

# ON THE RECOVERY OF TRANSCRIPTION AFTER INHIBITION BY ACTINOMYCIN D

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## ABSTRACT

After pulse exposure to concentrations of actinomycin D (AMD) sufficient to abolish transcription, Vero cells recover RNA synthesis much more rapidly than most other cell types. This is only in part attributable to the remarkable capacity of Vero very promptly to excrete bound AMD, elimination of which, although necessary, is not a sufficient condition for resurgence of RNA synthesis. After elimination of higher concentrations of AMD from Vero, although over-all RNA synthesis resumes a normal rate within 24 hr, protein synthesis lags, and a long period of division-delay ensues. Division-delay lasting 2-3 days results from exposure of Vero to doses of AMD greater than those that suppress RNA synthesis by greater than 90% (e.g. 1  $\mu\text{g}/\text{ml}$  for 2 hr) but not by lower doses, which permit almost immediate reentry into the cell cycle. In contrast, although L cells recover over-all RNA synthesis very slowly after pulse treatment with AMD, resumption of protein synthesis or cell division is not comparably delayed thereafter. These and other data suggest that the early restoration of RNA synthesis in Vero after relief of inhibition by AMD is qualitatively imperfect. The results reported herein are explainable by the hypothesis that the synthesis of those species of RNA which are involved, directly or indirectly, in reactivating the transcription of genes controlling progression in the cell cycle is relatively resistant to suppression by AMD. Decay of such RNA templates and their products, which differs in different cell types during inhibition by AMD, determines the duration of division-delay.

## INTRODUCTION

The suppression of RNA synthesis for some hours by actinomycin D (AMD), or by anthracyclines, results in irreparable damage in most cell lines, manifested either acutely or after one or more days (1). Although transcription is virtually suppressed by moderate concentrations of AMD (1  $\mu\text{g}/\text{ml}$ ), Vero cells are remarkably more resistant to cytotoxication than are other cell types, even by much higher doses (e.g. 10-50  $\mu\text{g}/\text{ml}$ ) (1, 2). In contrast to other cell lines (e.g. HeLa, L, WI38, WIL2, MDBK), Vero are capable of resuming RNA synthesis very rapidly after withdrawal of AMD (1, 2), an observation since verified in other laboratories (3, 4). We had also indicated that

Vero can eliminate or excrete bound AMD very rapidly, faster than do other cell lines (1, 2). In this communication we report the results of experiments on the binding and release of AMD in relation to RNA synthesis, and the relation of recovery of transcription to protein synthesis and cell division. It will be shown that elimination of drug, although a necessary antecedent of recovery, is not per se a sufficient condition for restoration of transcription, and the apparent prompt recovery of over-all RNA synthesis in Vero is not followed by commensurate restoration of protein synthesis, or cell division.

## MATERIALS AND METHODS

The cells and their maintenance have been described previously (1). Monolayer cultures of HeLa, Vero, and L cells were maintained in a growth medium consisting of Eagle's minimal essential medium (Auto-Pow MEM, Flow Laboratories, Rockville, Md.) supplemented with newborn or fetal calf serum (10% v/v), MEM nonessential amino acids, streptomycin (75  $\mu\text{g}/\text{ml}$ ), and penicillin (75 units/ml) (Grand Island Biological Co., Grand Island, N.Y.), and in an atmosphere of 5%  $\text{CO}_2$ . The cells were kept in exponential growth by passage twice or thrice weekly. Doubling times of HeLa, Vero, and L cells were 19 hr, 21 hr, and 17 hr, respectively. Once a month the cultures were grown for 2 days in the presence of Tylocine (Anti-PPLO Agent, Grand Island Biological Co.), 50  $\mu\text{g}/\text{ml}$  of aureomycin or 20  $\mu\text{g}/\text{ml}$  of chloramphenicol; periodic tests for mycoplasma were always negative. Experiments were performed on cells explanted to 35 mm diameter Petri dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) 1 or 2 days before beginning the experiments when the cell number per dish was between  $0.7$  and  $1.2 \times 10^6$ .

Actinomycin D (Merck Sharp & Dohme, West Point, Pa.) was dissolved in dimethyl sulfoxide, diluted in saline, and sterile filtered. Since AMD binds to nitrocellulose filters, the exact concentration of the AMD stock solution was determined by measuring the optical density of 441 nm ( $\epsilon = 25.7 \times 10^3$ ) after filtration. Direct exposure to light of solutions containing AMD and of cells treated with AMD was avoided. The stock solution was diluted with fresh growth medium to the desired drug concentration and administered in a volume of 2 ml/ $10^6$  cells. After removal of AMD, the cells were rinsed once with balanced saline and refed with fresh drug-free growth medium; the cells were refed again 3-5 hr later to remove the AMD that had diffused back into the medium, and every day thereafter.

RNA and protein syntheses were measured by determining the amount of uridine- $^{14}\text{C}$  (50.5-58 mCi/mole, New England Nuclear Corp., Boston, Mass. or Schwarz Bio Research, Orangeburg, N.Y.) and amino acids- $^{14}\text{C}$  (1.5 mCi/mg, New England Nuclear Corp.), respectively, incorporated during a given period of time into the cold acid-insoluble (5% trichloroacetic acid, [TCA]) cellular material. The uridine- $^{14}\text{C}$  was diluted to 0.1-0.3  $\mu\text{Ci}/\text{ml}$  ( $2-6 \times 10^{-6}$  M uridine) with fresh growth medium containing  $2 \times 10^{-5}$  M deoxycytidine and thymidine to prevent incorporation of the label into DNA. The amino acids- $^{14}\text{C}$  were diluted to 1  $\mu\text{Ci}/\text{ml}$  with fresh growth medium that had one tenth the normal concentration of MEM amino acids and did not contain MEM nonessential amino acids. The labels were administered in a volume of 1 ml per 35-mm Petri dish. After

