Degradation of RNA in Liver of Rats Treated with Actinomycin D

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SUMMARY

Actinomycin D (2 mg/kg) was given to rats after administration of a labeled RNA precursor (orotic-6-^{14}C acid); thereafter, labeled RNA was extracted from nuclear and cytoplasmic fractions of liver and sedimented in sucrose gradients. Following treatment with actinomycin, newly synthesized RNA (i.e., labeled 40 min or less before the drug was given) was lost and labeled RNA failed to appear in the cytoplasm. This loss also occurred in adrenalectomized rats. There was no apparent loss of older RNA (i.e., labeled 3 hr before drug) or massive destruction of unlabeled ribosomes and polysomes. The results suggest that the transfer of labeled RNA from the nucleus to the cytoplasm was blocked because of destruction of precursors of ribosomal RNA. Since these and other forms of RNA are synthesized in the nucleus, it is likely that the loss occurred here, possibly at nucleolar sites which are thought to participate in ribosomal synthesis.

INTRODUCTION

Actinomycin D inhibits DNA-directed synthesis of RNA with remarkable specificity. The rapid onset of maximal inhibition with this antibiotic has made it feasible to study turnover of RNA in bacteria (1–5) and in mammalian cells in culture (6–11). However, few such studies have been reported in animals. Previous workers (12, 13) have described net losses of nuclear RNA in liver of rats after treatment with actinomycin; the evidence presented here indicates that it is rapidly labeled nuclear RNA which is lost, presumably in the form of ribosomal precursors. Further, the drug blocks the appearance of newly labeled RNA in the cytoplasm. These results have added to our understanding of the synthesis and stability of RNA in rat liver; even more significantly, these studies provide a model which may explain the selective cytotoxicity of actinomycin D in sensitive tumors.

MATERIALS AND METHODS

Young, mature, male Sprague-Dawley rats (180–225 g) of the Charles River CD line were used intact or 4–5 days after adrenalectomy (maintained with free access to 1% NaCl solution). Actinomycin D was generously provided by Merck, Sharp & Dohme Research Laboratories. Stock solutions contained actinomycin D (200 μg/ml) in 0.9% NaCl and animals were given 1 ml/100 g body weight intravenously (2 mg/kg) under light ether anesthesia. Control rats were given saline. Orotic-6-^{14}C acid (8 μC/μmole), purchased from New England Nuclear Corporation, was dissolved in a slight excess of 0.1 M NaOH and diluted with isotonic saline and phosphate buffer (0.01 M, pH 7.0) to a final concentration of 40 μC/ml. One milliliter per kilogram of body weight was given intravenously to prelabel RNA.

RNA was isolated from both nuclei and cytoplasm of rat liver by methods described by DiGirolamo *et al.* (14) and modified slightly in this laboratory (15). Nuclear fractions were sedimented and washed twice ($600 \times g$ for 10 min) in 0.25 M sucrose, 0.003 M $CaCl_2$, and 0.03 Tris buffer at pH 7.6. Cytoplasmic fractions were obtained from the upper two-thirds of the super-

natant of the first sedimentation of nuclei. Aqueous suspensions of nuclei and cytoplasm were extracted 3 times with 90% phenol containing 1% sodium lauryl sulfate and 0.5% naphthalene disulfonate. During each extraction, samples were shaken vigorously in bottles that were placed within plastic bags packed with crushed ice. After centrifugation of the extracts, aqueous and interphase components were collected as described by DiGirolamo *et al.* (14). Removal of DNA and oligonucleotides from nuclear RNA, and precipitation and washing of both cytoplasmic and nuclear RNA were also as described. In addition, however, final preparations were washed 3 times with cold ethanol to ensure removal of traces of naphthalene disulfonate. Isolated nuclear and cytoplasmic RNA was dissolved in 0.1 M NaCl and 0.01 M sodium acetate, pH 5.0. Approximately 12 optical density units (260 mμ) in a volume of 1 ml was layered over a 27 ml 5–20% linear sucrose gradient (prepared in 0.1 M NaCl–0.01 M Na acetate). Tubes were centrifuged (Beckman, Spinco, Model L2) for 17 hours at 18,000 rpm (5°C) in the SW 25.1 rotor. Twenty-drop fractions (approximately 0.75 ml) were collected automatically from a syringe-type needle inserted into the bottom of each tube. Fractions were diluted with 0.5 ml H_2O and assayed for absorption at 260 mμ and for radioactivity in a liquid scintillation counter after precipitation of RNA on Millipore filters with 100 μg bovine serum albumin and 5% trichloroacetic acid. To minimize variability, livers from two rats were combined for each determination. Sedimentation coefficients are those used by DiGirolamo *et al.* (14).

