 12 h as do wild type cells. At 33°C they bind more drug than at 22°C, though the increase is not as great as that found in wild type cells. The strains are listed in Table I in order of their resistance to AMD at 33°C. There is a correlation between the amount of drug bound at 33°C and the ability of the cells to survive. Those ART mutants which are least affected by AMD at 33°C (ART-10 and ART-8) bind less drug than do the more sensitive strains. However, the data in Table I clearly show that the reduction in binding is not sufficient to explain the resistance of the mutants.

Two ART mutants which were tested do not excrete the drug more rapidly than wild type cells do. Fig. 1 shows that the rate of excretion of AMD in all of the strains is slightly slower at 33°C than at 22°C. This may reflect the fact that binding of the drug to DNA is stronger at higher temperature (6). One mutant, ART-10A, excretes AMD at the same rate as wild type cells, the other, ART-6A, is much slower.

Cells of ART-6A and CW-15 were labeled at 22°C and 33°C with ^{3}H-AMD. After 8 h the cells were washed and extracted with chloroform. The silica gel thin layer chromatogram of the 33°C extract is illustrated in Fig. 2. The majority of the counts extracted from mutants and wild type cells migrated as AMD, although some material remained at the origin. Chromatography of the extract from cells incubated at 22°C yielded the same results (data not shown).

Actinomycin D inhibits RNA transcription in the mutants. In Table III cells were preincubated in AMD for 3 h at 22°C or 33°C, and pulse

Figure 1



Excretion of AMD at 22°C and 33°C. Cells were washed and resuspended in HSMA at 2×10^6 cells/ml. After 2 h preincubation at either 22°C or 33°C they were labeled with ^{3}H-AMD (2 µCi/ml, 40 µgm/ml). After 8 additional hours at their respective temperatures the cells were washed and resuspended in HSMA and further incubated at their respective temperatures. The amount of AMD bound to the cells was measured at the times indicated during the chase. CW-15: 22°C, O——O; 33°C, ●——●. ART-6A: 22°C, Δ—•—Δ; 33°C, ▲—•—▲. ART-10A: 22°C, □---□ ; 33°C, ■---■ .

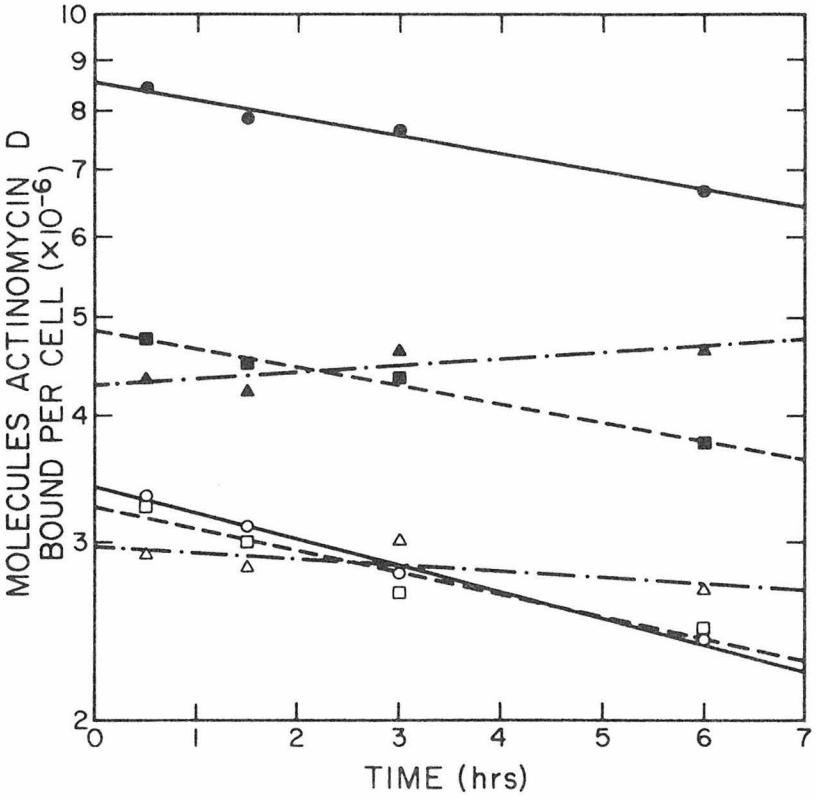

MOLECULES ACTINOMYCIN D
BOUND PER CELL (×10⁻⁶)
10
9
8
7
6
5
4
3
2
0
1
2
3
4
5
6
7
TIME (hrs)

Figure 2

Chromatography of $CHCl_3$ extract of cells labeled with ^3H-actinomycin D at 33°C. Cultures of CW-15 and ART-6A were incubated in ^3H-AMD for 8 h at 33°C as described in Fig. 1. The cells were washed, extracted with $CHCl_3$, and the extract was chromatographed in silica gel thin layer plates as described in Methods. CW-15: —— ; ART-6A: ••• . The chromatogram is outlined at the top of the figure. The circles represent yellow spots. The large circles coincide with known AMD.

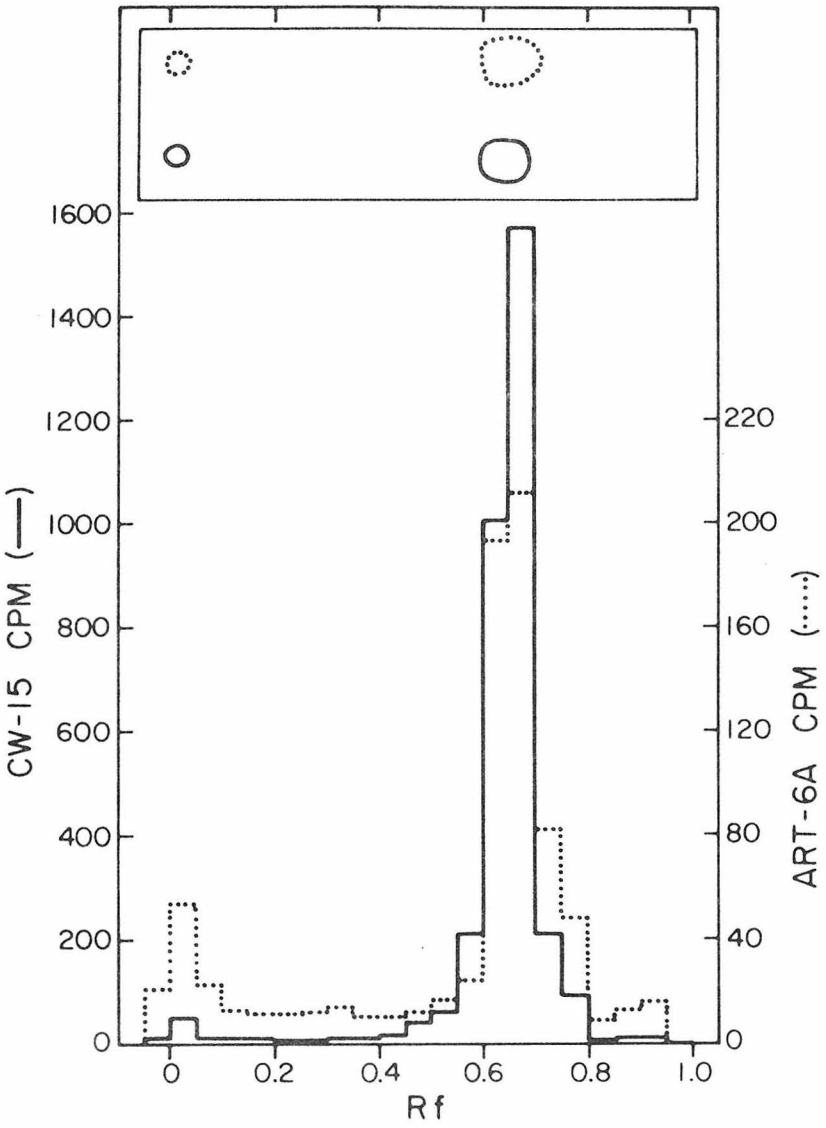

1600
1400
1200
1000
800
600
400
200
0
CW-15 CPM (—)
220
200
160
120
80
40
0
ART-6A CPM (·····)
0
0.2
0.4
0.6
0.8
1.0
Rf

TABLE III. Incorporation of $^{32}PO_4^{-2}$ into RNA and DNA at 22°C or 33°C in the Presence or Absence of Actinomycin D.

