ABSTRACT

The effects of aclacinomycin (ACM; NSC 208734) on cell viability, growth, and colony formation were investigated in suspension (Friend leukemia and L1210) and adherent (Chinese hamster ovary) cell systems. Cell cycle progression and the effect of the drug on various transition points in the cell cycle (i.e., G1 to S phase, through a window in early S phase and G2 phase to mitosis) were monitored by flow cytometry.

Formation of Chinese hamster ovary cell colonies was inhibited by 50% following 24 hr of exposure to 0.05 μg ACM per ml whereas 1 hr of exposure to 1.0 μg ACM per ml reduced colony formation by only 30%. Stationary cultures required a drug concentration more than 5 times higher to reduce colony formation by an equivalent amount when present for 24 hr. Short-term (1-hr) exposure to drug concentrations up to 1.0 μg/ml had no effect on colony formation of stationary-phase Chinese hamster ovary cells. Cell growth was inhibited by 50% in suspension cultures of Friend leukemia and L1210 cells when exposed for 24 hr to 0.024 and 0.053 μg ACM per ml, respectively. Continuous drug exposure of Friend leukemia and L1210 cells to ACM concentrations of 0.05 to 0.1 μg/ml led to a slow down in cell progression manifested as an accumulation of cells in G2 + M phase by 24 hr and then in G1 phase by 48-hr culture. However, brief (1-hr) exposure of L1210 cells to 0.5 μg/ml resulted in an irreversible accumulation of cells in G2 + M phase. A more detailed examination of drug effects on the cell cycle determined that 0.1 μg ACM per ml resulted in a slow down in L1210 cells leaving G1 phase and entering mitosis and an accumulation of cells in G2 phase, although early S-phase cells appeared unaffected. At a 5 times higher drug concentration, exit of cells from G1 was almost completely halted, passage of cells through early S was slowed, and the entrance of cells into mitosis plateaued 3.5 hr after addition of the drug; G2-phase cells were only mildly affected. The RNA content of all cells examined was reduced by 35 to 50% depending upon dose and time of exposure. These findings are discussed in terms of the known biochemical effects of ACM on RNA and protein synthesis.

INTRODUCTION

ACM<sup>3</sup> is an anthracycline antibiotic with antitumor activity. The effects of aclacinomycin (ACM; NSC 208734) on cell viability, growth, and colony formation were investigated in suspension (Friend leukemia and L1210) and adherent (Chinese hamster ovary) cell systems. Cell cycle progression and the effect of the drug on various transition points in the cell cycle (i.e., G1 to S phase, through a window in early S phase and G2 phase to mitosis) were monitored by flow cytometry. Formation of Chinese hamster ovary cell colonies was inhibited by 50% following 24 hr of exposure to 0.05 μg ACM per ml whereas 1 hr of exposure to 1.0 μg ACM per ml reduced colony formation by only 30%. Stationary cultures required a drug concentration more than 5 times higher to reduce colony formation by an equivalent amount when present for 24 hr. Short-term (1-hr) exposure to drug concentrations up to 1.0 μg/ml had no effect on colony formation of stationary-phase Chinese hamster ovary cells. Cell growth was inhibited by 50% in suspension cultures of Friend leukemia and L1210 cells when exposed for 24 hr to 0.024 and 0.053 μg ACM per ml, respectively. Continuous drug exposure of Friend leukemia and L1210 cells to ACM concentrations of 0.05 to 0.1 μg/ml led to a slow down in cell progression manifested as an accumulation of cells in G2 + M phase by 24 hr and then in G1 phase by 48-hr culture. However, brief (1-hr) exposure of L1210 cells to 0.5 μg/ml resulted in an irreversible accumulation of cells in G2 + M phase. A more detailed examination of drug effects on the cell cycle determined that 0.1 μg ACM per ml resulted in a slow down in L1210 cells leaving G1 phase and entering mitosis and an accumulation of cells in G2 phase, although early S-phase cells appeared unaffected. At a 5 times higher drug concentration, exit of cells from G1 was almost completely halted, passage of cells through early S was slowed, and the entrance of cells into mitosis plateaued 3.5 hr after addition of the drug; G2-phase cells were only mildly affected. The RNA content of all cells examined was reduced by 35 to 50% depending upon dose and time of exposure. These findings are discussed in terms of the known biochemical effects of ACM on RNA and protein synthesis.