In experiments with adrenalectomized rats, liver DNA and RNA were assayed by methods described by Schneider (16). Whole homogenates were precipitated and washed 4 times with cold 10% trichloroacetic acid, defatted, and extracted twice with hot 10% trichloroacetic acid (15 min at 90°). Extracts were washed 3 times with ether in preparation for liquid scintillation counting and for colorimetric determination of RNA (orcinol reaction) and DNA (diphenylamine). Specific activities were calculated as disintegrations per minute (dpm)/optical density of orcinol-reactive material. Ratios of RNA:DNA were calculated only as optical densities of orcinol-reactive to diphenylamine-reactive materials in the hot trichloroacetic acid extracts.

RESULTS

Isolated RNA. Orotic-6-^{14}C acid was given intravenously to rats which were killed 5, 10, 20, or 40 min later. These animals received no further treatment and are designated as controls. A second series of animals was also given orotic acid, and at 5, 10, 20, or 40 min they were injected with either saline solution or actinomycin D. The rats in this series were killed after an additional 175, 170, 160, or 140 min, so that the total time of exposure to the radioactive label was 180 min. In a third series, animals were given orotic acid; at 180 min some were killed (controls) and others were injected with saline or drug and sacrificed 140 min later. RNA from nuclei and cytoplasm of pooled liver homogenates was isolated as described in Materials and Methods, and centrifuged on sucrose gradients.

Figure 1 shows profiles of nuclear and cytoplasmic RNA isolated from controls at 40 min after injection of labeled orotic acid. The radioactivity of the nuclear RNA was disperse though there was evidence of early peaking in the regions of 35–45 S (tubes 10–15), 28 S (tubes 17–24), 18 S (tubes 25–29), 5–10 S (tubes 30–32) and 4 S and smaller (tubes 33 and higher). In the 28 S and 18 S regions, the peaks of early radioactivity did not correspond exactly with the optical peaks, indicating that as newly formed 28 S and 18 S RNA organized they sedimented slightly faster than older 28 S and 18 S. By contrast with the nuclear fraction, the cytoplasmic RNA (Fig. 1D and Table 1) had relatively little radioactivity at 40 min and it contained a prominent 4 S optical density peak.

Radioactivity was lost from the nuclear fraction in animals receiving actinomycin at 40 min after orotic acid (compare Fig. 1B with Figs. 1A and 1C). Despite the loss of more than half the activity, optical peaks

