ON THE DIFFERENTIAL CYTOTOXICITY OF ACTINOMYCIN D

STANLEY G. SAWICKI and GABRIEL C. GODMAN

From the Department of Pathology, Columbia University, New York 10032

ABSTRACT

Actinomycin D (AMD) at concentrations that inhibit cellular RNA synthesis by 85% or more causes an acute phase of lethal cell degeneration in HeLa cultures beginning as early as 3 hr after drug exposure, resulting in the nearly complete loss of viable cells by 12 hr. The loss of cells during this acute phase of lethality is closely dose dependent. Vero, WI38, or L cells are not susceptible to this early acute cyto-intoxication by AMD, and may begin to die only after 1-2 days. Differential susceptibility to acute cyto-intoxication by AMD, or other inhibitors of RNA synthesis (daunomycin or nogalamycin), among different types of cultured cells is analogous to that observed in vivo in certain tissues and tumors, and cannot be accounted for by differences in the effect of AMD on RNA, DNA, or protein syntheses, or by the over-all loss of preformed RNA. Actinomycin D in a dose that inhibits RNA synthesis causes an equivalent loss of the prelabeled RNA in all the cell types studied. Inhibition of protein synthesis with streptovitacin A or of DNA synthesis with hydroxyurea does not cause acute lethal injury in HeLa cells as does inhibition of RNA synthesis. Furthermore, since Vero or L cells divide at about the same rate as HeLa cells, no correlation can be drawn between the rate of cell proliferation and susceptibility to the cytotoxicity of AMD. Susceptible cells are most vulnerable to intoxication by AMD in the G1-S interphase or early S phase. Inhibition of protein synthesis (which protects cells against damage by other agents affecting DNA) does not protect against AMD-induced injury. Although HeLa cells bind more AMD at a given dose than Vero or L cells, the latter cell types, given higher doses, can be made to bind proportionally more AMD without succumbing to acute cyto-intoxication. It is suggested that the differential susceptibility of these cell types to acute poisoning by AMD may reflect differences among various cells in the function or stability of certain RNA species not directly involved in translation whose presence is vital to cells. In HeLa cells, these critical species of RNA are presumed to have a short half-life.

INTRODUCTION

Although actinomycin D (AMD) inhibits transcription in nearly all animal cells in consequences of its binding to DNA (18, 36), certain cell types, e.g. intestinal, lymphocytic, and haematopoietic, are much more susceptible to cytotoxic injury by this drug in vivo (44). The carcinostatic action of AMD on such susceptible tumors as renal or genital embryonal carcinomas also depends upon its selective cytoidal toxicity. Some of the vulnerable tissues, especially intestinal crypt epithelium, suffer acute degeneration as early as 4 hr after injection of a single dose of AMD, while other susceptible tissues (including both proliferating systems as well as stationary cells such as dorsal root ganglion and salivary acinar epithelium) manifest toxic damage only much later (19, 44). We have observed that among different cell types in culture there is an analogous selective cytotoxicity
of AMD (and of some other inhibitors of transcription) to that described in vivo (13). A single (pulse) dose of AMD, daunomycin, or nogalamycin that suppresses RNA synthesis by 85% kills a large proportion of the HeLa population, but not WI38, Vero, or L cells, in an acute early phase beginning about 3 hr after exposure. Smaller numbers of WI38 and L cells may succumb only much later, and Vero cells are relatively even more resistant. AMD inhibits RNA synthesis in all of these cells; and although susceptibility to cytostatic agents has sometimes been correlated with rate of cell proliferation (3), all of the cell lines that we have studied have similar generation times and cell cycles. Nevertheless they exhibit marked differences in their susceptibility to acute intoxication by AMD.

Definitive studies have been published on the ultimate effects of AMD on the potential for proliferation of particular cell lines in terms of reproductive survival, such as have been widely employed in assessing the effects of radiation or radiomimetic and other cytostatic agents in vitro (2, 8–10, 23). Such information does not distinguish between acute or delayed cell degeneration, prolonged "metabolic" survival with inability to divide, proliferative latency with eventual recovery, and arrest after one or more divisions. In this study we are concerned with acute and subacute lethal toxic cell degeneration, a form of early premitotic cytocidal injury analogous to the "interphase death" observed in vitro after high doses of radiation (14).

The early acute degeneration of HeLa is probably the same process as that which occurs in intestinal crypt epithelium, lymphocytes, and probably embryonal carcinoma. The relative resistance of Vero cells is like that of hepatocytes, dimethylbenzanthracene (DMBA) transplantable tumors, and some other tissues (44) which, like Vero cells, can survive high single (pulse) doses of AMD fatal to susceptible cells. The reasons for the differential susceptibility to cytosis by AMD of these various tissue, tumor, and cultured cell types are mostly not understood. We have investigated the basis of the selective acute cytotoxicity of AMD, and some aspects of resistance in cell culture, by comparing the effects of AMD in different cell lines in relation to growth rate, age in the cell cycle, inhibition of macromolecule synthesis, depletion of RNA, and binding of AMD-3H. It has also been our object to study the mechanism of the acute cell injury effected by this agent, important in experiment and in therapy, many of the effects of which on biosynthesis are already well known.