MATERIALS AND METHODS

Cells. FL cells, strain 745, were obtained from the Medical Research Institute, Camden, N. J. The L1210 cells used in this study were kindly provided by Dr. F. Kingsley Sanders of this Institute. Both cell lines, grown in suspension cultures, were maintained as described previously (23). In preparation for the addition of drug, the cultures were split 1:3 with fresh pre-warmed medium on 3 successive days to insure asynchronous growth.

CHO originally obtained from Dr. T. T. Puck by Dr. L. Chasin were maintained as growing monolayer cultures. Asynchronous growth was assured by daily resuspension in fresh medium.

Drugs. ACM (NSC 208734) was provided by the Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute. Drug solutions were prepared fresh on the day of the experiment by first dissolving ACM at a concentration of 1.0 mg/ml in 0.1 N HCl followed by appropriate dilutions in HBSS (Grand Island Biological Co., Grand Island, N. Y.). Serial dilutions were made in HBSS at room temperature to 10 times the final concentration desired in the cell culture medium. Vinblastine sulfate was balanced salt solution; AO, acridine orange; t<sub>50</sub>, time required for 50% of the cells to exit the indeterminate portion of G1 phase.
obtained from Sigma Chemical Co. (St. Louis, Mo.) and was stored frozen in stock solutions at a concentration of 50 μg/ml in HBSS.

**Clonability Studies.** Exponentially growing CHO cells were seeded at a concentration of 200 and 2000 cells in a volume of 2 ml in 35-mm-diameter wells (Costar, Cambridge, Mass.). Two hr were allowed from cell reattachment before addition of ACM. The drug was administered directly to the cultures by addition of 0.2 ml of stock solution to obtain the desired concentration. Following 24 hr of incubation, the cells were washed 3 times with HBSS and refed with fresh prewarmed medium.

Stationary CHO cell cultures were prepared by growing cells to confluence, followed by addition of fresh medium to the confluent cultures for 2 successive days. These cultures were distinguished from exponentially growing CHO cells inasmuch as greater than 80% of the cells contained diploid (G₁) amounts of DNA as compared to generally fewer than 45% G₁ cells in low-density cultures. Stationary cultures were treated with ACM at the appropriate concentration for 24 hr, washed free of drug, trypsinized, and replated at a concentration of 200 and 2000 cells/well for each drug concentration in drug-free medium.

All survival studies were carried out in triplicate, and each experiment was repeated at least once. The plating efficiency of control CHO cells varied but was typically 80% for exponentially growing and 40 to 60% for stationary CHO cell cultures. In each case, all drug results were compared to the equivalent controls and expressed as a percentage of control colony formation. When the plating efficiency in drug-treated cultures fell below 10% of control values, wells plated at the higher cell concentration were counted.

**Drug Effect on Growth of Suspension Cultures.** Suspension cultures of FL and L1210 cells in exponential growth were split and treated with varying concentrations of ACM. Cell growth was monitored by counting viable cells as defined by their ability to exclude trypan blue. The relative cell number following 24 hr of continuous incubation in the presence of the drug was expressed as a percentage of control cell growth.

Cultures were tested in triplicate, and each count was performed twice; the points in the charts represent the mean of 3 counts in a single representative experiment in which control cell growth was exponential.

**Cytokinetic Studies.** Exponential cultures of FL and L1210 cells were established to which the appropriate drug concentration was added. Aliquots (0.2 ml) were removed at 6, 24, and 48 hr, stained with the metachromatic dye AO (see below), and analyzed by flow cytometry as described previously (23). In each instance, the frequency distribution histograms of DNA content from a minimum of 5 × 10⁵ cells were obtained and compared to the appropriate controls. The stoichiometry of the staining reaction for DNA has been described previously by us (20, 21) and others (2). All cytokinetic experiments were run in duplicate and repeated at least 3 times with similar results.

The staining technique used in this study (see below) also allows for the simultaneous measurement of relative cellular RNA content (21). The stoichiometry of the RNA measurement has been documented by Bauer and Dethlefsen (1).