incubation of the cells in the label for the times specified in the Results section of this article, the medium containing label was removed by suction and the cells were rinsed thrice with balanced saline which contained  $5 \times 10^{-4}$  M uridine or MEM amino acids and the cells were detached by incubation for 3 min in 1 ml of 0.25% trypsin-0.15% Versene in balanced saline. After dispersion of the cells, a sample of a known number of cells was solubilized with Soluene-100 (Packard Instrument Co., Inc., Downers Grove, Ill.), or was precipitated and washed with ice cold 5% TCA. The acid-insoluble precipitates were either collected on membrane filters (Bac-T-Flex, B-6, Schleicher & Schuell Inc., Keene, N.H.) or solubilized with Soluene-100. The samples were counted in a toluene base scintillant (Omnifluor, Pilot Chemicals, Inc., Watertown, Mass.) with a Packard Liquid Scintillation Spectrometer. The counting efficiency was determined using either a channel ratios method or an automatic external standard; the counting efficiency varied between 78% and 82%. Greater than 94% of the radioactivity associated with the acid-precipitable material during a 1 hr labeling period with uridine- $^{14}\text{C}$  was hydrolyzed with 0.3 N KOH at 37°C for 18 hr; and the rate of incorporation of radio-labeled uridine is linear for at least 2 hr at the concentrations of uridine used.

The binding of AMD- $^{14}\text{C}$  (11.4 mCi/mole, New England Nuclear Corp.) by cells has been described previously (1). Briefly, dilutions of AMD- $^{14}\text{C}$  were administered to the cells, and at specified times the label was removed, the cells were rinsed thrice with balanced saline, and refed with fresh drug-free growth medium. The cells were trypsinized and collected for scintillation counting as described above. Radioautography of cell cultures treated with AMD- $^3\text{H}$  (8.4 Ci/mole; Schwarz Bio Research) showed the label to be associated only with the nucleus and removable by heating the cells to 98°C for 10 min in 4% formaldehyde buffered at pH 7.0.

Morphological examination and mitotic indices were performed on cells grown on cover glasses and fixed in cold methanol, acetic acid, Formalin (85:5:10), and stained with azure and eosin.

## RESULTS

### *Recovery of RNA Synthesis after AMD*

Vero cells begin to recover their capacity to incorporate radiolabeled uridine into acid-precipitable material within 2-3 hr after removal of 10  $\mu\text{g}/\text{ml}$  of AMD after having been exposed to the drug for 1 or 4 hr; in contrast, L cells treated for 1 hr with 5  $\mu\text{g}/\text{ml}$  begin to reestablish RNA synthesis only after a much longer interval (9-12 hr) and at a much slower rate (Fig. 1). Thus, Vero cells can restore RNA synthesis much more promptly after

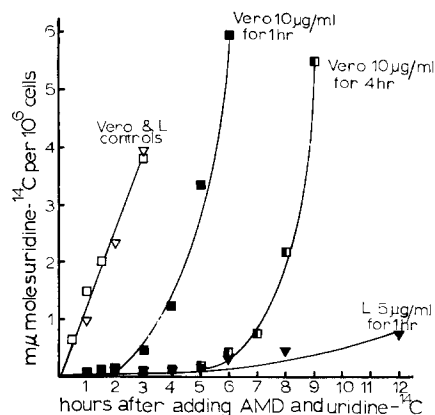


FIGURE 1 Recovery of RNA synthesis after treatment with AMD. Vero cells were exposed to 10  $\mu\text{g}/\text{ml}$  of AMD in the presence of uridine- $^{14}\text{C}$  (0.2  $\mu\text{Ci}/\text{ml}$ , 4  $\mu\text{Ci}/\text{mmole}$ ) for 1 hr (■) or for 4 hr (■), and L cells to 5  $\mu\text{g}/\text{ml}$  of AMD for 1 hr (▼), after which times the AMD was removed and the cells were rinsed and refeed with fresh drug-free medium containing the above concentration of uridine- $^{14}\text{C}$ . At the points indicated on the graph, duplicate sets of cells were prepared for liquid scintillation spectrometry as described in Materials and Methods. □, untreated Vero; ▽, untreated L cells. The slope of lines cannot be used to compare the exact rates of RNA synthesis after recovery from AMD in this experiment because of the smaller intracellular pool of nucleotides in the control as compared to the AMD-treated cells (see text).

removal of AMD than other types of animal cells in culture, even though Vero have been treated for longer periods and with higher drug concentrations.

In the experiment described in Fig. 1, radio-uridine was administered continuously from the beginning of the AMD exposure period and after it, and therefore during an interval when no RNA synthesis was taking place; however, the uptake of radiouridine into acid-soluble material ("pool") was proceeding at a rate unaffected by AMD (Table I). This must have resulted in an enlarged pool of radioactive precursor (i.e. a pool of higher specific activity) in the AMD treated cells, as compared with the untreated cells. The latter incorporate exogenously supplied uridine at a rate dependent on the concentration in the medium (within the range of concentrations of uridine used in our experiments, viz. less than  $5 \times 10^{-5}$  M). When RNA synthesis of the cells exposed to AMD began to recover after 3-4 hr of exposure to radio-uridine, the presence of an enlarged pool of radio-

TABLE I  
Recovery of RNA Synthesis in Vero after 2 Hr  
Exposure to 10  $\mu\text{g}/\text{ml}$  of AMD

Hours after adding AMD or refeeding controls	uridine- $^{14}\text{C}$ taken up by $10^6$ cells	uridine- $^{14}\text{C}$ incorporated by $10^6$ cells	corresponding control
	$\mu\mu\text{moles}$	$\mu\mu\text{moles}$	%
Untreated control cells			
0	1297	280	100
2	890	163	100
4	1390	208	100
6	1340	219	100
12	1340	298	100
24	1030	252	100
10 $\mu\text{g}/\text{ml}$ of AMD for 2 hr			
2	1390	3	<2
4	1500	19	9
6	1577	85	39
12	1600	198	66
24	1500	279	111