MATERIALS AND METHODS

Monolayer cultures of HeLa cells (CCL 2, American Type Culture Collection), HeLa S3 cells, L cells (a subline of NCTC clone 929 mouse cells), WI38 cells in 18th–26th passage, and Vero cells (a continuous cell line derived from African green monkey kidney) were grown in milk dilution bottles at 37°C in Eagle's minimal essential medium (MEM) supplemented with 10% (v/v) fetal or newborn calf serum, 75 units/ml penicillin, and 75 μg/ml streptomycin, in an atmosphere of 5% CO in air. The cultures were periodically tested for mycoplasma with negative results. Eagle's MEM was modified in some experiments by replacing bicarbonate with 0.035 × N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid (HEPES) (45). All experiments were performed on cultures in exponential growth, at least 18 hr after passage. Either 30-ml flasks or 60-mm Petri dishes (Falcon Plastics Div. of B-D Laboratories, Inc., Los Angeles, Calif.) containing from 106 to 2 × 106 cells each were used in the cell enumeration and radioactive labeling studies. Cytological examinations, including the mitotic and moribundity indices and radioautography, were performed with cells growing on coverglasses in Leighton tubes or in 35-mm plastic Petri dishes. All experiments were performed in duplicate.

Actinomycin D (Merck, Sharp & Dohme, West Point, Pa.); daunomycin (National Cancer Institute.); and nogalamycin (Upjohn Co., Kalamazoo, Mich.) were dissolved in dimethyl sulfoxide (10 mg/ml) and a stock solution (100 μg/ml) was prepared by dilution with distilled water. The exact concentration of the AMD stock solution was determined by reading the optical density at 441 nm (ε = 257 × 109). Direct exposure of antibiotic solutions to light was avoided at all times. The stock solutions were diluted with fresh growth medium to the desired concentration and applied in a volume of 2 ml/106 cells. Unless otherwise stated, the exposure period to AMD was 1 hr, followed by rinsing and refeeding of the cultures with fresh drug-free medium; the cultures were refed again 3 hr later to remove the AMD that had diffused back into the medium either from the cells or from the plastic surface (10).

The number of viable cells was determined in replicate cultures by the dye exclusion method (24). After a 10 min incubation in 0.1% trypan blue, the cell sheet was rinsed twice with Earle's balanced salt solution (EBSS), detached by incubation in 0.25% trypsin in EBSS containing 0.15% Versene (Dow Chemical Co., Midland. Mich.) and dispersed to a
single cell suspension. The viable, or unstained, cells were counted in a hemacytometer. Some experiments were performed using a Coulter electronic particle counter (Model B). The number of mitotic cells detached by rinsing represented less than 1% of the total cell population and was considered insignificant in evaluating the number of viable cells. RNA synthesis or degradation was measured by the amount of incorporated 14C-labeled uridine (55.2 mCi/m mole, New England Nuclear Corp., Boston, Mass.) present in the acid-insoluble cell residue. The label (0.1-0.3 µCi/ml) was applied for the times stated in the Results in fresh growth medium containing 2 × 10^{-6} M thymidine and deoxycytidine to prevent incorporation into DNA. After removing the excess label and rinsing with EBSS, the cells were detached and suspended as described for cell enumeration, and a known number of cells were precipitated and extracted for 10 min with cold 5% trichloroacetic acid (TCA), the precipitates were collected and washed on membrane filters (Bac-T-Flex No. B-6, Schleicher & Schnell, Inc., Keene, N. H.), dried, and counted in a toluene based scintillant (Omnifluor, Pilot Chemicals Inc., Div., Watertown, Mass.) with a Packard Liquid Scintillation Spectrometer at an efficiency near 80%. The data are expressed without quench correction or background subtraction.

The binding of 14C-labeled AMD (11.4 mCi/m mole, New England Nuclear Corp.) was assayed as above with exceptions as detailed in the Results. In addition to collecting TCA-extracted cells on filters, duplicate samples were dissolved in NCS (Amersham-Searle Corp., Des Plains, Ill.) without TCA-extraction. These different methods of sample preparation gave equivalent amounts of radioactivity. This finding differs from those of Magee and Miller (22), who found 23% of the cell-associated AMD-3H susceptible to methanol and TCA extraction (15% was extracted by methanol alone) and of Bachetti and Whitmore (2), who reported that washing with TCA resulted in a dissociation of DNA-AMD-3H complexes. Bachetti and Whitmore (2) also found high background radioactivity when using AMD-3H, while in the experiments described here with AMD-14C the background radioactivity was negligible.

Moribundity indices were performed on coverslip cultures that had been given 1.0 µCi/ml of thymidine-3H (14 Ci/m mole, Schwartz Bio Research Inc., Orangeburg, N. Y.) for 1 hr during the AMD treatment and fixed in cold methanol at hourly intervals. After extraction with cold 5% TCA, the cover glasses were mounted on clean glass slides, dipped in K-5 emulsion (Ilford Ltd., Ilford, Essex, England) and exposed for 1 wk at 4°C. After development the cells were stained with azure B to differentiate viable from moribund cells. Mitotic indices were performed on formalin-fixed cells stained by the Feulgen reaction. Mitoses were synchronized at the beginning of S phase by applying two consecutive thymidine (5.0 mm) treatments (25).

**RESULTS**

**Dose-Dependent Degeneration of HeLa Cells; a Comparison with Other Cell Types**

Actinomycin D, at concentrations commonly employed to inhibit RNA synthesis in cell cultures (1-10 µg/ml), is acutely cytotoxic to HeLa cells. As shown in Fig. 1, the continuous exposure of HeLa cells to concentrations of AMD greater than 0.1 µg/ml causes the detachment from monolayer and the lysis of about 75-80% of the cells within 12 hr. The injured or moribund cells, which are in interphase and are easily identified by a distinctive kind of pyknosis, begin to appear only after 2 hr and then accumulate rapidly during the next 6-8 hr. With continuous exposure to lower doses (between 0.1 and 0.01 µg/ml), the acute phase of cell degeneration is delayed. When AMD is administered for 1 hr, concentrations greater than 0.35 µg/ml cause an acute phase of cell destruction; and the fraction of the population that survives this acute phase is inversely related to the drug concentration (Fig. 2). A drug concentration, 0.35 µg/ml, that is not acutely cytotoxic when administered for 1 hr becomes so when administered for 2 hr (Fig. 2, broken line). Thus, the extent of degeneration in HeLa culture during the acutely lethal phase is closely dose dependent for drug concentrations higher than 0.01 µg/ml but lower than 2.5 µg/ml. AMD concentrations in excess of 2.5 µg/ml cause a maximum loss of HeLa cells and do not increase the rate at which cells are detached from monolayer. Pyknotic cells are never seen in HeLa culture sooner than 2 hr, even after treatment with high drug concentrations.