Strain	Analysis	CPM/10^5 Cells					
		RNA			DNA		
		22°C	33°C	33°C/22°C	22°C	33°C	33°C/22°C
CW-15	Control	882.1	3102.8	3.52	83.0	134.0	1.61
	+ AMD	45.4	69.3	1.53	20.7	11.7	.57
	%*	5.15	2.23	.43	24.9	8.7	.35
ART-8	Control	758.1	2117.2	2.79	65.2	173.7	2.66
	+ AMD	73.3	81.3	1.11	9.2	8.8	.96
	%*	9.67	3.84	.40	14.1	5.1	.36
ART-6A	Control	953.7	1978.1	2.07	88.6	85.3	.96
	+ AMD	112.2	259.2	2.31	14.2	12.5	.88
	%*	11.72	13.09	1.12	16.0	14.6	.91
ART-7	Control	894.5	895.4	1.00	88.3	36.3	.44
	+ AMD	127.3	213.3	1.69	8.6	15.9	1.85
	%*	14.23	23.82	1.67	9.7	43.8	4.52
ART-10A	Control	972.9	918.1	.94	85.0	78.9	.93
	+ AMD	140.1	238.6	1.70	14.1	14.0	.99
	%*	14.38	25.99	1.81	16.6	17.7	1.07
ART-10	Control	828.8	1082.0	1.31	71.4	89.8	1.26
	+ AMD	26.7	522.6	19.57	9.4	38.7	4.12
	%*	3.22	48.3	15.0	13.2	43.1	3.27

Cells were treated and labeled as described in Fig. 7., except that the $^{32}PO_4^{-2}$ concentration was 0.5 μCi/ml. Strains are listed in order of % RNA synthesis at 33°C in the presence of AMD.

*The "%" represents the percentage incorporation in the presence of AMD as compared to control.

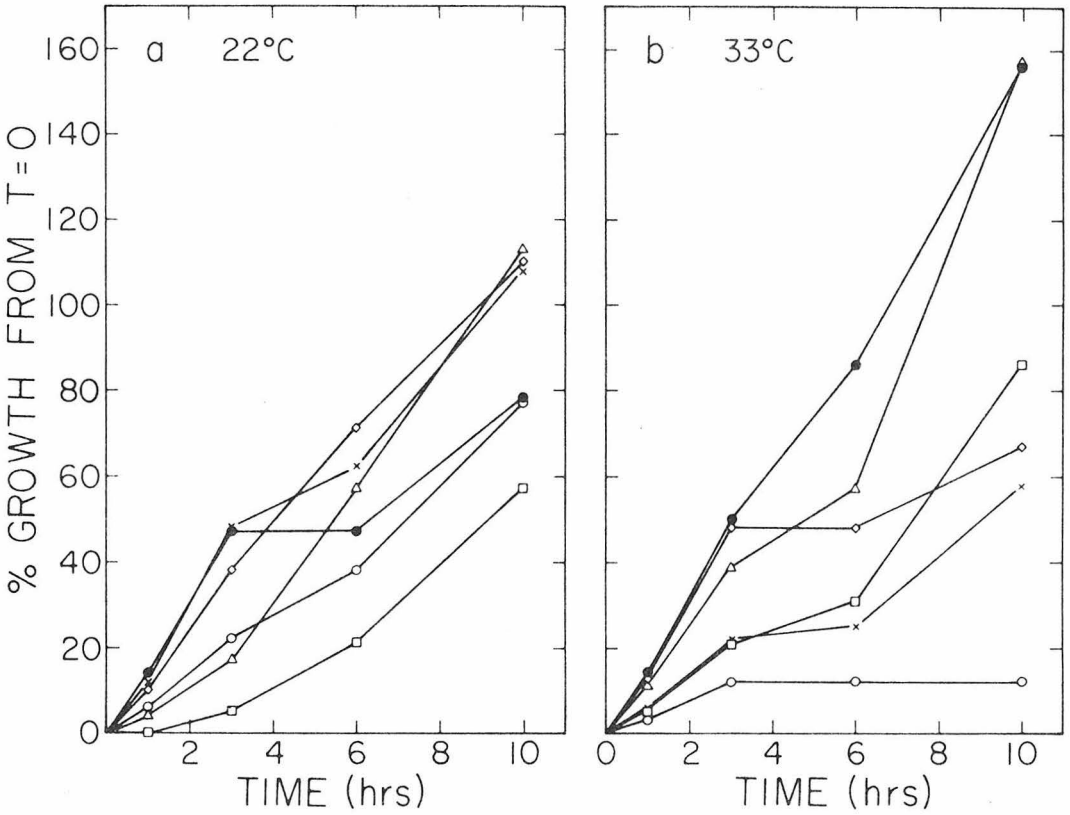
labeled for 3 additional hours with $^{32}PO_4^{-3}$. Incorporation into RNA and DNA was measured in the presence and absence of AMD. Actinomycin D inhibits RNA synthesis in the ART mutants by 85% to 97% (CW-15 = 95%) at 22°C and by 52% to 96% (CW-15 = 98%) at 33°C. The synthesis of DNA is inhibited in the mutants and in CW-15. At 33°C there is consistently more incorporation into the RNA of each of the ART mutants in the presence of AMD than into the RNA of CW-15. With the exception of ART-10, this is also true at 22°C, although the relative increase is not so dramatic as it is at 33°C. The mutants are also partially temperature sensitive in RNA synthesis and (with the exception of ART-8) in DNA synthesis. The strains are listed in ascending order of the percentage of RNA synthesis at 33°C in the presence of AMD. A comparison of Tables I and III shows that there is no correlation between the ability to synthesize RNA in the presence of AMD at 33°C and resistance to the drug. Most notably, ART-8 which is the most resistant to AMD killing is as sensitive as CW-15 to inhibition of nucleic acid synthesis by AMD.

Temperature Sensitivity of Growth, Nucleic Acid Synthesis and Protein Synthesis

All of the ART mutants do not form visible colonies on agar plates at 33°C. Fig. 3 shows the relative rate of growth of CW-15 and the ART mutants for 10 h at 22°C (Fig. 3a) and 33°C (Fig. 3b). This, and other experiments, indicate that at 22°C there is no consistent difference in the rate of growth between CW-15 and the ART mutants. At 33°C, however, all of the mutants, except ART-8, grow at a slower rate than does CW-15. ART-7 stops growing immediately, but ART-6A, -10, and -10A will

Figure 3

Temperature sensitivity of growth. The relative growth of cells at 22°C (a) and 33°C (b) as measured in the experiment described in Fig. 4. CW-15 (initial concentration = 3.6×10^5 cells/ml) ● ; ART-6A (4.0×10^5 cells/ml) X ; ART-7 (3.2×10^5 cells/ml) O ; ART-8 (2.3×10^5 cells/ml) Δ ; ART-10 (4.2×10^5 cells/ml) □ ; ART-10A (2.1×10^5 cells/ml) ◇ .


a 22°C
b 33°C
% GROWTH FROM T=0
0
20
40
60
80
100
120
140
160
2
4
6
8
10
TIME (hrs)
TIME (hrs)

grow for one to two generations before stopping. ART-8 grows for several generations before stopping, and small colonies (8 to 16 cells) can be observed with a microscope on HSMA agar plates kept for several days at 33°C.

Nevertheless, the ability of the ART mutants to survive treatment with AMD is related to their temperature sensitivity of growth. Cultures of ART mutants were mutagenized with N,N-nitrosogranidine as described in Materials and Methods, and revertants were selected by plating cells and incubating them at 33°C. The frequency of reversion was one in 10^4. When the revertants were tested for resistance to AMD we found they were more sensitive than their parent strain. Although not as sensitive as wild type cells (Table II), their sensitivity to killing by AMD increased by 300-1600 times.