**Terminal Point of Drug Action.** Vinblastine sulfate was added to exponentially growing L1210 cells at a final concentration of 0.5 μg/ml. A 5-ml sample of cells was taken immedi-

**RESULTS**

**Inhibition of Colony Formation**

**Exponentially Growing CHO Cells.** As shown in Chart 1, exponentially growing CHO cells exposed to ACM for 24 hr (a length of time in excess of the doubling time) were inhibited from colony formation at drug concentrations in excess of 0.01 μg/ml. Under these conditions, 0.05 μg drug per ml inhibited CHO cell colony formation by 50% (Table 1). If, however, exponentially growing cultures were exposed to the drug for 1 hr, colony formation decreased by only 30% relative to untreated CHO cells even at 1.0 μg ACM per ml (Chart 1).

**Stationary-Phase CHO Cells.** When stationary CHO cells were treated with ACM, a 5 times higher concentration of the drug (0.26 μg/ml) was required to inhibit colony formation by 50% (Chart 1; Table 1). If stationary cultures were exposed to ACM for only 1 hr, no decrease in colony formation could be
The effect of continuous drug exposure on the cell cycle distribution of the suspension cultures, FL and L1210 cells, was analyzed by comparing the DNA distributions obtained by flow cytometry of AO-stained cells after various times in culture following drug treatment. In all instances, the histograms and values in the tables refer to the cell cycle distribution of viable cells only. Cells were considered viable by 2 criteria, namely, exclusion of trypan blue and resistance to cell lysis by the detergent present in the staining solution. Since lysed cells contain no RNA and, therefore, have no red fluorescence, they are easily distinguished from viable cells in scattergrams (7, 21).

**FL Cell Kinetics.** Table 2 illustrates the cell cycle distribution of FL cells exposed continuously to 3 concentrations of ACM and monitored at 6, 24, and 48 hr. A slight increase in G1 cells was observed following 6 hr of treatment with 0.01 µg ACM per ml. However, drug concentra-
Effects of ACM on Cell Cycle Kinetics

The lowest concentration of ACM (0.01 μg/ml) had little or no effect on the cell cycle distribution. At a concentration of 0.05 μg ACM per ml, cells began to accumulate in G1, the percentage of cells in G1 increasing with increasing time of exposure to the drug (Table 3). However, higher concentrations of the drug...
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(0.1 μg/ml) caused an early (6-hr) accumulation of cells in S-phase (Chart 4; Table 3). By 24 hr, this population had appeared to have moved to G2 + M phase. Finally, after 48-hr continuous exposure to 0.1 μg ACM, L1210 cells accumulated almost exclusively in G1 phase (Chart 4; Table 3).

Short-term exposure (1-hr pulse) of L1210 cells followed by removal of the drug and growth in fresh drug-free medium resulted in a somewhat different effect on cell kinetics. Thus, 0.05 and 0.1 μg ACM per ml for 1 hr resulted in only minor perturbations in the cell cycle distribution of L1210 cells (Table 4). However, a 0.5-μg/ml pulse of the drug resulted in an early accumulation of cells in S-phase (by 6 hr). By 24 hr, cells were observed to have accumulated in G2 + M phase (Chart 5; Table 4).

RNA Content

Table 5 illustrates the change in relative RNA content of G1 cells of FL and L1210 cultures either exposed to a pulse (1 hr) followed by growth in drug-free medium or treated continuously with ACM. Continuous exposure to 0.01 μg ACM per ml for 24 hr had no effect on the RNA content of either FL or L1210 cells. Higher concentrations of the drug, however, decreased the mean RNA content of G1 cells by 36 to 49% in both cell lines (Table 5). A 1-hr pulse of the drug (at concentrations of 0.05 to 0.5 μg ACM per ml) lowered the RNA content of the G1 population by 39 to 44% (Table 5).

Cell Cycle Point of Action of ACM

The data in Charts 4 and 5 and Tables 3 and 4 suggest that ACM at higher concentrations acts on L1210 cells to slow cell progression through S phase and results in their accumulation in either G2 + M or G1 phase depending upon the drug concentration. In order to study the kinetics of this phenomenon in more detail, a stathmokinetic experiment has been performed in which L1210 cells were exposed to vinblastine and 1 hr later to various concentrations of the drug (22).