Vero cells ( $9 \times 10^6$  cells/35-mm Petri dish) were given 10  $\mu\text{g}/\text{ml}$  of AMD at 0 hr and at 2 hr the AMD medium was removed, and the cells were rinsed with EBSS and refeed with fresh drug-free growth medium; the cells were refeed again at 4 hr. The untreated control cells were refeed at 0 hr. At 0, 2, 4, 6, 12, and 24 hr, duplicate samples were pulse labeled for 30 min with 0.3  $\mu\text{Ci}/\text{ml}$  of uridine- $^{14}\text{C}$  (50.5 mCi/mmole) and the amount of uridine taken up by the whole cells (the acid-soluble plus acid-insoluble fractions) and into the acid-insoluble fraction was determined as described in Materials and Methods. After refeeding the control cells there is a decrease in the amount of uridine taken up by the cells which results in a decreased rate of incorporation of label into acid-insoluble material 2 hr after refeeding; this does not occur in the presence of AMD.

active nucleoside gave the appearance of a normal, or faster than normal, rate of RNA synthesis as compared to untreated cells. The pulse labeling experiments illustrated in Table I show that, in Vero, RNA synthesis does not, in fact, fully recover until 18-24 hr after removal of a dose of 10  $\mu\text{g}/\text{ml}$  for 2 hr. Restoration of RNA synthesis in L cells is much slower than in Vero even though L cells were exposed to a lower dose of AMD. Pulse labeling experiments show that after treatment for 1 hr with 5  $\mu\text{g}/\text{ml}$ , L cells do not recover RNA synthesis until more than 48 hr after removal of the

drug (Fig. 8B). By 12 hr after removal of this dose, RNA synthesis in L cells is still less than 20% of that of the untreated control cells. Note also in Table I that the uptake of uridine-<sup>14</sup>C is not impaired or altered by AMD treatment. The transitory decrease in the uptake of uridine from the medium shown in control Vero cells in Table I at 2 hr after refeeding with fresh medium is noteworthy. We have no explanation for this phenomenon; it occurs regularly and reproducibly in Vero and WI-38, but does not occur in HeLa or L cells.

#### *Dose Response of Inhibition of RNA Synthesis in Relation to Binding of AMD*

The dependency of the rate of radiouridine incorporation into RNA upon concentration of AMD in HeLa, Vero, and L cells is illustrated in Fig. 2, from which it is apparent that RNA synthesis in HeLa is inhibited at lower concentrations of AMD than in Vero or L cells. This can be correlated with the higher uptake of AMD, at any given concentration, in HeLa than in Vero or L cells, as shown in Fig. 3. HeLa bind more than twice as much AMD-<sup>14</sup>C during a 1 hr exposure than Vero or L cells, both on a per cell basis (Fig. 3A) and on a per DNA basis (Fig. 3B). However, although L cells take up more drug per cell at a

given concentration than do Vero (Fig. 3A), both Vero and L take up approximately the same amount of drug on a DNA basis (Fig. 3B). Fig. 3 also illustrates that, in the range of concentrations of AMD generally employed to block transcription, i.e. less than 20  $\mu\text{g}/\text{ml}$ , the amount of drug taken up by all cell lines is similarly dependent on the concentration in the medium. Thus, there is a direct correlation between the extent of inhibition of RNA synthesis effected by a given concentration of AMD and the amount of AMD taken up per unit DNA in all of these cell types. However, Vero can be made to bind more AMD than HeLa and L cells but they nevertheless recover RNA synthesis more rapidly after removal of these higher drug doses.

#### *Dose Response of Recovery of RNA Synthesis in Relation to Retention of AMD*

Fig. 4A shows that RNA synthesis in Vero recovers progressively more slowly after removal of a given concentration (10  $\mu\text{g}/\text{ml}$ ) of AMD after increasingly longer exposure periods, i.e. 2, 4, or 6 hr. Fig. 5 shows that during continuous exposure to AMD-<sup>14</sup>C, the amount of drug taken up by Vero reaches an equilibrium after about 2 hr at higher drug concentrations (5–20  $\mu\text{g}/\text{ml}$ ) and after about 1 hr at lower concentrations (1 and 2.5  $\mu\text{g}/\text{ml}$ ).

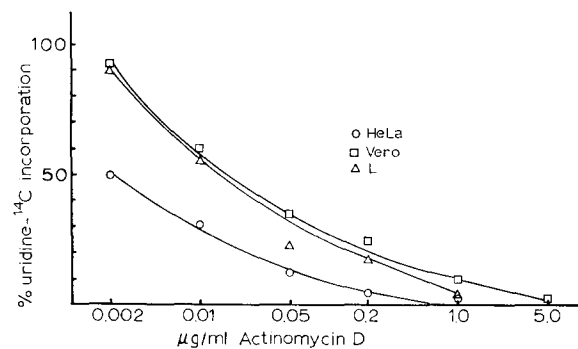


FIGURE 2 Concentration dependent inhibition of RNA synthesis by AMD. Replicate cultures of Vero, HeLa, and L cells in 35-mm Petri dishes were given 1 ml of uridine-<sup>14</sup>C (0.2  $\mu\text{Ci}/\text{ml}$ ; 50.5 mCi/mmmole), and 15 min later 1 ml of 0.004, 0.02, 0.1, 0.4, 2.0, or 10  $\mu\text{g}/\text{ml}$  of AMD was added. 1 and 2 hr after adding AMD, duplicate samples were prepared for liquid scintillation spectrometry as described in Materials and Methods. The amount of radioactivity present in the 1 hr acid-insoluble precipitates was subtracted from the amount in the corresponding 2 hr sample to give the amount of uridine-<sup>14</sup>C incorporated between 1 and 2 hr after the addition of varying concentrations of AMD. The labeled uridine was added 15 min before the AMD in order to label the rapidly metabolized RNA, and thereby ensure that all of the nucleotide pools, including those derived from degraded, or metabolized, RNA, were labeled at the time the drug was added. The results are expressed as the present inhibition of uridine-<sup>14</sup>C incorporated by untreated control cells.  $\square$ , Vero;  $\Delta$ , L; and  $\circ$ , HeLa.