HeLa S3 cells are a little more resistant to early acute injury induced by AMD than wild-type HeLa cells, in that they do not begin to degenerate until 6-8 hr after the addition of AMD (Fig. 3). In contrast to HeLa cells, Vero, WI38, or L cells are not susceptible to early acute cell killing by AMD. Fig. 3 shows that WI38 and L cells treated for 1 hr with 2.5 µg/ml are not killed during the 12 hr in which 75-80% of the HeLa cells are destroyed by this dose, while Vero cells continuously treated with 10 µg/ml of AMD do not succumb to acute lethal injury. These doses effectively abolish 95% or more of the RNA synthesis in all of these cell types. Other inhibitors of RNA synthesis are also acutely lethal to HeLa
Comparison of the Inhibition of RNA Synthesis by AMD in Different Cell Lines

To determine whether there is a direct or an immediate relation between the suppression of RNA synthesis and the early occurrence of lethality, RNA synthesis, i.e., incorporation of radio-uridine into the acid-insoluble cell residue, was measured during or after treatment with various doses of AMD. Table I and Fig. 5 show that a range of drug concentrations between 0.1 and 2.5 µg/ml inhibits RNA synthesis in HeLa cells by 85% or more by 1 hr. Although RNA synthesis eventually returns in the surviving cells after removal of AMD, at a rate inversely proportionate to dose, it remains at a very low level for at least 6 hr, even after treatment with sublethal doses (between 0.1 and 0.5 µg/ml administered for 1 hr).

Since early acute lethality occurs in HeLa cells only after doses of AMD (or other RNA synthesis inhibitors) that suppress 85% or more of over-all RNA synthesis, it would appear that acute cyto-intoxication results from the near complete inhibition of RNA synthesis. If early acute lethality in HeLa cells is due to inhibition of RNA synthesis, only the final 10–15% of RNA synthesis remaining after lower nontoxic doses of AMD would seem to be implicated in acute cyto-intoxication. HeLa cells withstand inhibition of up to 85% of over-all

**Figure 1** The effect of various concentrations of actinomycin D on the number of surviving HeLa cells in monolayer culture. AMD was given at time 0, and the number of adherent living cells was counted at intervals thereafter as described in Methods. 0.01 µg/ml, - ■ - ; 0.05 µg/ml, - △ - ; 0.10 µg/ml, - ○ - ; 0.50 µg/ml, - × - ; 1.0 µg/ml, - □ - ; and 5.0 µg/ml, - ○ - .
RNA synthesis without early acute lethality; but as RNA synthesis is further depressed either with higher drug concentrations or with longer exposure periods, acute cell death begins to occur after a latent interval whose duration is proportional to dose. Doses of AMD in excess of those that completely block RNA synthesis do not increase the extent of lethal injury (Fig. 1) nor do they shorten the latent interval of at least 2 hr before the appearance of pyknotic cells. Thus, once RNA synthesis is inhibited by more than 85%, HeLa cells begin to undergo an acute degenerative process beginning 2–3 hr after drug treatment.

Vero, WI38, and L cells suffer comparable inhibition of RNA synthesis (more than 90% after 1 hr of 2.5 µg/ml) (Table I). Recovery of RNA synthesis in L cells begins about 18 hr after the removal of a 1 hr dose of 5 µg/ml of AMD, while Vero cells regain 50% of their capacity to synthe-
size RNA by 3 hr after removal of 10 µg/ml, and by 24 hr uridine-\(^{14}\)C incorporation is near the control rate. For this reason it is necessary to expose Vero cells continuously to AMD when comparing Vero to HeLa cells with respect to acute lethality and suppression of RNA synthesis. Although Vero cells reinstate RNA synthesis, within 6 hr after removal of a 1 or a 4 hr pulse dose of AMD (10 µg/ml), cell division does not resume until after 48–72 hr. W138 cells do not recover their capacity to synthesize RNA after removal of 2.5 µg/ml. Thus, RNA synthesis is completely inhibited by AMD in all the cell types studied, but of these only HeLa undergoes acute degeneration within a few hours after inhibition of RNA synthesis. The other cell types remain viable in monolayer for at least 12 hr or more usually some days longer, before finally succumbing to cyto-intoxication by transcription-inhibiting doses of AMD.

Therefore, HeLa cells are uniquely susceptible to early acute injury resulting from complete inhibition of RNA synthesis, while some other cell types, although suffering comparable inhibition of RNA synthesis, are not susceptible to this early cell degeneration.