Table 4

<table>
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<th>Hr</th>
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<td>37.5</td>
<td>36.4</td>
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<tr>
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<tr>
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<tr>
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</tr>
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</table>

* Length of time in culture. After 1 hr of exposure to ACM cells were grown in drug-free medium.
* Cell cycle phase distribution was calculated from green (DNA) fluorescence histograms by interactive computer analysis.
Effects of ACM on Cell Cycle Kinetics

Hr L1210 Cells

Chart 5. Two-dimensional (DNA versus RNA) and one-parameter DNA histograms of L1210 cells exposed to a 1-hr pulse of 0.5 μg ACM per ml. Displays are described in the legend to Chart 3. Following 1 hr of exposure to the drug, cells were washed with HBSS and resuspended in drug-free medium. Note the accumulation of cells in G2 + M phase and compare with the continuous exposure of L1210 cells to a lower ACM concentration illustrated in Chart 4.

Table 5

<table>
<thead>
<tr>
<th>Drug (μg/ml)</th>
<th>Exposure hr</th>
<th>Cell line</th>
<th>Mean a</th>
<th>D/C b</th>
<th>Mean</th>
<th>D/C</th>
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<td></td>
<td>29.2</td>
<td>0.59</td>
<td>37.8</td>
<td>0.64</td>
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</table>

a The RNase-sensitive fraction of red fluorescence (mean values of the G1 population in arbitrary units).
b D/C, the relative mean RNA content of drug-treated cells (D) compared to cultures not exposed to drug (C).

Mitotic Cells. Except for a short delay, due to the time required for vinblastine to enter the cells and interact with the mitotic spindle, control cells accumulated exponentially in mitosis (Chart 6) during the experiment. From the slope of the straight portion of the curve, the cell cycle duration of this culture was estimated to be 10.2 hr.

Cultures treated with 0.1 μg ACM per ml 1.0 hr after addition of vinblastine accumulated in mitosis at a slower rate (Chart 6). The cell cycle duration for such culture could be estimated to be approximately 16 hr. At a drug concentration of 0.5 μg/ml, cells accumulated even more slowly until by 4.5 hr (3.5 hr after addition of the drug) the cells ceased entering mitosis (Chart 6).

The relative proportion of cells in mitosis was determined by flow cytometry as described previously (8-11), and the principles of this technique are illustrated in Chart 7. Note that the difference in susceptibility of chromatin to acid denaturation in mitotic versus interphase cells (α) permits a complete discrimination of the mitotic cells, allowing for a highly accurate determination of the mitotic index. In addition, the cell cycle distribution of the remaining interphase cells can be derived from the intensity of the total cell fluorescence which, in effect, is the sum of native and denatured DNA per cell (Chart 7). Thus, 25.3% of cells in control cultures accumulated in mitosis by 5 hr, and the remaining interphase cells were distributed throughout the cycle (G1, S, and G2) although with many more cells in S and G2 phase than would normally be found in exponentially growing cultures (e.g., see Chart 4). ACM-treated cultures had fewer cells in mitosis and at 0.5 μg/ml many more cells in G1 phase than in control cultures (Chart 7).

G2-Phase Cells. When the percentage of cells in both G2 and mitosis are plotted versus time after vinblastine treatment as in Chart 6, it is evident that at 0.1 μg ACM per ml the accumulation of cells in G2 exactly offsets the slower accumulation of cells in mitosis such that the control and drug curves overlap. The
The effect of aclacinomycin on the passage of cells through G2 phase and a window in early S phase

Table 6

<table>
<thead>
<tr>
<th>Hr</th>
<th>S0c/S0a</th>
<th>G20/G2c</th>
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</thead>
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</tr>
<tr>
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<td>0.95</td>
<td>1.06</td>
</tr>
<tr>
<td>4</td>
<td>1.14</td>
<td>1.33</td>
</tr>
<tr>
<td>6</td>
<td>1.44</td>
<td>1.60</td>
</tr>
</tbody>
</table>

*Sc/Sc*, the proportion of cells in a window in early S phase (i.e., from the G1-S boundary to approximately one-third of the way through S) in drug-treated (Sc) relative to control (Sc) cultures as calculated from histograms as in Chart 7 by interactive computer analysis. G20/G2c, the proportion of G2-phase cells in drug-treated (G20) as compared to control (G2c) cultures.