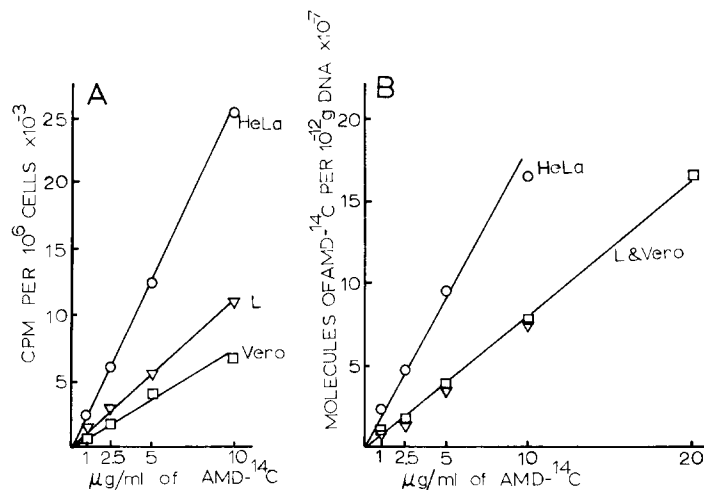


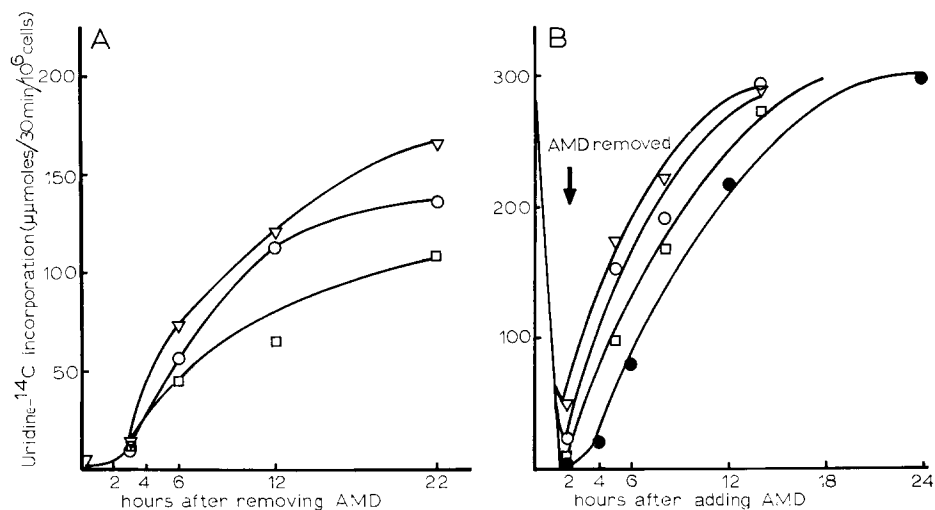
FIGURE 3 Concentration dependent uptake of AMD-<sup>14</sup>C. Replicate cultures of Vero, HeLa, and L cells were exposed for 1 hr to 1, 2.5, 5, 10, and 20  $\mu\text{g/ml}$  of <sup>14</sup>C-labeled AMD (3.8 mCi/mmmole, or  $3 \times 10^{-3}$   $\mu\text{Ci}/\mu\text{g}$ ), and the amount of radioactivity taken up by the cells was determined as described in Materials and Methods. Fig. 3A expresses the results as cpm of AMD-<sup>14</sup>C per  $10^5$  cells; and Fig. 3B as molecules of AMD per picogram of DNA.  $\square$ , Vero 12.6  $\mu\text{g}$  DNA/ $10^6$  cells;  $\nabla$ , L, 21.0  $\mu\text{g}$  DNA/ $10^6$  cells; and  $\circ$ , HeLa, 18.2  $\mu\text{g}$  DNA/ $10^6$  cells.

Therefore, treatment of Vero with AMD for periods longer than 2 hr does not result in an increase in the ratio of AMD to DNA in the cell. Vero are unique in this regard compared with HeLa and L cells because HeLa and L continue to take up AMD with longer exposure. Although the rate of uptake of drug in HeLa and L slows after an initial period of rapid binding in contrast to Vero, it does not reach an equilibrium (1). This equilibrium signifies that after 2 hr the rate of drug ingress and binding in Vero equals the rate of drug detachment and excretion; in HeLa and L cells, however, the rate of uptake and binding is always greater than the rate of drug dissociation and elimination.

Fig. 6 depicts the rate of excretion of AMD-<sup>14</sup>C from HeLa, L, and Vero cells. Two main points are made by these data: (a) Vero eliminate AMD approximately twice as fast after removal of the drug as HeLa and L cells; (b) the rate at which Vero eliminate AMD is independent of the drug concentration, and of the length of exposure to the drug. Thus, the more rapid recovery of RNA synthesis by Vero as compared with L cells after removal of AMD can be correlated with the faster elimination of the drug by Vero. However, this is by no means the only determinant of recovery. RNA synthesis in Vero comes back more quickly after removal of a given concentration of AMD

after shorter exposure periods even though no more AMD is bound or retained with longer exposure. Furthermore, Vero recover their RNA synthesis much more rapidly after removal of 10  $\mu\text{g/ml}$  applied for 6 hr than do L cells treated for only 1 hr with 5  $\mu\text{g/ml}$ . Since Vero take up about four times as much AMD when treated for 6 hr with 10  $\mu\text{g/ml}$  than do L cells treated for 1 hr with 5  $\mu\text{g/ml}$ , and because Vero eliminate the drug only about twice as rapidly, it would be expected, if recovery of RNA synthesis were due solely to elimination of AMD, that L cells should be able to recover RNA synthesis after this dose (5  $\mu\text{g/ml}$ ) to about the same extent and at approximately the same time as Vero. This is clearly not the case as is apparent in Fig. 1.