**Loss of Cellular RNA**

The possibility was examined that a greater loss of RNA occurs in HeLa cells after acutely cytotoxic concentrations of AMD (but not with non-cytotoxic concentrations), and that this loss is somehow responsible for the special susceptibility

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**Table I**

Inhibition of RNA Synthesis in HeLa, Vero, W138, and L Cells Treated with AMD

| Cell type | µg AMD/ml | Hr of exposure | Hr after AMD removal | cpm/10^6 cells | % of control |
|-----------|-----------|----------------|----------------------|----------------|--------------|
| HeLa      |           |                |                      |                |              |
| Control   |           |                |                      |                |              |
| 0.05      | 1         | 0              | 1050                 | 58             |              |
|           | 1         | 1              | 965                  | 54             |              |
|           | 1         | 2              | 740                  | 41             |              |
| 0.10      | 1         | 0              | 250                  | 14             |              |
|           | 1         | 1              | 445                  | 25             |              |
|           | 1         | 2              | 600                  | 30             |              |
| 0.50      | 1         | 0              | 85                   | 5              |              |
|           | 1         | 1              | 115                  | 6              |              |
|           | 1         | 2              | 123                  | 7              |              |
| 1.00      | 1         | 0              | 85                   | 5              |              |
| 2.50      | 1         | 0              | 40                   | 3              |              |
| W138      |           |                |                      |                |              |
| Control   |           |                |                      |                |              |
| 2.5       | 1         | 0              | 25                   | 4              |              |
|           | 1         | 23             | 20                   | 3              |              |
| L cells   |           |                |                      |                |              |
| Control   |           |                |                      |                |              |
| 2.5       | 1         | 0              | 320                  | 12             |              |
| 5.0       | 1         | 0              | 150                  | 6              |              |
|           | 1         | 23             | 550                  | 22             |              |
| Vero      |           |                |                      |                |              |
| Control   |           |                |                      |                |              |
| 10.0      | 1         | 0              | 69                   | 3              |              |
|           | 1         | 3              | 1200                 | 50             |              |
|           | 1         | 23             | 2000                 | 83             |              |
| 1.0       | 1         | 0              | 230                  | 10             |              |
|           | 3         | 0              | 240                  | 10             |              |
| 24        | 0         |                | 260                  | 11             |              |

The cultures were incubated for 20 min in uridine-\(^{14}\)C-containing medium that was drug-free at intervals after exposure to various doses of AMD. The radioactivity in the acid-insoluble cell precipitate was assayed as described in Methods. In other experiments (to be reported elsewhere), RNA synthesis in Vero returns to control levels by 4 hr after removal of a 1 hr or a 4 hr pulse dose of AMD (10 µg/ml).
TABLE II
The loss of RNA from Cultures after Exposure to AMD

% of uridine-14C remaining in acid-insoluble fraction at times after AMD addition

| Cell type | Dose (µg AMD/ml) | 1 hr | 2 hr | 3 hr | 1 hr | 3 hr |
|-----------|-----------------|------|------|------|------|------|
|           | Nuclear RNA     |      |      |      |      |      |
| HeLa      | 0.05            | 63   | 60   | 62   | 95   | 90   |
|           | 0.50            | 56   | 53   | 53   | 94   | 90   |
|           | 1.00            | 53   | 52   | 53   | 94   | 90   |
|           | 2.50            | 52   | 49   | 48   |      |      |
|           | 5.00            | 48   | 47   | 44   | 52   | 90   |
| Vero      | 10.0            | 52   | 48   | 46   |      |      |
| WI38      | 2.50            | 52   | 52   |      | 85   |      |

Cultures were prelabeled with uridine-14C for 1 hr (nuclear RNA) or for 12 hr (cytoplasmic RNA), before addition of AMD; the radioactivity of the acid-insoluble residue was determined 1, 2, and 3 hr after treatment with AMD as described in Methods.

of HeLa cells to early acute injury as compared with Vero and WI38 cells. The cellular RNA was labeled by means of a 1 hr pulse with uridine-14C (0.2 µCi/ml) given either 1 hr ("nuclear" RNA) or 12 hr ("cytoplasmic" RNA) before the addition of various concentrations of AMD. 1, 2, and 3 hr after the addition of AMD, the cells were collected and the radioactivity in the acid-insoluble cell precipitate was determined. Cytoplasmic RNA is lost to the same extent (10% after 3 hr) after treatment of HeLa cells with 0.05, 0.5, and 5 µg/ml of AMD and of WI38 cells with 2.5 µg/ml (Table II). The decay of the rapidly labeled nuclear RNA is shown in Table II: there is a nearly equivalent loss of label (RNA) after a treatment of HeLa cells with 0.05, 0.5, 1.0, 2.5, and 5 µg/ml as with 10 µg/ml given to Vero cells and 2.5 µg/ml given to WI38 cells. Exposure to lower drug concentrations results in somewhat smaller loss of RNA (Table II); this probably reflects both the slower rate of inhibition and the incomplete suppression of RNA synthesis caused by these lower doses (Fig. 5). The decay of prelabeled RNA observed after AMD administration is unlikely to be the factor primarily responsible

![Figure 5](image-url)
for the acute degeneration of HeLa cells since an equivalent loss of prelabeled RNA occurs in WI38 and Vero cells and especially because a dose of AMD that immediately reduces RNA synthesis causes a nearly equivalent loss of prelabeled RNA of HeLa cells but not an equivalent amount of acute lethality (Figs. 1 and 2).

Effect of Protein Synthesis

Is the acute cyto-intoxication of AMD to HeLa cells due to a dose-dependent inhibition of protein synthesis? Any dose of AMD that inhibits RNA synthesis by more than 85% also reduces protein synthesis by about 50% at 4 hr after drug treatment. (Thereafter, it becomes difficult to assess protein synthesis because of the variable numbers of dead cells that begin to accumulate in HeLa cultures.) However, inhibition of protein synthesis by certain inhibitors is not acutely cytotoxic to HeLa cells. Less than 20% of a HeLa culture is killed at 12 hr after the virtually immediate and complete stoppage of protein synthesis by streptovitacin A (10 µg/ml), whereas a 1 hr exposure to 2.5 µg/ml of AMD kills 75% of a HeLa culture by 12 hr. In contrast to the protective effect of inhibitors of protein synthesis against injury caused by irradiation or radiomimetic agents, the addition of streptovitacin A (10 µg/ml) before, during, and after the administration of AMD (2.5 µg/ml) augments the early fatal injury to HeLa cells. By 8 hr no viable cells are left after this treatment.