slope of the best fit line through the control points (G2 + M) as determined by a regression analysis was 2.816 and that for the ACM-treated cells 2.817. Both slopes are almost the same as the slope of the mitotic accumulation curve for the control culture, i.e., 2.879. The data in Table 6 illustrate that the relative number of cells in G2, in cultures treated with 0.1 μg ACM per ml, increased with increased time of exposure. This was not the case for cultures treated with 0.5 μg ACM per ml (Table 6). Thus, the curve of accumulation of cells in G2 and mitosis quite clearly parallels the mitosis accumulation curve (Chart 6), suggesting that transit of cells through G2 phase was basically unaffected following treatment with 0.5 μg ACM per ml.

G1-Phase Cells. Assuming that no cells reenter G1 phase, due to the action of vinblastine, the G1 compartment will empty with time, and the kinetics can be analyzed from the change in the cell cycle distribution as in Chart 7.

The rate of cell exit from G1 is biphasic (Chart 8). The first phase (0 to 3 hr) is linear (a shoulder on the log plot), while the second (3 to 8 hr) is exponentially declining. These phases are believed to represent the cell exit rates from the deterministic and indeterminate G1 compartments, respectively (6). The addition of 0.1 μg ACM per ml affected only the second phase of the emptying of the G1 compartment (Chart 8). The kinetics of significantly from untreated control cultures, the fraction of cells in G2 + M is equivalent, indicating cells accumulate in G2 phase at that drug concentration. Cells are blocked from entering mitosis altogether 3.5 hr following addition of 0.5 μg ACM per ml.

Table 6

<table>
<thead>
<tr>
<th>Hr</th>
<th>0.1 μg/ml</th>
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<td>1.01</td>
<td>1.44</td>
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</table>
the second phase can be expressed as the $I_{50}$, i.e., an estimate of the halftime of cell residence in the indeterminate state of G1 (6). The $I_{50}$ for control cultures was approximately 1 hr 40 min, whereas the $I_{50}$ for cells treated with 0.1 $\mu$g ACM per ml was 1 hr 48 min, as determined from the slopes of the curves.

Cultures exposed to 0.5 $\mu$g ACM per ml have very different kinetics from control cultures. Thus, within 1 hr of the addition of 0.5 $\mu$g ACM per ml, the drug-treated cultures exit G1 phase at a greatly reduced rate. This would result in an accumulation of cells in G1, as was evident in the cell cycle distribution in Chart 7.

**S-Phase Cells.** Transit of cells through a window in early S phase (i.e., from the G1-S boundary to approximately one-third of the way through S) was also calculated. In control cultures, the percentage of cells in the early S window increased between 0 and 3 hr, leveled off between 3 and 4 hr, and then decreased (Chart 9). Relative to control cultures, cultures treated with 0.1 $\mu$g ACM per ml showed little or no change in kinetics (Table 6). However, treatment with 0.5 $\mu$g ACM per ml caused an increase in S-phase cells within the window several hr after addition of drug (Chart 9; Table 6). Since cells were leaving G1 phase in these cultures at a reduced rate (Chart 8), the increase in relative proportion of S-phase cells could only be due to a slow down or block in the transit of cells through the window.

**Chromatin Structure**

Interaction of intercalating agents may lead to alterations in chromatin structure which are often manifested as a change in the sensitivity of chromatin in situ to acid or thermal denaturation. While ACM is thought to interact with DNA in cells by intercalation, stabilizing it against thermal denaturation (15), no significant change was observed in the sensitivity of cells to acid denaturation in drug-treated culture. Thus, the $\alpha$ values for interphase and mitotic cell chromatin of drug-treated samples did not shift relative to control cells following partial acid denaturation (Chart 7). Apparently, the amount of intercalated drug (per unit of DNA) in the ACM-treated cells was not enough to be detected by the present method.

**DISCUSSION**

The present study was undertaken to determine the kinetic events occurring in vitro in mammalian cell lines following treatment with aclacinomycin. These data provide information on the action of the drug that may aid in predicting its effects in vivo and in planning dosage schedules of ACM as a single drug or in a multidrug regimen.