It appears from Fig. 4B that the rate of recovery of RNA synthesis is relatively independent of the drug concentration within the range from 0.5 to 10  $\mu\text{g/ml}$ . RNA synthesis is depressed proportional to dose up to 2.5  $\mu\text{g/ml}$ ; at concentrations higher than 2.5  $\mu\text{g/ml}$ , RNA synthesis is maximally inhibited, notwithstanding that more AMD continues to be bound at concentrations higher than this (Figs. 3 and 5). After withdrawal of AMD after an exposure period of 2 hr, the rate of restoration of RNA synthesis is independent of drug concentration, once the level of intracellular AMD has diminished below that necessary to



**FIGURE 4** Dependence of recovery of RNA synthesis on AMD dose. Fig. 4A, Dependence of the rate of recovery of RNA synthesis on the length of the drug exposure period. Vero cells growing in 35-mm Petri dishes ( $1.5 \times 10^6$  cells/dish) were treated for 2 ( $\nabla$ ), 4 ( $\circ$ ), or 6 ( $\square$ ) hr with  $10 \mu\text{g/ml}$  of AMD, after which times the drug was removed and the cells were rinsed and refed; the cells were refed again 3 hr later. At 0, 3, 6, 12, and 22 hr after removal of the drug, duplicate dishes were pulse labeled for 30 min with uridine- $^{14}\text{C}$  ( $0.2 \mu\text{Ci/ml}$ ,  $4 \mu\text{M}$  uridine) and the amount of radioactivity associated with the acid-insoluble precipitates of a known number of cells was determined as described in Materials and Methods. The rate of incorporation of uridine- $^{14}\text{C}$  into acid-insoluble material was corrected for changes in the rate of uptake of uridine. The rate of incorporation of uridine- $^{14}\text{C}$  in untreated control cells was  $204 \mu\text{moles}/10^6 \text{ cells}/30 \text{ min}$ . Therefore, in this experiment, the rate of uridine incorporation had not returned to the control rate of 24 hr after removal of a 2 hr pulse dose of  $10 \mu\text{g/ml}$ , but was at 75–80% in contrast to the experiment reported in Table I in which the rate of uridine incorporation 24 hr after removal of the same dose of AMD slightly exceeded the control value. The cell density of the monolayer in this experiment was approximately twice that of the experiment reported in Table I. Fig. 4B. Dependence on drug concentration of the rate of restoration of uridine- $^{14}\text{C}$  incorporation. Vero cells ( $1.25 \times 10^6$  cells/35-mm Petri dish) were treated with  $0.5 \mu\text{g/ml}$  ( $\nabla$ );  $1.0 \mu\text{g/ml}$  ( $\circ$ );  $2.5 \mu\text{g/ml}$  ( $\square$ ); and  $10 \mu\text{g/ml}$  ( $\bullet$ ) of AMD. After 2 hr the AMD was removed, the cells were rinsed and refed with drug-free medium; the cells were refed again 3 hr after removing the AMD. Immediately after removing the AMD, and 3, 6, 12, and 24 hr later, duplicate samples were pulsed for 30 min with uridine- $^{14}\text{C}$  ( $0.3 \mu\text{Ci/ml}$ ;  $58 \text{ mCi/mole}$ ) and the amount of label taken up by the cells and the amount incorporated into the acid-insoluble fraction was determined as described in Materials and Methods. Since, 3 hr after feeding, control Vero cells take up 50% less uridine (and therefore incorporate 50% less uridine into acid-insoluble material) than cells fed 12 hr before or cells treated with AMD (see Table I), the rate of uridine incorporation used as the control (100% =  $305 \mu\text{moles}/10^6 \text{ cells}/30 \text{ min}$ ) was the 0 hr value which was obtained from cells fed 12 hr before the beginning of the experiment.

inhibit RNA synthesis maximally. Thus, after removal of  $10 \mu\text{g/ml}$  after 2 hr, RNA synthesis remains suppressed for an additional 2 hr before recovery begins; but RNA synthesis begins to come back immediately after removal of  $2.5 \mu\text{g/ml}$  or lesser concentrations. If the apparent rates of RNA synthesis are compared at a fixed time, e.g. at 12 hr after treatment of Vero for 2 hr with 0.5, 1, 2.5, and  $10 \mu\text{g/ml}$  of AMD, recovery appears not to follow in strict concentration-dependent fashion. After exposure to the lower drug concentrations

(less than  $2.5 \mu\text{g/ml}$ ), the over-all rate of uridine incorporation returns to the control level within 12 hr, but after treatment with  $10 \mu\text{g/ml}$  restoration of RNA synthesis is delayed until 20–24 hr.

Fig. 7 shows that after treatment for 12 hr with  $1 \mu\text{g/ml}$ , a dose that quickly reduces uridine incorporation to less than 10% of untreated cells, Vero rapidly recover, and by 24 hr after drug removal RNA synthesis is near the rate of untreated cells. However, after removal of 5 and  $10 \mu\text{g/ml}$  applied for 12 hr, recovery of RNA synthesis is

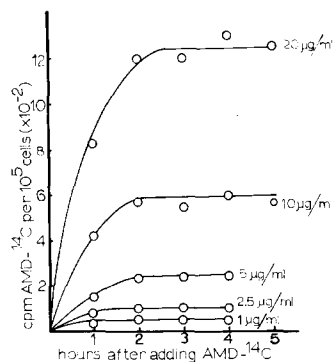


FIGURE 5 Uptake of AMD-<sup>14</sup>C with time by Vero. Vero cells were exposed to 1, 2.5, 5, 10, and 20 µg/ml of AMD-<sup>14</sup>C ( $3 \times 10^{-3}$  µCi/µg) continuously, and at hourly intervals the amount of radioactivity taken up by the cells was determined as described in Materials and Methods. An equilibrium or steady state is attained by 2 hr at every concentration.