Fig. 4 plots the rates of temperature dependent incorporation of $^{32}PO_4^{-3}$ into RNA. In order to detect any affects on incorporation which were in excess of the effects temperature has on cell growth we divided the incorporation by the cell concentration at each point and temperature. When CW-15 cells are placed at 33°C there is a rapid acceleration of the incorporation of $^{32}PO_4^{-3}$ into RNA (Fig. 4). In the ART mutants, however, the rate of accumulation of $^{32}PO_4^{-3}$ in RNA remains essentially constant. In no case is the relative increase in incorporation as great as that found in CW-15.

Incorporation of $^{32}PO_4$ into DNA is also faster at 33°C in wild type cells, although the relative increase is not as great as for the incorporation of $^{32}PO_4^{-3}$ into RNA (Fig. 5). The ART mutants are somewhat temperature sensitive in their ability to accumulate $^{32}PO_4^{-3}$ into their

Figure 4

Temperature sensitivity of the incorporation of $^{32}PO_4^{-3}$ into RNA. Cells were washed and resuspended in labeling media [0.133 mM PO_4^{-3} -20 nM Tris·HCl-HSMA (pH 7.2) (11)]. At time 0, the cultures were labeled with 0.5 µCi/ml $^{32}PO_4^{-3}$ and split in two with one-half being incubated at 33°C while the other half remained at 22°C. Incorporation into RNA and DNA (Fig. 5), and the cell concentration (Fig. 3) were measured at the indicated times. Incorporation into RNA was divided by the cell concentration at each time point. 22°C: ●——● ; 33°C: ●---● .

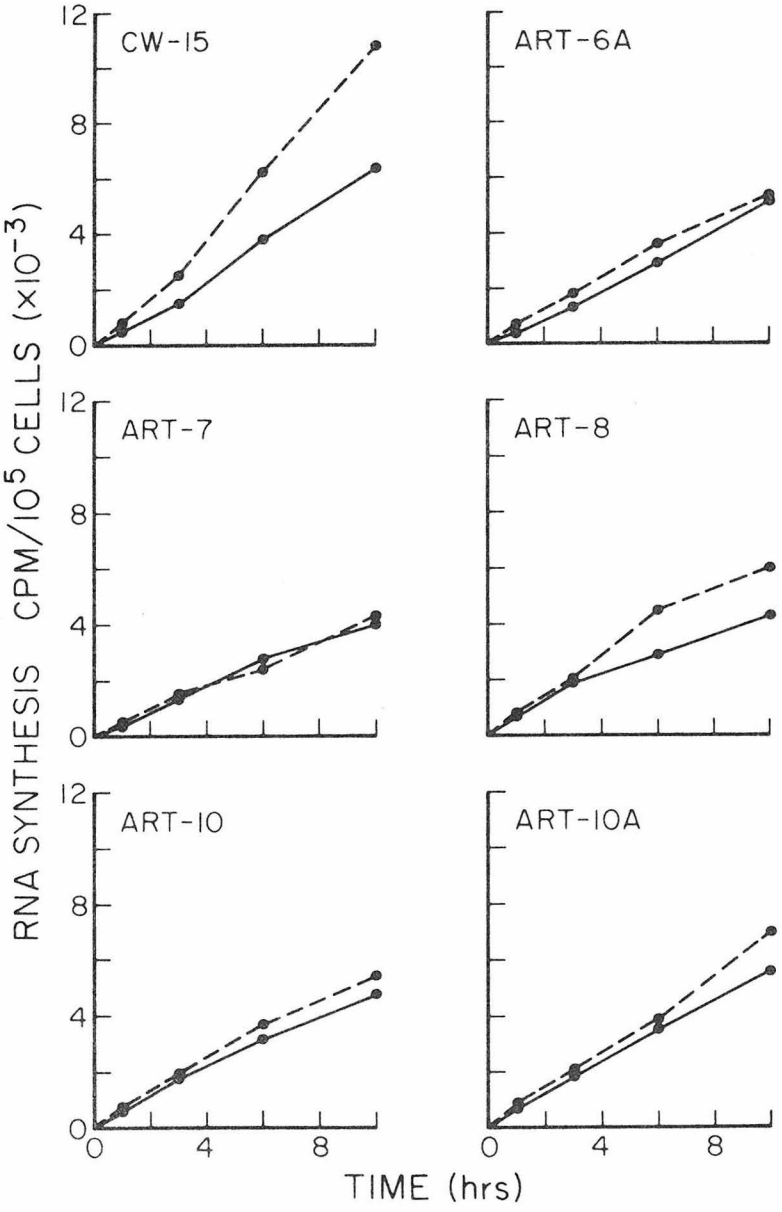
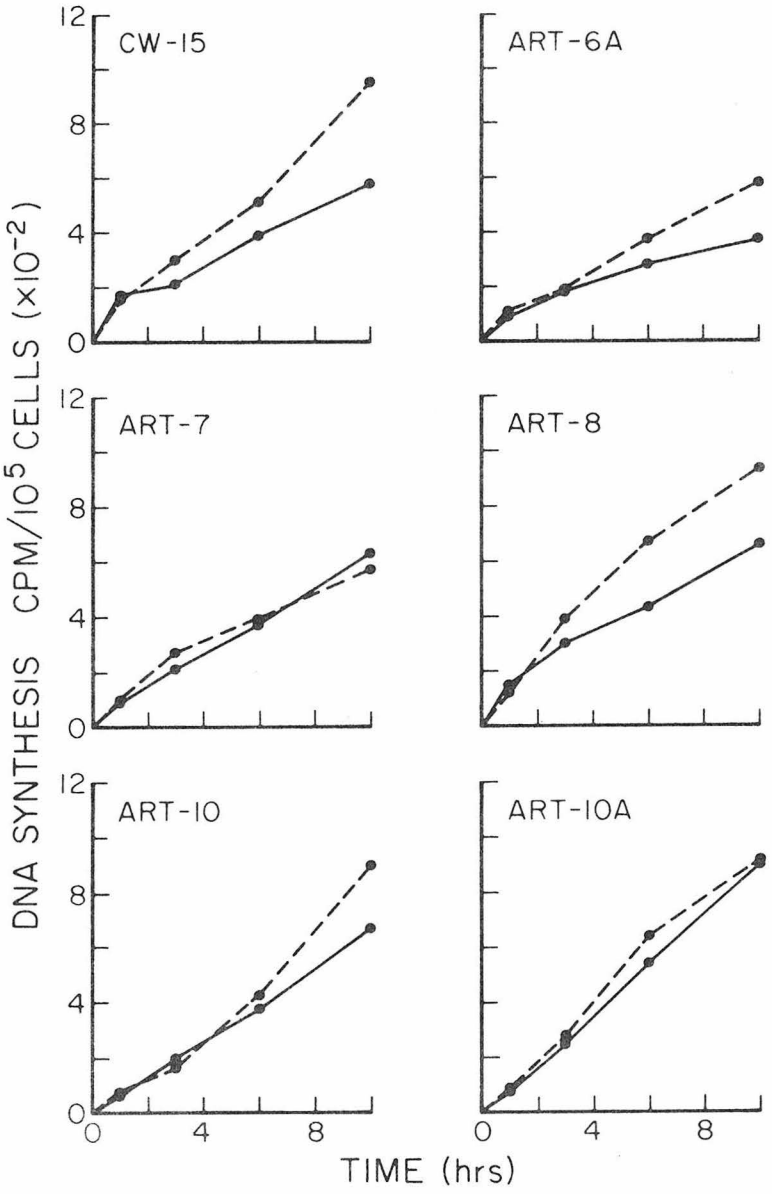

CW-15
ART-6A
ART-7
ART-8
ART-10
ART-10A
RNA SYNTHESIS CPM/10^5 CELLS (×10^-3)
TIME (hrs)
0
4
8
12

Figure 5

Temperature sensitivity of the incorporation of $^{32}PO_4$ into DNA. The synthesis of DNA at 22°C and 33°C was measured in the experiment described in Fig. 4. Incorporation was divided by the cell concentration at each time point.
22°C ●——● ; 33°C: ●---● .