Binding and Retention of AMD-14C

Is the early vulnerability of HeLa cells related to a difference in the amount of drug taken up and retained by these cells as compared with the other cell types? This was assessed by determining the amount of AMD-14C bound into the acid-insoluble form of lethal injury that occurs in all of the other cell types. Vero cells are resistant to the more delayed killing, even though more AMD is bound in the cell and has been removed by washing and refeeding. Vero cells treated with 10 µg/ml for 24 hr to ensure drug retention and continued suppression of RNA synthesis do not show an acute phase of cell killing even though they have taken up more AMD than HeLa cells treated with cytocidal doses (1–2.5 µg/ml). Thus, the vulnerability or resistance of various cell lines to acute injury is not entirely determined by the amount of AMD taken up or retained by the cells. The dose dependence of acute killing of HeLa cells may be correlated with the amount of AMD maximally bound. However, acute cell killing of HeLa cells follows several hours after exposure to AMD for 1 hr and occurs after more than half of the drug has diffused out of the cell and has been removed by washing and refeeding. Acute killing of HeLa cells is dose dependent, and can be correlated with the amount of AMD maximally bound, only in that dose range within which there is some variation in the response of RNA synthesis to inhibition by AMD. Doses exceeding those that maximally inhibit RNA synthesis do not augment acute killing, even though more AMD is bound in the cell proportionally with higher drug concentrations. Also, acute killing of HeLa cells follows several hours after a 1 hr exposure to AMD, and occurs after more than half of the drug has diffused out of the cell and has been removed by washing and refeeding. Vero, WI38, and L cells do not succumb to early killing even when continuously exposed to high concentrations of AMD at which doses they bind more AMD than HeLa cells. Vero cells are resistant to the more delayed form of lethal injury that occurs in all of the other

S. G. Sawicki and G. C. Godman  Cytotoxicity of Actinomycin D  753
Figure 6  Binding and retention of $^{14}$C-labeled AMD. AMD-$^{14}$C was given to HeLa (○), Vero (□), or L cells (△) for 4 hr (A) or for 1 hr followed by rinsing and refeeding with drug-free medium (B). The radioactivity in the acid-insoluble cell precipitate was assayed at hourly intervals as described in Methods. Fig. 6, A: A comparison of the amount of AMD-$^{14}$C bound and retained during a continuous 4 hr exposure to 1 µg/ml. Fig. 6, B: A comparison of the amount of AMD-$^{14}$C bound and retained after a 1 hr exposure to 2.5 µg/ml (---) or 10 µg/ml (---). The AMD-$^{14}$C was given at 0 hr and removed at 1 hr.

TABLE III
Vulnerability of HeLa in DNA Synthesis (S Phase) to AMD

| Time | Viable cells | Moribund cells |
|------|--------------|----------------|
|      | Labeled      | Unlabeled      |
|      | Labeled      | Unlabeled      |
| Control | 1 hr | 560 | 440 | 0 | 0 |
| AMD    | 1 hr | 554 | 446 | 0 | 0 |
|        | 4 hr | 522 | 411 | 24 | 43 |
|        | 6 hr | 288 | 431 | 234 | 47 |

HeLa monolayers were labeled with TdR during treatment with AMD (1.25 µg/ml) for 1 hr, and were fixed at 1, 4, and 6 hr after addition of AMD as described in Methods. Radioautograms were scored to determine number of labeled cells per thousand.

Cell Cycle and DNA Synthesis

Does a correlation exist between the position of HeLa cells in the division cycle and susceptibility to early acute killing by AMD? This question was examined in two ways: (a) by determining if the cells dying during the phase of acute lethality are located in a particular phase of the cell cycle while exposed to AMD, and (b) by determining whether different rates of acute cell killing occurred in synchronized cultures treated with AMD while in different phases of the cell cycle. HeLa cells in DNA synthesis (S) were labeled with thymidine-$^3$H (1.0 µCi/ml) for 1 hr during a 1 hr AMD (1.25 µg/ml) treatment, and the cultures were fixed at intervals after the combined labeling and AMD treatment. The viable and moribund cells were scored in radioautograms as labeled or unlabeled, i.e. cells in S or in some other phase of the cell cycle while AMD was administered. Table III shows that nearly all of the moribund cells which accumulate at 6 hr are derived from the population of cells that were synthesizing DNA during exposure to AMD, while the cells that were not in S during exposure to AMD constitute the viable fraction. Hence, the population of HeLa cells most susceptible to being
killed during the early acute phase of AMD-induced lethality are those in S during the exposure period. A 1 hr AMD dose of 2.5 µg/ml was administered to thymidine-synchronized HeLa cells at various times after the removal of the second thymidine block, and the number of viable cells was counted at intervals thereafter. As shown in Table IV, when this dose is applied to cells in S, i.e. 2 hr after removal of thymidine, 46% and 90% of the population are killed by 6 and 12 hr, respectively, as compared to 15% and 75% killed in unsynchronized cultures. A culture in G2-Mitosis, i.e. 8 hr after removal of thymidine, when treated with this dose of AMD shows no cell killing at 6 hr and only 50% at 12 hr. When this dose of AMD is administered to HeLa cultures held in the second thymidine block, i.e. the HeLa cultures kept in thymidine-containing medium before, during, and after AMD exposure, 65% of the culture is killed by 6 hr, and less than 1% of the original cell number remains attached to the surface of the flask by 12 hr. Since cells held in the second thymidine block are near the beginning of S (G1-S interphase) and DNA synthesis is virtually completely inhibited, HeLa cells need not be actively synthesizing DNA to be susceptible to AMD while in S. In contrast to HeLa cells, thymidine-synchronized Vero cultures exposed to AMD (10 µg/ml given continuously) while in the second thymidine block, or while in G2-Mitosis, i.e. 10 hr after removal of thymidine, do not show any difference in the number of cells killed before 24 hr as compared to unsynchronized cultures treated with the same dose (Table IV).