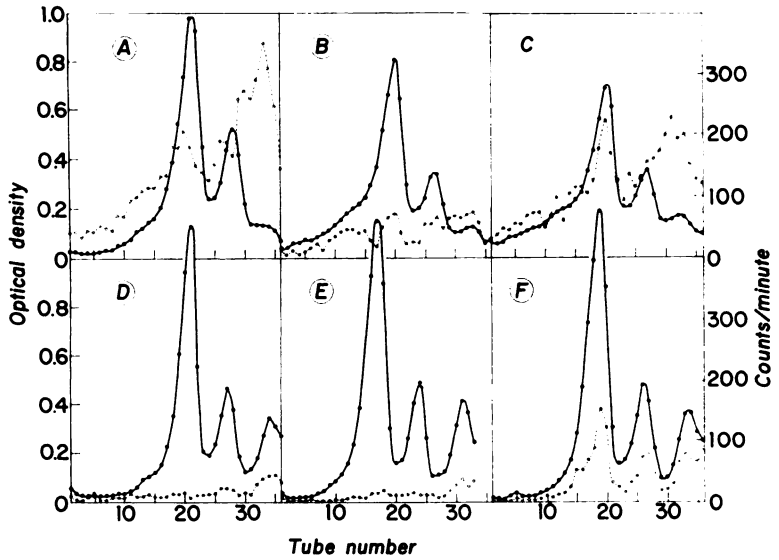

FIG. 1. *Sedimentation profiles of phenol-extracted RNA in 5–20% sucrose gradients*

Liver RNA was prepared from nuclear (A, B, C) and cytoplasmic fractions (D, E, F). Control rats were labeled with orotic-6-^{14}C acid for 40 min and killed (A, D). Experimental animals were similarly labeled, then given either actinomycin D (B, E) or saline (C, F) and killed 140 min later. Filled circles show optical density at 260 mμ; open circles show radioactivity of each 20-drop fraction.

TABLE 1

Cytoplasmic RNA from liver of control and saline and actinomycin D-treated rats

Subjects	Time (min) of		Fractions[a]		
	Incorporation	Chase	28S	18S	4S
Control	5	—	1.18	0.42	0.27
Act. D	5	175	1.25	0.55	0.32[b]
Sal	5	175	**169**	**223**	**314**
Control	10	—	0.95	0.40	0.19
Act. D	10	170	1.12	0.57	0.31[b]
Sal	10	170	**200**	**232**	**382**
Control	20	—	1.23	0.64	**97**
Act. D	20	160	1.35	0.63[b]	**97**
Sal	20	160	**159**	**215**	**234**
Act. D	20	160	1.02	0.43	0.33[b]
Sal	20	160	**194**	**254**	**385**
Control	40	—	1.11	0.47[b]	**124**
Act. D	40	140	1.15	0.49[b]	**93**
Sal	40	140	**86**	**154**	**183**
Control	180	—	**131**	**198**	**306**
Act. D	180	140	**217**	**173**	**354**
Sal	180	140	**356**	**330**	**573**
Act. D	180	140	**206**	**236**	**928**
Sal	180	140	**392**	**454**	**699**
Sal	180	140	**340**	**255**	**496**

[a] Boldface designates cpm/$OD_{260\ m\mu}$; others indicate $OD_{260\ m\mu}$ where the cpm were less than 15 cpm above background (background was 26–32 cpm for all experiments).

[b] Cpm ranged from 16 to 37 above background.

at 28 S and 18 S showed little change. In liver cytoplasm optical peaks were also unaltered in rats treated with the drug but these remained unlabeled (compare Fig. 1E with Figs. 1D and 1F). Thus there was a loss of newly formed nuclear RNA and an inhibition of the transfer of this RNA to the cytoplasm.

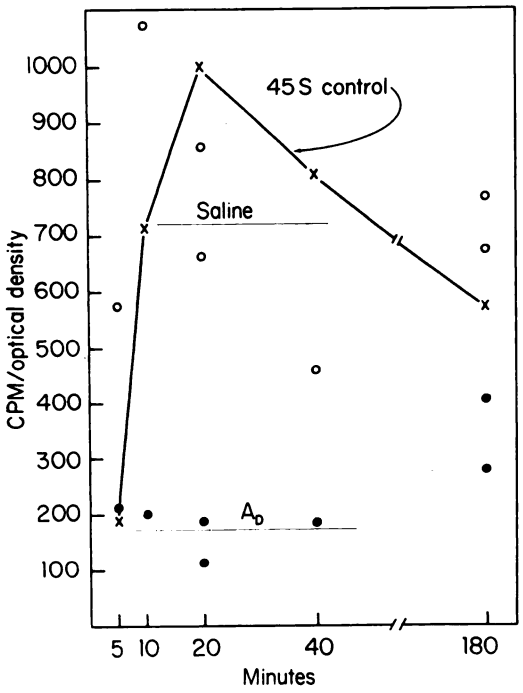

FIG. 2. *Specific activity of nuclear 45 S RNA from livers of control rats* (×), *and of experimental rats given actinomycin* (●) *or saline* (○)