Growth of mammalian cell lines was inhibited at concentrations of 0.02 to 0.05 $\mu$g/ml following long (24-hr) exposure and 0.1 to 3.6 $\mu$g/ml following short (1-hr) exposure to the drug (Table 1). ACM was approximately 5 times more effective in inhibiting colony formation of exponentially growing as compared to stationary CHO cells (Table 1).

With exponentially growing cells under continuous exposure to ACM at 0.05 to 0.1 $\mu$g/ml, there was first an accumulation of cells in S phase, then in G2 + M, and finally in G1 phase (Tables 2 and 3; Charts 3 and 4). As the drug concentration was increased, passage of cells through S and G2 + M was slowed (Tables 2 and 3). Thus, for example, L1210 cells accumulated in G1 following 24 hr of exposure to 0.05 $\mu$g ACM per ml whereas cultures exposed to 0.1 $\mu$g ACM per ml contained cells accumulating in G2 + M at this time (Table 3). However, by 48 hr, both cultures contained mostly G1-phase cells (Table 3; Chart 4). The drug concentrations used in these studies were not cytotoxic (Chart 2). Therefore, it may be assumed that cell accumulation in various cell cycle compartments was due not to cell death and loss of cells from other compartments but rather to a slow down or cessation of cell transit through particular cell cycle phases.

A brief 1-hr pulse of ACM followed by growth in drug-free medium had only a transient effect on the cell cycle distribution of L1210 cells at low concentrations (i.e., 0.05 to 0.1 $\mu$g/ml). However, at 0.5 $\mu$g ACM per ml, a 1-hr pulse resulted in a dramatic accumulation of cells in S phase by 6 hr which by 24 hr accumulated in G2 + M phase and remained there over the next 24 hr (Table 4; Chart 5).
A detailed study of the kinetics of the action of ACM on exponentially growing L1210 cells has helped explain the effect of dose on the cytostatic action of the drug. At an ACM concentration of 0.1 µg/ml, cells entered mitosis more slowly (Chart 6) due to the transient block in G2 phase (Table 6). Cell transit through early S phase was unaffected at this drug concentration (Table 6) although exit of cells from G2 was slowed slightly (Chart 8).

Exposure of L1210 cells to 0.5 µg ACM per ml resulted in a considerably different set of events. Thus, the accumulation of mitotic cells was slowed markedly between 0 and 3.5 hr after addition of the drug and halted entirely from that point on (Chart 6). The transit of cells through G2 phase was unaffected until about 6 hr after the addition of the drug (Table 6). In contrast to the lower drug concentration, 0.5 µg ACM per ml caused an accumulation of cells in early S phase and a dramatic decrease (within 1 hr of addition of drug) in the exit rate of cells from G1 phase (Chart 6).

Therefore, the effect of ACM on cell cycle progression appeared to depend on cell type and drug dose more than length of exposure. L1210 cells were more sensitive than FL cells to continuous exposure at low drug concentrations (1.0 µg ACM per ml or less) as witnessed by the dramatic accumulation of L1210 cells in G1 phase by 48 hr (Tables 2 and 3). However, L1210 cells treated with a 5 times higher dose of drug accumulated in the preceding G2 phase (Table 4). Thus, whereas the lower drug concentration led to an early accumulation of G2-phase L1210 cells (Table 6), these cells were eventually able to divide (Chart 6) and reach, but not exit, the following G1 phase. The higher drug concentration slowed cell transit through S phase (Table 6; Charts 6 and 9) and prevented cells from entering mitosis within 3.5 hr of addition of drug (Chart 6).

The effect of ACM on cells entering and passing through S phase may be secondary to its action on RNA synthesis (25). In the present study, the RNA content of G1-phase cells was lowered by 35 to 50% following treatment with ACM (Table 5). As shown previously (6), cells in G1 must accumulate a threshold amount of RNA to progress into S phase. The rate of cell exit from the indeterminate state of G1 appears to be correlated with RNA content (12). Indeed, as presently observed, this exit from the indeterminate state of G1 appears to be correlated with RNA content (12). In contrast to the lower drug concentration, 0.5 µg ACM per ml caused an accumulation of cells in early S phase and a dramatic decrease (within 1 hr of addition of drug) in the exit rate of cells from G1 phase (Chart 6).

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