### Table IV

Relative Susceptibility of HeLa and of Vero Cells to Cytocidal Effects of AMD after Synchronization with Thymidine

|          | 6 hr | 12 hr | 24 hr |
|----------|------|-------|-------|
|          | HeLa | Vero  | HeLa  | Vero  | HeLa  | Vero  |
| No TdR (asynchronous) | 85   | 100   | 25    | 90    | <15   | 85    |
| Continuous TdR (blockaded) | 34   | 100   | <5    | 90    | <0    | 85    |
| S phase  | 54   | —     | 10    | —     | <1    | —     |
| G2-M phase | 100  | 100   | 50    | 90    | 40    | 85    |

HeLa or Vero monolayers were synchronized with excess thymidine and then treated with AMD (HeLa, 2.5 µg/ml for 1 hr; Vero, 10 µg/ml continuously) either during the TdR blockade, 2 hr after removal of TdR (S phase), or 6 hr after removal of TdR (G2-M phase). The number of living cells was determined at 6, 12, and 48 hr and is expressed as per cent of the initial cell number.

### Table V

Effect of Hydroxyurea, Actinomycin D, or Hydroxyurea and Actinomycin D on the Number of Viable HeLa Cells

|          | 6 hr | 12 hr | 24 hr |
|----------|------|-------|-------|
| Hydroxyurea (25 mM) | 105  | 110   | 98    |
| Actinomycin D (0.10 µg/ml) | 98   | 70    | 3     |
| Actinomycin D (0.10 µg/ml) and hydroxyurea (25 mM) | 95   | 55    | 3     |

HeLa cells were exposed for 24 hr to hydroxyurea (25 mM), actinomycin D (0.1 µg/ml), or hydroxyurea (25 mM) and actinomycin D (0.1 µg/ml), and the viable cells were counted at 6, 12, and 24 hr. The results are expressed as per cent of the 0 hr cell number.

Another indication that inhibition of DNA synthesis per se does not cause cell degeneration is afforded by the following experiment. HeLa cells in exponential growth were exposed to 25 mM hydroxyurea in the presence or absence of 0.1 µg/ml of AMD. Table V shows that this concentration of hydroxyurea, which completely and immediately inhibits DNA synthesis, does not cause a significant loss of HeLa cells for 24 hr. When the hydroxyurea is given to HeLa cells in the presence of 0.1 µg/ml of AMD, there is an equivalent loss of cells as compared to that observed after the same concentration of AMD given alone (0.1 µg/ml for 24 hr). Hence, the early acute cytotoxicity of AMD observed in HeLa cells is not

S. G. SAWICKI AND G. C. GOODMAN  Cytotoxicity of Actinomycin D  755
due to the inhibition of DNA synthesis, but is nevertheless somehow related to the cell cycle.

**DISCUSSION**

We have shown that HeLa cells are killed during an early rapid cytotoxic phase after exposure to AMD, i.e. after 3–12 hr, whereas most cell types such as Vero, WI38, or L cells, treated with comparable or higher doses of AMD, are not susceptible to an early phase of lethality, but only after 1–3 days does a gradual loss of these cells occur. Since AMD inhibits transcription as effectively in Vero, WI38, or L cells as in HeLa cells, and since the amount of acute killing in HeLa cells is proportional to the dose after depression of 85% of RNA synthesis, the acute cytotoxicity of AMD might not at first appear to be an immediate consequence of the inhibition of RNA synthesis per se. Such a conclusion had been reached by Schwartz and his collaborators (44) on the basis of comparative studies of different tissues and tumors of animals. However, the toxicity of AMD, and that of analogous DNA-binding RNA synthesis inhibitors, is dependent upon their ability to suppress RNA synthesis.

**RNA Synthesis**

Only doses of AMD sufficient to inhibit RNA synthesis almost completely in HeLa cells bring about acute cytotoxicity in which 75–80% of the viable cells are lost within 12 hr. Only HeLa cells of the several lines observed succumb in this way. Concentrations of AMD that partially block RNA formation are known to inhibit preferentially the production of ribosomal RNA while permitting the continued synthesis of other species of RNA (12, 28, 37). That acute killing of HeLa cells might be due to the loss of some critical species of non-ribosomal RNA whose synthesis is inhibited only at higher concentrations of AMD is suggested by: (a) the 2.5–3 hr latent period between the addition of AMD and the onset of acute cell death, and (b) the occurrence of acute lethality only after 85% of over-all RNA synthesis has been inhibited. Doses that almost immediately and completely inhibit RNA synthesis do not shorten the latent period before acute killing; however, lower doses of AMD that inhibit RNA synthesis at a slower rate cause a proportional delay in the onset of the acutely lethal phase. Furthermore, the other inhibitors of RNA synthesis, daunomycin and nogalamycin, cause a similar phase of acute cell death in HeLa cells. If a species of RNA with a short natural half-life, or one subject to degradation after AMD, is vital to HeLa cells, it must be assumed to function in some manner other than in the production of protein(s). The virtually complete inhibition of protein synthesis by streptomycin A does not cause early acute injury in HeLa cells as does inhibition of RNA synthesis. Various species of RNA of unknown function, either confined to the nucleus (1, 7, 17, 26, 34, 39, 52, 53) or in the cytoplasm (27), have been identified. Certain of these RNA species may function in some unknown manner, perhaps not directly related to protein synthesis, and their decay results in the acute degeneration of cells.