CW-15
ART-6A
ART-7
ART-8
ART-10
ART-10A
DNA SYNTHESIS CPM/10^5 CELLS (x10^-2)
TIME (hrs)
0
4
8
12

DNA, but in the case of ART-6A, -8, and -10 the relative decrease is less than that found for incorporation into RNA. For ART-7 and -10A the temperature sensitive inhibition of DNA synthesis is about the same as that found for RNA synthesis.

The incorporation of $^{35}SO_4^{-3}$ into protein also accelerates when wild type cells are placed at 33°C. With the exception of ART-8, the synthesis of protein in the ART mutants does not increase at the non-permissive temperature (Fig. 6). Indeed, the relative inhibition of protein synthesis is very similar to the relative inhibition of RNA synthesis found in each of the mutants.

Synthesis of Individual Species of RNA

As we noted above, synthesis of RNA is partially protected from inhibition by AMD in the ART mutants, while RNA synthesis is partially temperature sensitive. Therefore we examined the synthesis of various species of RNA at the permissive and nonpermissive temperatures in the presence and absence of AMD.

Fig. 7a and b shows that when wild type cells are placed at 33°C, there is a significant increase of incorporation of $^{32}PO_4^{-3}$ into all species of RNA. Actinomycin D reduces ribosomal RNA (rRNA) synthesis to an undetectable level at both 22°C and 33°C (Fig. 7c and d). Some incorporation into the low molecular weight species of RNA is evident but discrete species are not apparent. The amounts of incorporation were determined by summing the counts under each peak and subtracting the background as determined from the baseline. The counts were corrected for the decay of $^{32}PO_4^{-3}$ between the day of the experiment and

Figure 6

Temperature sensitivity of the incorporation of $^{35}SO_4$ into protein. Cells were washed and resuspended in 16 µM SO_4^{-2} -HSMA. At time 0 the cultures were labeled with 0.5 µCi/ml $^{35}SO_4^{-2}$ and split in two with one-half being incubated at 33°C while the other half remained at 22°C. Incorporation into protein was divided by the cell concentration as measured at the indicated times. 22°C: ●——● ; 33°C: ●---● .

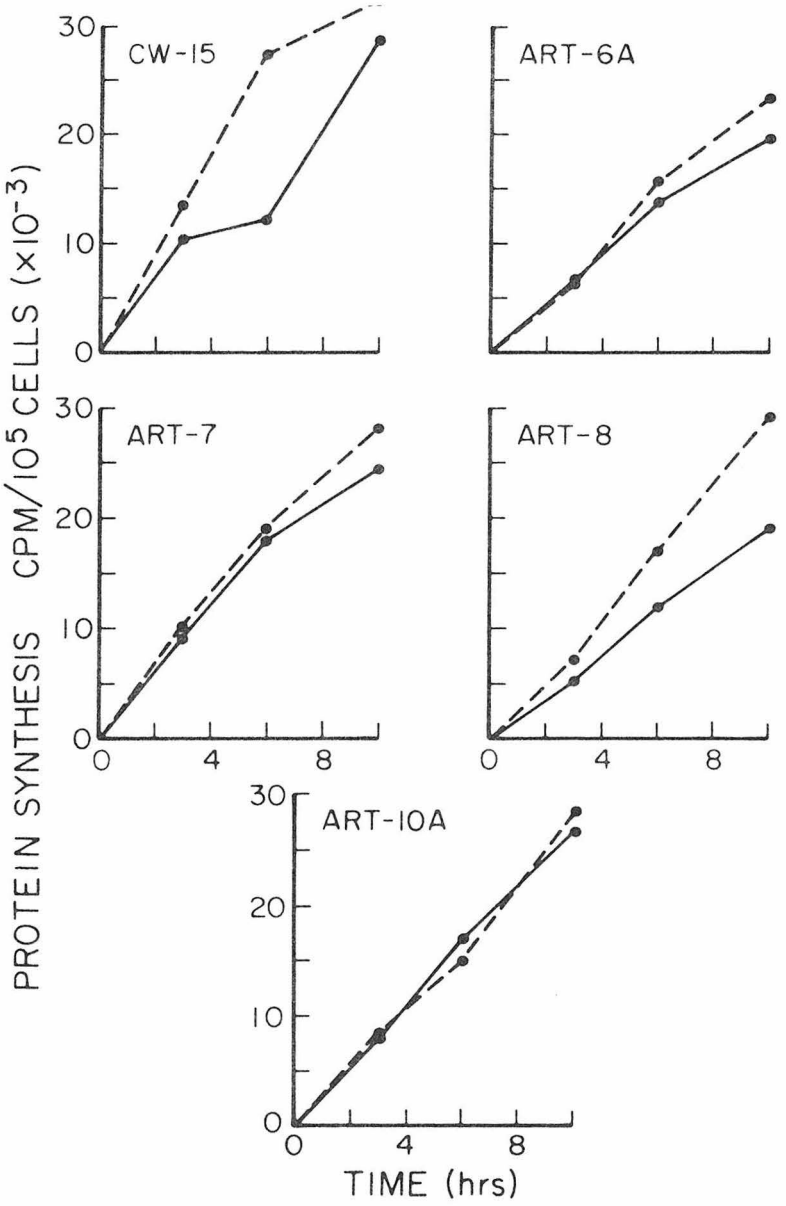
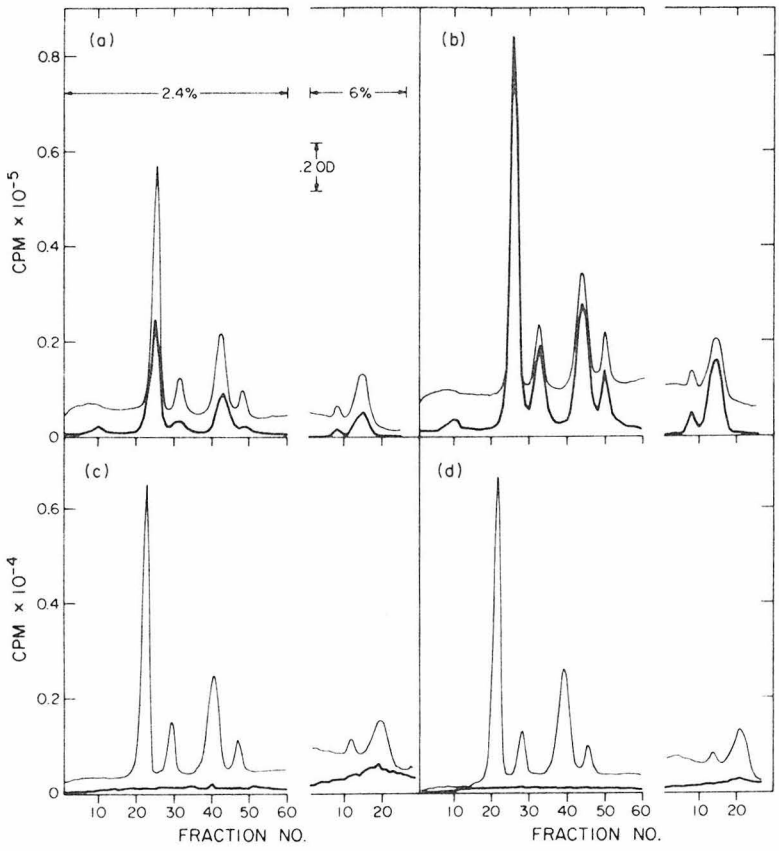

CW-15
ART-6A
ART-7
ART-8
ART-10A
PROTEIN SYNTHESIS CPM/10^5 CELLS (×10^-3)
TIME (hrs)
0
10
20
30
4
8

Figure 7

Synthesis of RNA species in CW-15 at 22°C or 33°C in the presence or absence of actinomycin D. CW-15 cells were washed and resuspended in 0.133 mM phosphate -20 mM Tris-HCL-HSMA (pH 7.2). The culture was split into four parts and two were placed at 33°C, the other two left at 22°C. After 2 h, AMD was added to one culture at each temperature to a final concentration of 40 µgm/ml. Three hours later the cells were labeled with 10 µCi/ml $^{32}PO_4^{-3}$. After 3 h of labeling, RNA was extracted (11) and portions were subjected to electrophoresis in both 2.4% acrylamide, 0.4% agarose gels, to separate ribosomal RNA species, and in 6% acrylamide gels to separate the 4S and 5S RNA species. The gels were scanned at 260 mµ in a Varian gel scanning spectrophotometer before being sectioned into 1.0 mm fractions on a Mickle gel slicer. The A_{260} scan is drawn with thin lines, the $^{32}PO_4^{-3}$ cpm with thick lines.
a) 22°C; b) 33°C; c) 22°C + AMD; d) 33°C + AMD.