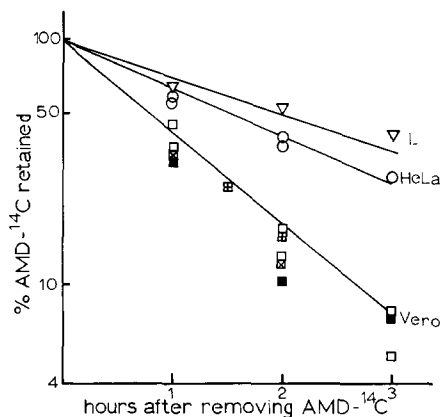


FIGURE 6 Rate of release of AMD-<sup>14</sup>C HeLa (○) and L (▽) cells were treated with 2.5 µg/ml of AMD-<sup>14</sup>C for 1 hr, and Vero with 2.5 µg/ml for 1 hr (⊞), or 10 µg/ml for 1 hr (□), 4 hr (■), or 12 hr (⊠). The medium containing AMD-<sup>14</sup>C was then removed (0 time) and the cells were rinsed three times and refed with drug-free medium. At the times indicated on the abscissa, the amount of radioactivity associated with the cells was determined as described in Materials and Methods.

slower, and its rate is independent of concentration; 24 hr after removal of 10 µg/ml for 12 hr, it is at the same level as after removal of 5 µg/ml for 12 hr, i.e. approximately 65% of that of the untreated control cells. We have observed that RNA synthesis is more readily reinstated in all types of cells after prolonged treatment with concentrations of AMD that do not completely suppress RNA synthesis, although uridine incorporation may be only

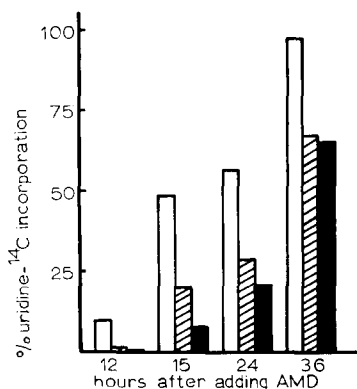


FIGURE 7 Recovery of RNA synthesis in Vero after 12 hr of exposure to 1, 5, and 10 µg/ml of AMD. Vero cells were exposed to 1, 5, and 10 µg/ml of AMD for 12 hr, after which the cells were rinsed and refed with drug-free medium; the cells were refed again 4 and 12 hr later. At 0, 3, 12, and 24 hr after removing the AMD, duplicate cultures were pulse labeled for 30 min with uridine-<sup>14</sup>C (0.1 µCi/ml, 50.5 mCi/mmmole) and the incorporation into acid-insoluble material was determined as described in Materials and Methods. The results are expressed as per cent of control (100% = 83 µmoles/10<sup>6</sup> cells). 1 µg/ml, open; 5 µg/ml, striped; and 10 µg/ml, closed.

5–15% of the normal value. Apparently, the effect of AMD is more reversible if transcription had not been altogether extinguished during treatment.

#### Over-all Protein Synthesis and Cell Division

Since, after treatment with AMD, Vero recovered the capacity to incorporate radiolabeled uridine much more quickly than L cells, we inquired whether restoration of the capacity to incorporate amino acids was linked to recovery of RNA synthesis and was therefore restored more quickly in Vero than in L cells. Figs. 8A and 8B show that incorporation of amino acids-<sup>14</sup>C in L appears to be more sensitive to AMD than in Vero cells: 4–8 hr after treatment with suppressive concentrations, it declines to approximately 25% in L and 50% in Vero of the value of untreated cells. However, after 24 hr the rate of incorporation of amino acids returns to about the same level (60% of untreated cells) in both cell types. Although over-all RNA synthesis remains suppressed after removal of AMD for substantially longer times in L than in Vero cells, over-all incorporation of amino acid in L cells nevertheless returns to the

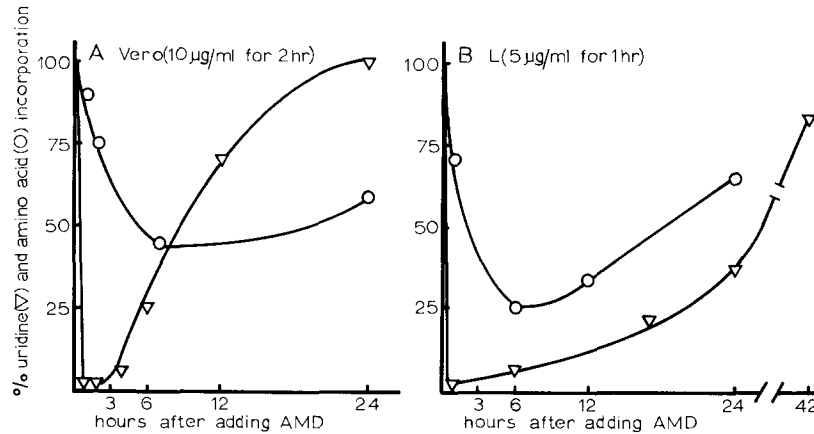


FIGURE 8 Recovery of RNA and protein syntheses in Vero and L cells. Vero (8A) and L(8B) cells were treated with 10  $\mu\text{g}/\text{ml}$  of AMD for 2 hr and 5  $\mu\text{g}/\text{ml}$  for 1 hr, respectively, after which times the AMD was removed, and the cells were rinsed with EBSS and refed again 4 hr later. At the intervals indicated on the graph, after beginning the treatment with AMD, duplicate dishes were pulse labeled for 30 min with 0.3  $\mu\text{Ci}/\text{ml}$  of uridine- $^{14}\text{C}$  (50.5 mCi/mmmole) ( $\nabla$ ) or 1  $\mu\text{Ci}/\text{ml}$  of amino acids- $^{14}\text{C}$  (0.1 mCi/0.067 mg) (O), and the amount of radioactivity taken up and incorporated by a known number of viable cells, i.e. cells excluding trypsin blue, was measured as described in Materials and Methods. Untreated Vero and L cells incorporated  $1.98 \times 10^5$  dpm and  $1.09 \times 10^5$  dpm per  $10^6$  cells, respectively, of amino acids- $^{14}\text{C}$ , and 304 and 294  $\mu\mu\text{moles}$  of uridine per  $10^6$  cells, respectively.