Actinomycin D and other inhibitors of RNA synthesis, at concentrations in excess of those that inhibit RNA synthesis, do not cause acute cell death in Vero, WI38, or L cells, although Vero and L cells divide (and probably synthesize RNA) at an approximately similar rate, and are maintained under the same conditions as HeLa cells. Certain differences must exist among cell types which would account for the selective susceptibility of HeLa cells to acute cytotoxicity induced by inhibitors of RNA synthesis. If HeLa cells require the continued synthesis of certain “critical” species of RNA, then these cell types either do not require these RNA species or have them in a more stable, or longer lived, form. That Vero cells might possess similar, except more stable, species of RNA whose existence is vital to cells is suggested by the data of Fig. 3 and Table I. Although RNA synthesis is completely inhibited in Vero cells following a 1 hr exposure to 10 µg/ml of AMD, it returns promptly after drug removal (Table I) and almost no cells are lost from the culture (Fig. 3). On the other hand, Vero cells given 10 µg/ml (or lower concentrations) of AMD for 24 hr become moribund between 48 and 72 hr (Fig. 3). Therefore, susceptibility and relative resistance to AMD-induced acute lethality among various types of cultured cells might be due to the presence of critical species of RNA, short-lived in some cell types, longer lived in others. If RNA synthesis is suppressed for long periods, all cell types eventually succumb to continued AMD poisoning, but some, e.g. HeLa cells, intestinal epithelium (44), avian myeloblasts (55), and KB and chick embryo cells (53), are killed much sooner than others.
RNA Degradation

Following the administration of AMD there is a significant loss of RNA, the bulk of which consists of the newly synthesized, rapidly labeled RNA of the nucleus (15, 17, 27, 40, 41, 47, 52, 53). This loss of RNA after AMD probably represents the unmasking of a physiologically normal decay of the so-called nuclear heterodisperse RNA (17, 27, 48, 52), but it may perhaps also be due, at least in part, to an abnormally induced degradation of newly synthesized RNA (38, 49). This decay and depletion of RNA after AMD have been implicated by some as a mechanism causing late cell death (42, 44, 54). However, the marked susceptibility of HeLa cells to early lethal injury cannot be demonstrably associated with a gross loss or breakdown of RNA during the first 3-4 hr. RNA depletion of nearly the same magnitude occurs in HeLa cells treated with a wide range of drug concentrations (0.05–5.0 µg/ml), all of which reduce RNA synthesis by at least 50% by 1 hr, in contrast to the strict dose dependence of acute lethality at these concentrations; furthermore, Vero and W138 cells lose equivalent amounts of prelabeled RNA as HeLa during the first 3-4 hr, without suffering acute degeneration.

Inhibition of Protein Synthesis

Since the nearly complete and immediate inhibition of protein synthesis by streptovitacin A does not cause early acute lethal injury in HeLa cells, the eventual partial inhibition of protein synthesis by AMD would not appear to be a direct cause of acute cell death. In the interval under consideration, since protein synthesis is only partly inhibited (by about 50%), there must be some imbalance in both the amounts and kinds of proteins being accumulated. Moreover, it is known that in some circumstances AMD can “paradoxically” stimulate the formation, i.e. translation, of certain proteins (chiefly enzymes) whose relatively stable messages have been released from translational repression (51). It is, therefore, at least theoretically possible to envisage the accumulation of abnormal amounts and/or kinds of polypeptides which might be toxic. This eventuality is unlikely because the simultaneous inhibition of protein synthesis and RNA synthesis by the combined administration of streptovitacin A and AMD does not protect HeLa cells but actually augments the acute cytotoxicity of AMD. Nor does inhibition of protein synthesis protect cells against the ultimate lethal effects of AMD as measured by reproductive survival analysis (8) as it does against irradiation (32), or the lethal effects of cytosine arabinoside (20) or mitomycin C (8).

Inhibition of DNA Synthesis and the Cell Cycle

Actinomycin D at high concentrations directly affects DNA synthesis (2, 22, 35); it has been argued that the toxic mechanism of AMD is related, at least in some part, to inhibition of DNA synthesis (2, 43). Schwartz et al. (43) attempted to correlate early acute injury of intestinal epithelial cells after AMD with inhibition of DNA synthesis rather than with inhibition of RNA synthesis, because RNA synthesis appears to be only moderately affected in these cells after cytotoxic doses of AMD while DNA synthesis is suppressed, and because inhibitors of DNA synthesis cause similar acute injury in the same cells. However, Farber and Baserga (11) have shown that inhibition of DNA synthesis per se is not sufficient to cause acute injury since proliferating cells other than intestine are not susceptible to fatal acute poisoning by inhibitors of DNA synthesis. Direct and complete inhibition of DNA synthesis by excess thymidine (10 mM) or hydroxyurea (25 mM) does not cause acute damage to HeLa cells such as occurs in certain proliferating cell populations of animals given hydroxyurea (31). Moreover, the simultaneous exposure of HeLa cells to AMD and hydroxyurea does not significantly alter either the time of first occurrence or the rate of cell degeneration from that observed after exposure to an equal dose of AMD. Thus, although HeLa cells appear analogous to intestinal epithelial cells in sensitivity to the acute cytotoxicity of AMD, they do not exhibit a similar sensitivity to inhibitors of DNA synthesis. Although HeLa cells in S are more susceptible to the acute cytotoxicity of AMD than cells in G2, cells blocked with excess thymidine, i.e. at or near the G1-S interphase, are destroyed even more rapidly by AMD than cells treated while actively synthesizing DNA. For this and other reasons, it appears that cells need not progress to a certain stage of the division cycle in order for the damage to be expressed. All HeLa cells, regardless of their position in the division cycle, if treated with sufficiently high doses of AMD die within several hours. Only at lower doses of AMD does a pref-
The differential killing of cells at the G1-S interphase or of cells in S occurs. Since HeLa cells increase their rate of RNA synthesis as they initiate DNA synthesis (30), the increased sensitivity to inhibitors of RNA synthesis during or immediately preceding DNA synthesis may reflect a critical requirement for RNA synthesis at this phase of the division cycle. A more probable hypothesis is that as the rate of RNA synthesis increases during late G1 or early S, the DNA in chromatin may serve as a better binding site for AMD, resulting in heightened sensitivity to inhibitors of RNA synthesis that bind to DNA. Acceleration of RNA synthesis in lymphocytes stimulated with phytohemagglutinin has been shown to be paralleled by an increased ability of chromatin to bind AMD (4, 5). Lower concentrations of AMD might more effectively inhibit RNA synthesis at certain stages of the division cycle due to increased availability of binding sites. Cells in G1 or S are apparently more vulnerable to disturbances in biosynthesis of nucleic acids or proteins (23), but certain cell types are obviously more susceptible to these perturbations. As another instance of the variation among cell types in response to the same drug, Pfeiffer and Tolmach (28) have shown that HeLa cells are able to withstand up to 19 hr of exposure to hydroxyurea without any lethal effects while Chinese hamster cells in S are killed during a 1 hr exposure (46).