(a)
(b)
(c)
(d)
2.4%
6%
.2 OD
CPM x 10⁻⁵
CPM x 10⁻⁴
0.8
0.6
0.4
0.2
0
10 20 30 40 50 60
10 20
FRACTION NO.

the day the samples were counted. The counts were normalized by dividing them by the activity of $^{32}PO_4^{-3}$ in the medium, and by the µgm of RNA layered on the gel (determined spectrophotometrically, assuming A_{260} = 23.0 for a 1.0 mg/ml solution of RNA). This data, along with the incorporation of $^{32}PO_4^{-3}$ into poly(A)-containing RNA (determined by poly(U) filtration), is summarized in Table IV. When wild type cells are placed at 33°C incorporation into their ribosomal and soluble RNAs increase 2-fold or more. Poly(A) containing RNA species increase by only 44%. The 34S precursor to cytoplasmic ribosomal RNA does not increase in amount. All of the species of rRNA are quite sensitive to AMD. However, soluble RNA is somewhat resistant and poly(A)-containing RNA quite resistant to AMD.

ART-6A incorporates slightly less $^{32}PO_4^{-3}$ into all of its RNA at 22°C, than does CW-15 (Table IV and Fig. 4). Chloroplast rRNA is synthesized to about the same level as is wild type cells. Incorporation into each RNA species except 4S decreases at 33°C. Compare these values also to those of CW-15 at 33°C. Synthesis of chloroplast rRNA is the most sensitive to 33°C, followed by poly(A) RNA, then cytoplasmic rRNA (Table V). The incorporation patterns in the presence of AMD also differ greatly from wild type cells (Fig. 8, Table IV). At 22°C AMD decreases cytoplasmic rRNA synthesis to a barely detectable level, and chloroplast rRNA synthesis is reduced by 95%. Only 4S RNA appears to be protected from inhibition by AMD. At 33°C, however, the synthesis of each RNA species is greatly protected from AMD inhibition (Table IV, Fig. 8d). Cytoplasmic rRNA is protected most spectacularly. Its relative amount of synthesis in the presence of AMD increases almost 400-fold (Table V), while the synthesis of other species increases only 5-15-fold. The synthesis of RNA is still inhibited by AMD, however. At 33°C cytoplasmic

Figure 8

Synthesis of RNA species in ART-6A at 22°C or 33°C in the presence or absence of actinomycin D. ART-6A cells were preincubated and labeled as described in Fig. 7. a) 22°C; b) 33°C; c) 22°C + AMD; d) 33°C + AMD.

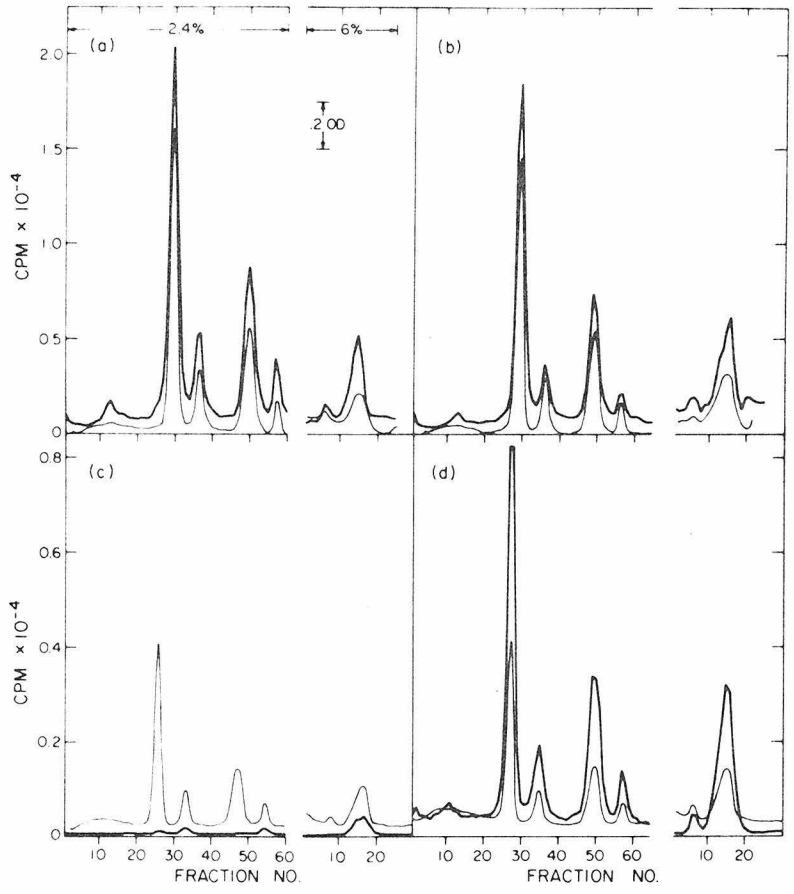

(a)
2.4%
6%
(b)
2.00
CPM x 10⁻⁴
2.0
1.5
1.0
0.5
0
(c)
(d)
0.8
0.6
0.4
0.2
0
10 20 30 40 50 60
FRACTION NO.
10 20
10 20 30 40 50 60
FRACTION NO.
10 20

TABLE IV

Incorporation of $^{32}PO_4^{-3}$ into Ribosomal, Transfer and Poly(A)-Containing RNA at 22°C and 33°C in the Presence and Absence of Actinomycin D

Strain	Temperature	Analysis	Cytoplasmic rRNA 34S	25S	18S	Chloroplast rRNA 23S	16S	Low MW RNA 5S	4S	Poly(A) RNA
CW 15	22°C	Control	116	1224	580	178	91.5	56.4	369	126.3
		+ AMD	<.2	<.2	<.2	<.8	<.5	<.6	9.1	16.5
		% *	<.2	<.02	<.03	<.4	<.5	<1.0	2.5	13.1
	33°C	Control	120	2426	1159	608	330	119	756	182.0
		+ AMD	<.2	<.2	<.2	<.2	<.2	<.3	5.6	5.8
		% *	<.2	<.01	<.02	<.03	<.06	<.3	.74	3.2
ART-6A	22°C	Control	53	788	394	163	82	30	213	94.6
		+ AMD	<.2	.6	.3	7.6	5.5	<.7	25.1	10.3
		% *	<.4	.07	.07	4.7	6.7	<2.3	11.8	10.9
	33°C	Control	39	758	354	98	35	28	294	68.5
		+ AMD	11.4	209	122	45.2	22.8	10.4	166	56.8
		% *	29.2	27.6	34.5	46.1	65.1	37.1	56.5	86.3
ART-10A	22°C	Control	94.8	1239	528	199	169	54.4	399	
		+ AMD	<.2	1.8	.3	34.1	25.1	<.2	45.3	
		% *	<.2	.15	.06	17.1	14.9	<.4	11.4	
	33°C	Control	73.1	917	465	155	108	32.3	406	
		+ AMD	2.8	43.3	20.8	10.2	5.8	1.4	33.0	
		% *	3.8	4.72	4.47	6.58	5.37	4.3	8.13	

Cells were treated and labeled as described in Fig. 7. Data is in cpm under a peak, after subtracting background counts and correcting for $^{32}PO_4$ decay. The data is normalized by dividing by the µgm of RNA layered on the gel and by the µCi/ml of $^{32}PO_4$ in the media.

*The "%" represents the percentage of incorporation in the presence of AMD as compared to control.

TABLE V

Ratio of Incorporation of $^{32}PO_4$ into RNA Species at 33°C vs. 22°C

Strain	Analysis	rRNA		Low MW RNA		Poly(A) RNA
		Cytoplasmic	Chloroplast	5S	4S	
CW 15	Control	1.93	3.48	2.11	2.05	1.44
	+ AMD	*	*	*	*	*
ART-6A	Control	.93	.54	.93	1.4	.72
	+ AMD	380.4	5.2	*	6.6	5.5
ART-10A	Control	.78	.71	.59	1.02	
	+ AMD	31.0	.27	*	.73	

Data are taken from Table IV, and represent the 33°C/22°C incorporation ratio into various species of RNA in the presence of actinomycin D.