TABLE II  
Recovery of Cell Division

Cell type	Dosage of AMD	Mitoses per 1000 cells after treatment with AMD						
		0 hr	12 hr	24 hr	36 hr	48 hr	60 hr	72 hr
Vero	10 $\mu\text{g}/\text{ml}$ for 2 hr	31	0	0	0	0	20	17
	5 $\mu\text{g}/\text{ml}$ for 1 hr	37	0	1	1	1	19	36
	2.5 $\mu\text{g}/\text{ml}$ for 2 hr	36	0	0	0	15	14	28
	1 $\mu\text{g}/\text{ml}$ for 2 hr	39	1	1	3	24	31	
	0.5 $\mu\text{g}/\text{ml}$ for 2 hr	42	7	10	15	41		
	0.25 $\mu\text{g}/\text{ml}$ for 9 hr	29	1	7	19	20		
L	5 $\mu\text{g}/\text{ml}$ for 1 hr	32	0	14	8	15		34

Vero and L cells were exposed to the various doses of AMD as indicated in the table, and the cells were fixed at 12 hr intervals after the beginning of the drug treatment. The mitotic figures present per 1000 viable cells were determined by microscopic examination. The drug was administered at 0 hr and removed at the time indicated under dosage.

same level as in Vero after 24 hr. The greater initial sensitivity of amino acid incorporation in L cells to AMD could reflect either a direct influence of the drug on protein synthesis (5, 6) or a faster turnover of message RNA in L cells compared to Vero, and/or a more rapid disappearance of acid-precipitable aminoacyl tRNA. A direct effect on protein synthesis (at least in Vero) appears unlikely since Vero were treated with a

higher dose of AMD, and consequently three to four times more AMD was taken up per Vero cell than per L cell. It might therefore be expected that, if protein synthesis were directly inhibited by AMD, the Vero cells would have been more influenced than the L cells. But, in fact, recovery of protein synthesis after AMD more closely parallels recovery of RNA synthesis in L than in Vero cells.

Vero do not recover their capacity for cell divi-



sion any faster after exposure for 1 hr to 5  $\mu\text{g}/\text{ml}$  of AMD than do L cells treated with the same dose (Table II). Table II illustrates the "division-delay", i.e. the lag in resumption of mitosis, which occurs after exposure to varying concentrations of AMD. At concentrations higher than 1  $\mu\text{g}/\text{ml}$ , there is no cell division between 12 and 36 hr. In Vero, beginning after 36–48 hr, mitoses gradually begin to appear until by 3 days there is a near normal mitotic index. However, exposure of Vero to concentrations less than 1  $\mu\text{g}/\text{ml}$  for periods up to 9 hr results in a short delay in cell division, even though RNA synthesis has been reduced to less than 20% of that of the control. The length of the period of division-delay after removal of pulse doses of AMD is, therefore, dependent on concentration only at those concentrations that suppress transcription less than completely. At concentrations greater than 1  $\mu\text{g}/\text{ml}$ , which inhibit RNA synthesis by more than 90%, the length of the interval before recovery of cell division is independent of concentration. For Vero this is 2–3 days, and for L cells it is 1–2 days. These data suggest that the apparent quicker recovery of over-all RNA synthesis in Vero as compared to L cells is merely quantitative, but that qualitative recovery, as judged by the restoration of apparent protein synthesis and cell division, takes much longer.

#### DISCUSSION

As we (1, 2) and others (3, 4) have noted, Vero cells can recover their capacity to incorporate radiolabeled uridine much faster than most other cell types after removal of inhibiting concentrations of AMD. This occurs at doses even exceeding those that fully suppress transcription. The mechanism responsible for the rapid detachment and elimination of AMD by Vero, as compared with HeLa or L cells is unknown. However, the rapid recovery of over-all RNA synthesis in Vero can only partly be explained by the remarkable capacity of these cells to eliminate bound AMD. It appears that cells derived from kidney of the African green monkey (*Cercopithecus*) have the capacity to recover RNA synthesis rapidly after its suppression by AMD, and are also relatively resistant to the toxicity of AMD (1, 2, 3, 7). Their resistance to cell injury is not due solely to their smaller uptake or more rapid release of AMD (1). Even when Vero cells are forced to bind more AMD than L and HeLa cells, they can nevertheless eliminate the drug, and restore RNA synthesis rapidly. Therefore, the relative resistance of Vero to AMD is not due to any diminished

capacity of these cells to bind AMD, nor does it result from metabolic inactivation of the drug by the Vero cell. We have observed that the AMD eliminated from Vero after having been bound can inhibit RNA synthesis in HeLa cells (unpublished observation). Voll and Leive (8) have isolated a mutant *Escherichia coli* which also eliminates bound AMD, recovers RNA synthesis more rapidly, and is more resistant to the lethal effects of AMD than its parent. The AMD is eliminated in a form indistinguishable by chromatography from authentic AMD. Schwartz et al. (9) have also reported that the AMD eliminated from liver tissue is unchanged. Whatever the mechanism that enables Vero to eliminate AMD so rapidly, it is not related to metabolic alteration of the AMD.