**Actinomycin Uptake and Retention**

Susceptibility of HeLa cells to early acute injury as compared to Vero, WI38, or L cells is not strictly dependent on differential uptake or retention of AMD among these cell types. Although HeLa cells bind more AMD-14C at a given dose, the other cell types given higher doses can bind as much or more AMD without succumbing to acute cytotoxicity. Furthermore, HeLa cells in G1 bind as much or more AMD-14C as cells in S, although cells in S are more susceptible to early fatal injury.

The results of our study show that Vero cells, as compared to HeLa, WI38, or L cells, are able to rapidly release and excrete AMD, which is promptly followed by reinstatement of RNA synthesis. However, Vero cells do not recover their capacity for cell division until 2 days or more after exposure to pulse doses of AMD. This ability to excrete bound AMD and to resume RNA synthesis quickly after removal of AMD from the medium enables Vero cells to recover after a 1 hr exposure to very high concentrations of AMD which would destroy other cell types. Vero cells thus appear to be analogous to liver cells with respect to their low retentiveness of AMD and their relative insusceptibility to its toxic effects (44).

Binding of AMD by DNA is a necessary condition for cytotoxicity as well as for inhibition of transcription. Exposure of HeLa cells to the non-binding derivative deamino-actinomycin C10 (10 μg/ml) did not result in acute killing in our experiments, nor does it affect the growth of HeLa cells (36). Other studies (2, 8–10, 13, 23) have drawn attention to the similarity in the shapes of dose-response curves of reproductive survival after AMD and after ionizing radiation or radiomimetic drugs. Some of these investigators (8, 9) have commented on the additive interaction of these agents, and on the similar “age” dependence of their dose-response curves, and have suggested that the toxic effects of both AMD and irradiation depend upon the interaction of each with DNA, but that they are not entirely the same.

**Recovery**

The ability or inability of cells to recover after AMD may reflect a cytoplasmic state caused by AMD, similar to that of mature hen erythrocytes which lack certain cytoplasmic or nuclear factors capable of initiating nuclear activity, but which factors are present in cells capable of division (16). This possibility gains credence from the experiment of Lorch and Jeons (21) who interchanged the nuclei and cytoplasms of AMD-treated and untreated amebae. Amebae reconstituted from AMD-treated nuclei and untreated cytoplasms recovered nuclear activity and became reproductive. Insertion of untreated nuclei into the cytoplasm of previously treated amebae, however, resulted in a loss of nuclear activity and of reproductive capacity. The stability or presence of cytoplasmic factors involved in the initiation of nuclear activity varies with different types of cells and throughout the cell cycle (6, 33, 50). As AMD diffuses out of Vero cells these factors, which might be longer lived or more stable in Vero, would contribute to the reinstatement of RNA synthesis and reproductive recovery. Other cell types, however, like WI38, although they do not bind as much AMD and eliminate much of what is bound, do not recover RNA synthesis, perhaps due to the more rapid physiological decay of and failure to re-

738 THE JOURNAL OF CELL BIOLOGY • VOLUME 50, 1971
plenish these cytoplasmic factors. This might serve to explain the selective susceptibility or resistance of certain animal tumors to chemotherapy with AMD.

In conclusion, we have demonstrated that AMD induces in HeLa cells an early acute degenerative process, similar to that observed in intestinal epithelial cells, which does not occur until much later in most other cell types. Other inhibitors of RNA synthesis initiate a similar phase of early injury in HeLa cells, while inhibitors of DNA or protein synthesis do not. For these and other reasons, we suggest that cells may require certain species of RNA that are lost rapidly from HeLa cells but more slowly from other cell types following inhibition of RNA synthesis and which are vital for maintaining cellular integrity. The differences in response of different cell types or of cells in different stages of the division cycle to inhibition of transcription may be due to differences in the quality, function or stability of the RNA species whose synthesis is suppressed. These species of RNA would not appear to function directly in protein synthesis and probably represent only a small fraction of the total nonribosomal RNA of cells. This possibility might be examined by centrifugal and electrophoretic analysis of the various species of nonribosomal RNA from different types of cells after inhibition of RNA synthesis, and their rates of decay.

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