* No significant incorporation at 22°C.

TABLE VI

Relative Incorporation of $^{32}PO_4^{-3}$ into RNA Species of ART-6A and ART-10A as Compared to CW-15 (%)

Strain	Temperature	Analysis	rRNA		Low MW RNA		Poly(A) RNA %
			Cytoplasmic %	Chloroplast %	5S %	4S %	
ART-6A	22°C	Control	64.3	90.9	53.2	57.7	74.9
		+ AMD	.05	4.9	1.2	6.8	8.2
	33°C	Control	31.1	14.2	23.5	38.9	37.6
		+ AMD	9.2	7.2	8.7	22.0	31.2
ART-10A	22°C	Control	97.0	137.0	96.4	108.1	-
		+ AMD	.11	22.0	1.1	12.3	-
	33°C	Control	39.3	28.0	27.1	53.7	-
		+ AMD	1.8	1.7	1.2	4.4	-

Data are taken from Table IV. Incorporation into various classes of RNA in the presence and absence of actinomycin D is compared to the incorporation into uninhibited CW-15. The data is presented as percent of incorporation into CW-15 at the respective temperature.

rRNA synthesis is only 30% that of uninhibited ART-6A cells (Table IV) and only 9-10% that of uninhibited CW-15 (Table VI). However, its relative rate of synthesis in the presence of AMD is 1700 times greater than is found in wild type cells at 33°C.

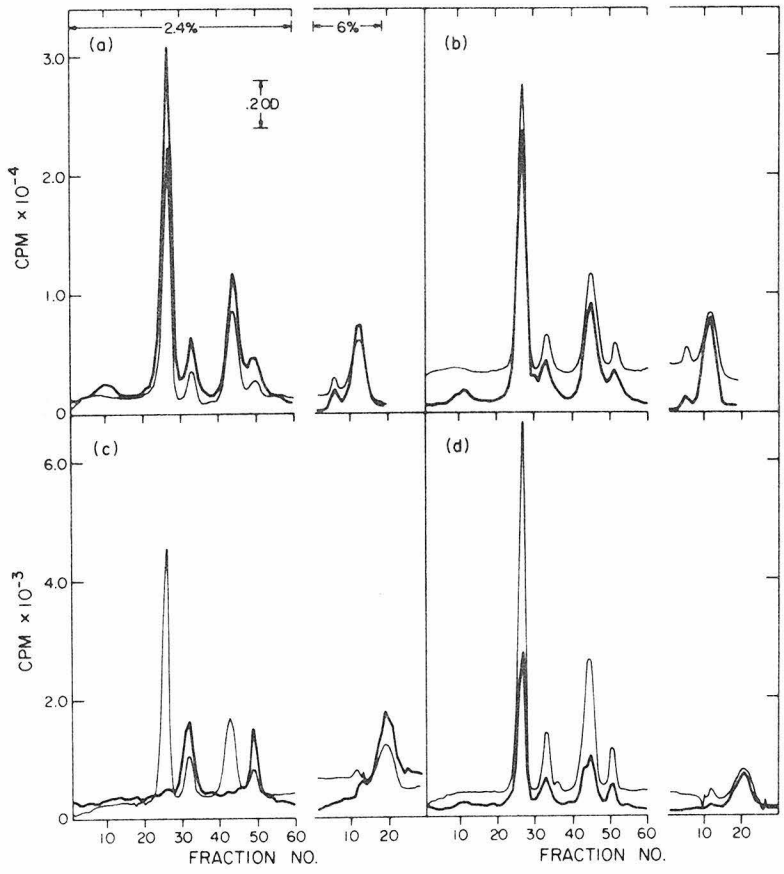
The synthesis of RNA in ART-10A is also temperature sensitive in the absence of AMD and temperature-protected in the presence of the drug (Fig. 9, Table IV). Unlike ART-6A, ART-10A incorporates the same amount of $^{32}PO_4^{-3}$ into its RNA as CW-15 does at 22°C. At 33°C ART-10A is temperature sensitive with respect to its ability to synthesize RNA. The ribosomal RNA species are somewhat more temperature sensitive than is 4S RNA (Table IV and V). Like CW-15 and ART-6A, AMD inhibits cytoplasmic rRNA synthesis by greater than 99% at 22°C. The synthesis of chloroplast rRNA and 4S RNA are much more resistant than in CW-15 (Table IV). At 33°C the synthesis of cytoplasmic rRNA is, like ART-6A, greatly protected from AMD, while the synthesis of chloroplast rRNA, unlike ART-6A, decreases (Table IV and V). Thus, for both ART-6A and ART-10A, increasing the temperature of incubation from 22°C to 33°C increases the protection of cytoplasmic rRNA synthesis significantly more than it protects the synthesis of chloroplast rRNA (Table V). However, the sensitivity of chloroplast rRNA at high temperature differs in the two mutants. It is important to note that the synthesis of all measured species of RNA in ART-10A, like ART-6A, are inhibited by AMD, although not so much as in wild type cells.

Chromomycin A_3 Killing of CW-15 and ART-6A

To examine the possibility that the chromatin of the mutants has an altered affinity for antibiotics, we examined the sensitivity of one

Figure 9

Synthesis of RNA species in ART-10A at 22°C or 33°C in the presence or absence of actinomycin D. ART-10A cells were preincubated and labeled as described in Fig. 7. a) 22°C; b) 33°C; c) 22°C + AMD; d) 33°C + AMD.


2.4%
6%
(a)
(b)
(c)
(d)
.200
CPM x 10⁻⁴
CPM x 10⁻³
3.0
2.0
1.0
0
6.0
4.0
2.0
0
10 20 30 40 50 60
10 20
FRACTION NO.

of the mutants to chromomycin A_3. Chromomycin A_3 (CA3) binds to GC rich regions of DNA and blocks the progress of RNA polymerase but does not intercalate into the double helix (23, 24). Its structure differs considerably from that of AMD. In the accompanying paper we demonstrated that CA3 kills wild type cells of C. reinhardi. We examined the sensitivity of ART-6A to CA3 since this mutant is conditionally resistant to AMD (see Table I). Fig. 10 shows that ART-6A is killed at about the same rate as CW-15 at 22°C but is significantly protected from chromomycin A_3 killing at 33°C.

Effect of Actinomycin D on the Nucleolus

Treatment with AMD causes the nucleolus to segregate its components into distinct regions generally referred to as granular or fibrillar (for a review, see ref. 2). Since ribosomal RNA synthesis is so protected from AMD inhibition at 33°C, we were interested in how AMD affected the nucleolus of an ART mutant and of wild type cells.

There is no difference between nucleoli of CW-15 cells incubated at 22°C and 33°C (Fig. 11a and b). After 4 h at 22°C in the presence of AMD, the nucleolus of CW-15 segregates into a fibrillar core region of uniform density, surrounded by a dark staning granular cortex (Fig. 11c). At 33°C the nucleolus appears to be breaking apart by this time. At 22°C the nucleolus of ART-10A appears essentially the same as that of CW-15 (Fig. 12a), but at 33°C one or more light regions, or nucleolar vacuoles, appear in the ART-10A nucleolus (Fig. 12b). In the presence of AMD (22°C) the nucleolus of ART-10A also segregates into a fibrillar and granular region (Fig. 12c). The granular region, in general, does not appear to be as thick as in CW-15. Also, although a definite granular

Figure 10

Survival of CW-15 and ART-6A in the presence of chromomycin A_3 at 22°C or 33°C. CW-15 or ART-6A cells were washed and resuspended in HSMA (1×10^6 cells/ml). Each culture was divided into two aliquots, one placed at 22°C, and the other at 33°C. After 2 h, an equal volume of 94 μgm/ml chromomycin A_3 (preincubated at 22°C or 33°C) was added. At intervals the cells were washed free of chromomycin A_3, counted on a Coulter counter and plated. CW-15: 22°C, -O- ; 33°C, -●- . ART--A: 22°C, —□— ; 33°C, —■— .