Although Vero are more proficient in excreting the bound drug, elimination of AMD is by no means the only important factor governing recovery of RNA synthesis after it has been suppressed by the drug, and, although removal of the drug from DNA is a precondition of recovery of transcription, it is not a sufficient condition. The capacity of Vero cells to incorporate labeled uridine is restored proportionately more slowly after removal of a given concentration of AMD after progressively longer exposure periods (Fig. 4A), even though periods of exposure longer than 2 hr do not result in a further increase in the amount of AMD in the cell (Fig. 5), and notwithstanding that the rate of drug elimination is independent of the length of exposure (Fig. 6). Furthermore, Vero cells, although exposed to ten times or more the concentration of AMD than L cells (with proportionately greater uptake by the Vero), still recover RNA synthesis much more rapidly than do L cells treated with lower doses. The binding of AMD to cells is clearly a reversible process (1, 9, 10, 11), but the effect of the drug on transcription is of considerably longer duration than would be expected from the length of the period of retention of the drug. Therefore, recovery of transcription must depend on other factors in addition to the release of AMD from the cell. Although Vero cells eliminate AMD rapidly and restore over-all RNA synthesis within a few hours after the removal of concentrations sufficient to inhibit transcription by more than 90%, a stationary period of 2–3 days ensues during which very few or no mitoses occur, but after which cell division resumes. This phenomenon of division-delay is observed in both Vero and L cells; however, L cells, which cannot instaurate over-all RNA synthesis as rapidly as

Vero, nevertheless resume cell division more promptly than Vero. It is of interest that the mutant *E. coli* described by Voll and Leive (8), which could eliminate AMD and restore transcription rapidly, apparently also suffered a period of division-delay. During the interval of division-delay and resurgent RNA synthesis after AMD, there is a parallel failure of premitotic semiconservative DNA synthesis (data to be published). After cessation of transcription the cells are stopped from progressing in the division cycle. Present evidence (see 12, 13) supports the concept that some species of RNA, which are rapidly lost after abolition of RNA synthesis, are required for nuclear activation leading to division. Production of these species of RNA is relatively resistant to AMD, because pulse exposure to high concentrations is required to induce more than transient division in Vero.

If RNA synthesis in Vero is blocked less than completely, i.e. by exposure to concentration of AMD less than 1  $\mu\text{g}/\text{ml}$ , a shorter period of division-delay ensues than when transcription is fully suppressed at concentrations greater than 1  $\mu\text{g}/\text{ml}$ . Concentrations in excess of those that extinguish RNA synthesis do not cause a concentration-dependent lengthening of division-delay. It would appear, therefore, that resynthesis of certain species of RNA, transcription of which is inhibited only by high doses of AMD, is required for cells to reenter the division cycle after suppression by AMD. Cell division is almost immediately restored after removal of low concentrations of AMD in contrast to the effects of high concentrations, which produce a long period of division-delay. We propose that RNA species, synthesis of which is relatively resistant to extinction by AMD, are involved either directly (14) or indirectly (15, 16) in the activation of genes controlling progression of the cell cycle and cell division. It is postulated that these RNA species continue to be synthesized during treatment with low, but not high, concentrations of AMD, and therefore those genes whose products are required for cell division are reactivated immediately after removing low drug concentrations. After relief of inhibition by high concentrations, however, the prolonged period of division-delay that ensues is at least partly attributable to the time required for resynthesis of RNAs necessary for the reactivation of genes whose products in turn govern the reentry of the cells into the division cycle. The fact that Vero treated for 2 hr with 2.5  $\mu\text{g}/\text{ml}$  suffer a lag in cell division of 2 days

after over-all uridine incorporation into RNA has returned to the control level, suggests that restoration of the qualitatively correct RNA-dependent sequence of steps in gene activation (13), rather than the over-all rate of RNA synthesis, determines when the cells will reenter the division cycle after treatment with AMD. In this regard, Schluenderberg and her colleagues (3) have reported that after removal of high concentrations of AMD from Vero a normal cytoplasmic profile of the major classes of labeled RNA does not reappear until after 48 hr. This finding and our data on resumption of protein synthesis indicate that while the over-all rate of RNA synthesis has come back to a normal level within 24 hr, the spectrum of RNAs synthesized is qualitatively abnormal or unbalanced.

It is a reasonable hypothesis that reinitiation of RNA synthesis after its suppression requires the operation of certain cytoplasmic and nuclear "factors", among them probably certain proteins of the class of the acidic chromosomal protein (13, 16). After the suppression of RNA synthesis these factors and their templates must be presumed to decay at variable rates, the rate depending on the cell type. Suppression of transcription for a sufficient time may eventuate in a state of nuclear repression similar to that, for example, of the nucleated avian erythrocyte in which the nucleus, although inactive, is capable of being reactivated by fusion with an active proliferating cell such as HeLa (17). The reactivation of chick-erythrocyte nuclei in heterokaryons composed of erythrocyte nuclei and HeLa cells is thought to be triggered by the migration of proteins from the HeLa cytoplasm into the erythrocyte nucleus (18). This is one among many examples and experiments demonstrating that factors (presumably proteins) fabricated or present in the cytoplasm control the activity of the nucleus (19, 20, 21); and the renewal of these depends in turn upon RNA synthesis. The reactivation of RNA synthesis after elimination of AMD from animal cells may, therefore, depend upon the stability of such factors, i.e. proteins or their templates, necessary for the initiation of transcription; and as these factors decay, during inhibition by AMD, faster in some cell types than others, the rate at which transcription recovers will progressively diminish. This hypothesis will be the subject of experiments to be reported later.

Actinomycin D, an antibiotic whose primary effect on cells has been so amply elucidated, can be a valuable probe for examining the processes of

gene reactivation after suppression of transcription, especially in Vero cells, because these cells are relatively resistant to the cytotoxicity of this agent.

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