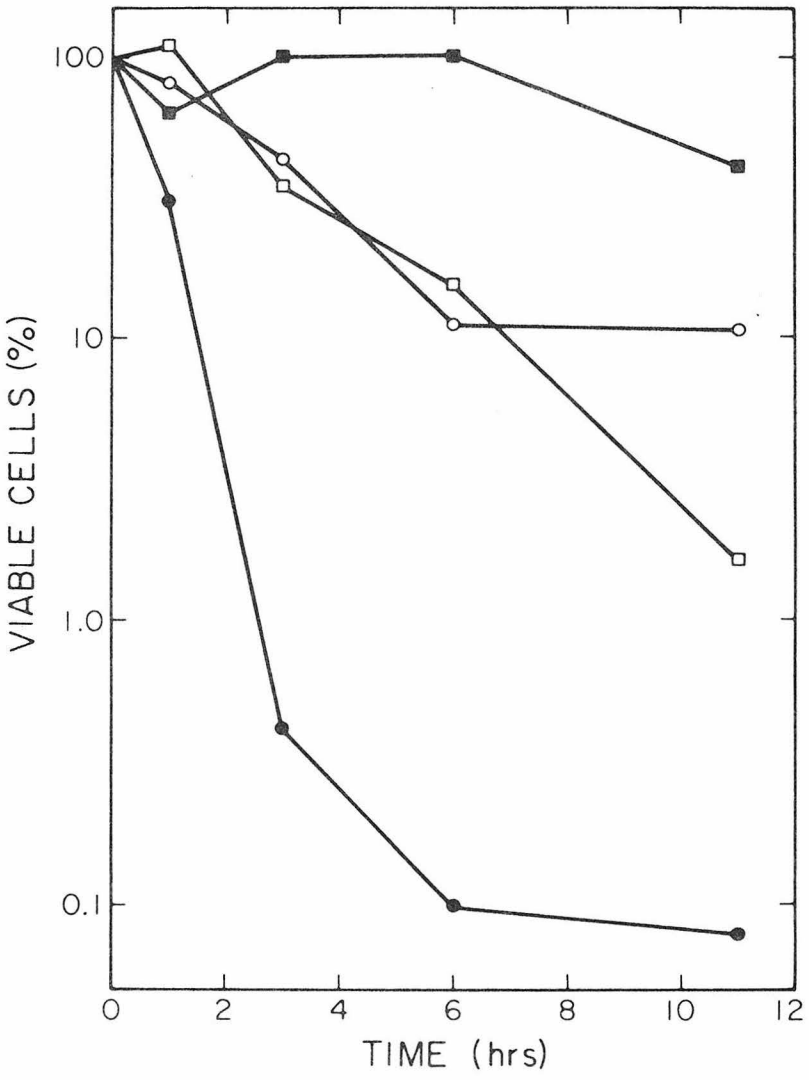

100
10
1.0
0.1
VIABLE CELLS (%)
0
2
4
6
8
10
12
TIME (hrs)

Figure 11

Nucleoli of CW-15 at 22°C and 33°C in the presence and absence of actinomycin D. CW-15 (and ART-10A) cells were washed and resuspended in fresh HSMA. After 2 h preincubation at 22°C or 33°C a culture at each temperature was treated with 40 µgm of AMD. The other set of cells were not inhibited. After 4 h incubation in the drug the cells were centrifuged and fixed as described in the preceding paper, except that they were embedded in straight epon instead of epon-avaldite. a) 22°C; b) 33°C; c) 22°c + AMD; d) 33°C + AMD. X43,000. Scale = 0.2 µM.

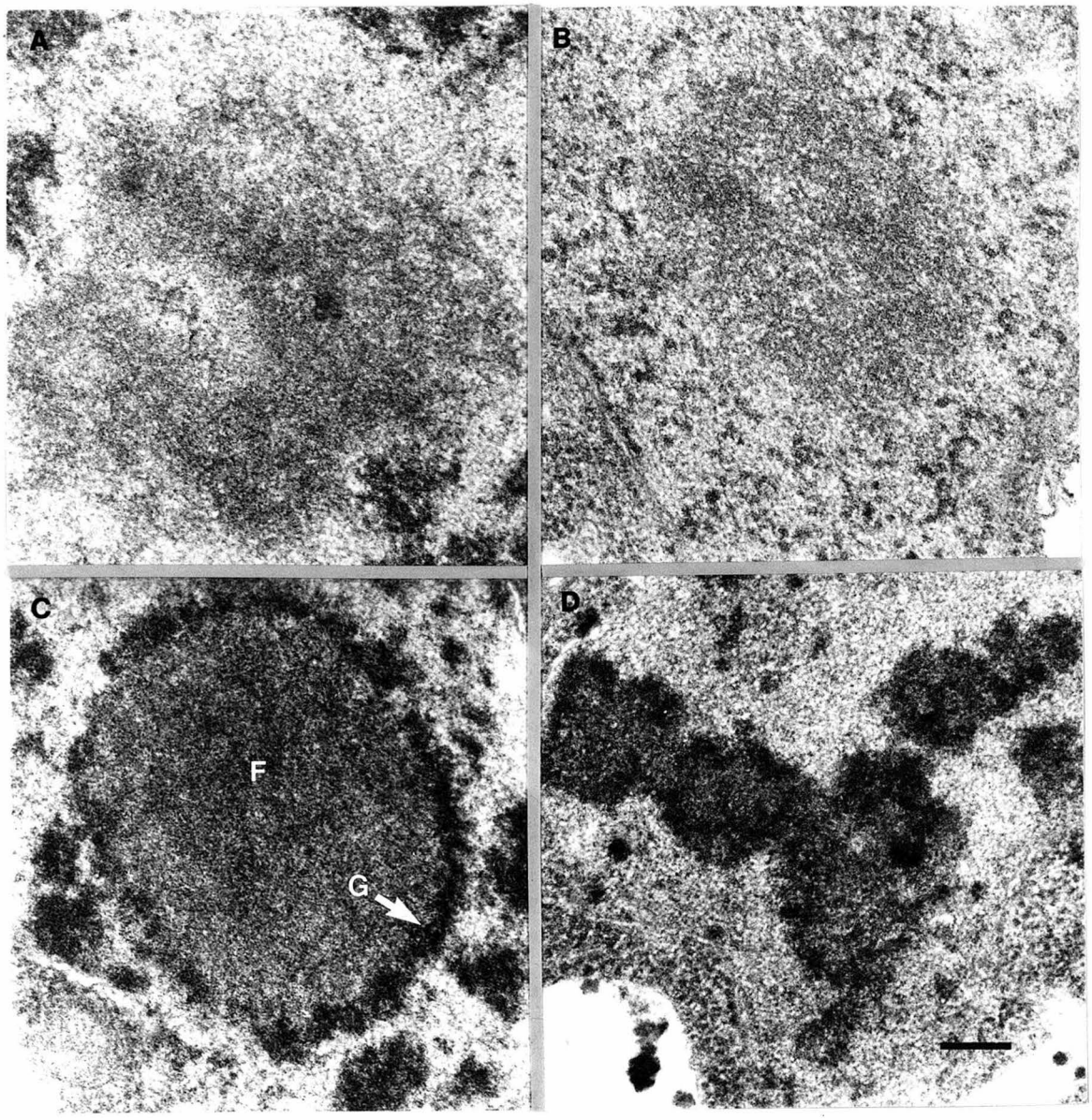
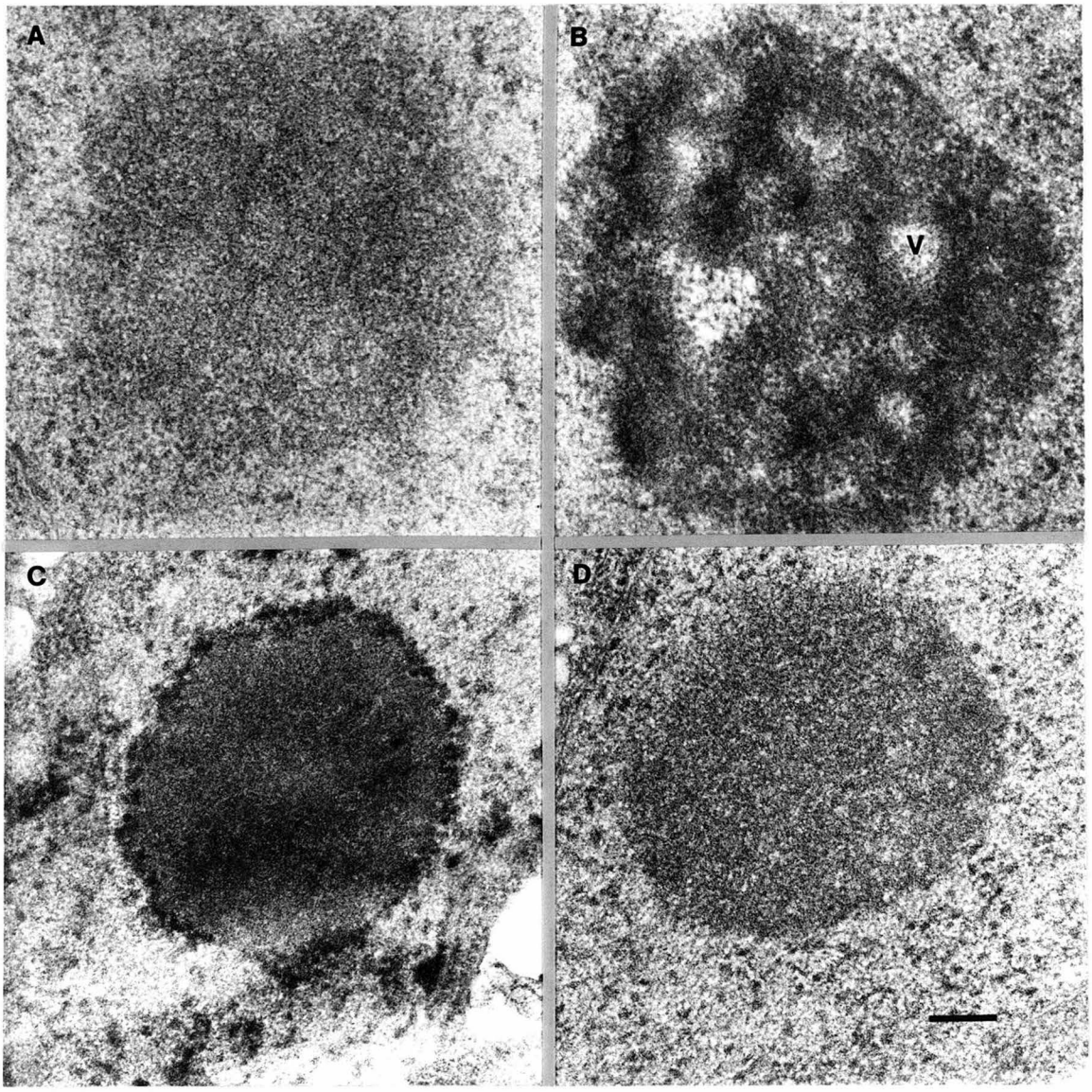

A
B
C
F
G
D

Figure 12

Nucleoli of ART-10A at 22°C and 33°C in the presence and absence of actinomycin D. ART-10A cells were treated and prepared as described in Fig. 11. a) 22°C; b) 33°C; c) 22°C + AMD; d) 33°C + AMD. X43,000. Scale = 0.2 μM.


A
B
V
C
D

region was observed in all of nine CW-15 nucleoli treated with AMD at 22°C , and eight treated at 33°C, it appeared in only nine out of 12 ART-10A nucleoli in cells treated with AMD at 22°C. At 33°C, in seven observed nucleoli, AMD did not induce segregation in ART-10A (Fig. 12d) neither did the nucleolus form the vacuoles noted in the absence of the drug. The electron density of the ART-10A nucleolus under these conditions is quite uniform and resembles the fibrillar core region of the nucleolus of cells treated with AMD at 22°C.

DISCUSSION

The mutants described in this paper are uniquely resistant to killing by actinomycin D. All of them are temperature sensitive in growth and in the extent of their resistance to AMD. Two, however, are very resistant to AMD even at a temperature which allows growth. The ART mutants are not impermeable to AMD since all of the strains bind more AMD at 33°C than at 22°C although the mutants show proportionately less incorporation than do wild type cells. The two mutants which were tested do not excrete AMD at an accelerated rate. AMD was not degraded in either the wild type or in the one mutant which was tested. Finally, AMD inhibits the synthesis of RNA in the mutants by up to 96% although they are not killed by the drug.

RNA synthesis is partially protected from AMD, but, at 33°C in the absence of the drug, RNA synthesis is partially inhibited in the mutants when they are compared to wild type cells. The decreased rate of RNA synthesis is characteristic of all of the ART mutants and extends to each of several different species of RNA measured in ART-6A and ART-10A,

although the extent of the inhibition varies. Similarly, all species of RNA (with the exception of chloroplast rRNAs in ART-10A) tested are synthesized at a greater relative rate in the presence of AMD at 33°C than at 22°C in ART-6A and ART-10A.

A mechanism to explain the phenotypes of the ART mutants must resolve the apparent contradiction that a temperature sensitive inhibition of RNA synthesis is coupled with a temperature sensitive protection of RNA synthesis from AMD. Since temperature sensitivity of growth and of resistance to AMD revert together it is likely that a single locus controls both processes although proof of this is contigent upon mapping of the loci. A simple explanation for these properties is that the cells contain a chromatin constituent(s) which simultaneously reduces the ability of the chromatin to bind AMD and to serve as a template for RNA synthesis. For example, one of the chromosomal proteins may have an altered structure or be present in an altered amount.

There is a considerable amount of work which indicates that the affinity of DNA for AMD is modulated by chromosomal proteins. Chromatin is known to bind less AMD than the DNA which can be isolated from it (15) and this protection appears to result from a chromosomal protein (8). However any protein which binds to DNA will not necessarily reduce its affinity for AMD. Thus chymotrypsinogen, which binds tightly to DNA, has no effect on its affinity for AMD (1). Histone F_1 binding to DNA also has little effect on the binding of AMD (8). Many studies of the binding of AMD to chromosomes or chromatin in vivo have led to observations that AMD is bound with different affinity to heterochromatin and euchromatin (5, 19, 20). However, these results are not easily

interpreted since either euchromatin or heterochromatin may have more affinity for AMD in cells of different organisms. Sieger et al. have suggested that this is a result of some structural difference between consititutive and faculative euchromatin which results in much greater binding of AMD by the former (19).

If ART mutants do alter the affinity of chromatin for AMD we would expect the mutants to be cross-resistant to a drug with a similar affinity for GC rich regions of DNA even if it had little else in common with AMD. Indeed, ART-6A is resistant to such an inhibitor, chromomycin A_3 (3, 23, 24). The variations in the resistance to AMD, the temperature sensitivity of the mutants and, most especially, the differential sensitivity of different kinds of RNA seen in ART-6A and ART-10A imply that different regions of the chromatin are affected to different extents in the mutants. Whether this results because of differential interactions of a single component with different regions of the genome in different mutants or because different components are altered in different mutants can only be answered by an examination of the chromatin constituents of the mutants.

Actinomycin D has been reported to induce nucleolar segregation in a wide variety of cells (2), and Schwartz et al. have suggested there may be a link between nucleolar segregation and the cytotoxic action of the drug (17). Our studies indicate, however, that nucleolar segregation does not cause death. Four hours of treatment with AMD kills only 20-30% of ART-10A cells at either 22°C or 33°C, but there is a significant difference in the level of nucleolar segregation observed at the two temperatures. At 22°C, 75% of the ART-10A nucleoli are seen to segregate while at 33°C no segregation is observed. In wild

type cells segregation occurred in all nucleoli at both temperatures, despite there being only 50% death after 4 h at 22°C and over 99.9% death at 33°C (see Fig. 1a of accompanying paper). However, nucleolar segregation does appear to be associated with the reduction of cytoplasm rRNA synthesis to an undectable level.

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