Data were calculated from sedimentation profiles such as those shown in Fig. 1. Abscissa indicates the duration of labeling time before control rats were killed or before experimental rats were given actinomycin or saline. Total labeling time for experimental animals was 180 min for those prelabeled 40 min or less and 320 min for those prelabeled 180 min. Horizontal bars indicate average specific activity for all actinomycin and saline-treated animals prelabeled 40 min or less.

Similar results were obtained when actinomycin was given at 5, 10, and 20 min after orotic acid. These are shown in Figs. 2 and 3 where the changes in nuclear RNA are illustrated separately at 45 S, 28 S, and 18 S. In the controls 45 S RNA (Fig. 2) was maximally labeled by 10–20 min after orotic acid while 28 S and 18 S RNA (Fig. 3) had steady increases in specific activity up to 320 min. When actinomycin was given at 5 min after orotic acid, it prevented the expected increases in specific activity seen in controls and saline-treated rats. When it was given at 10, 20, or 40 min, it caused a loss of label to the level found in 5-min controls. When the injection of the antibiotic was delayed until 180 min, there was

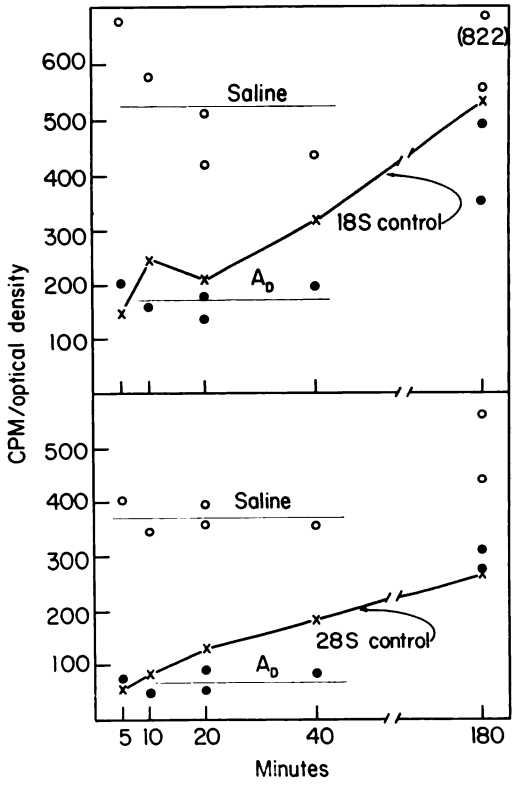

FIG. 3. *Specific activities of nuclear 18 S and 28 S RNA*

See Fig. 2 for description.

little or no significant loss of labeled nuclear 18 S and 28 S RNA. At this time, both nuclear and cytoplasmic components are organized into units which seem more resistant to degradation than do the newer forms of labeled RNA.

Table 1 lists analyses of cytoplasmic RNA from animals described above. The data show that little or no specific labeling appeared in cytoplasmic 28 S and 18 S RNA

of control animals during the first 40 min of incorporation. By 180 min after orotic acid this RNA was well labeled and gradient density profiles showed that optical and radioactivity peaks were in close register. When actinomycin was given to rats at any time up to 40 min after orotic acid, it prevented the expected labeling of ribosomal RNA in the cytoplasm. Since the radioactivity of RNA in the 4 S region was not increased over that of controls or that of saline-treated rats, it is unlikely that the antibiotic had caused degradation of cytoplasmic RNA.

This was also shown by the fact that actinomycin did not induce loss of radioactivity from cytoplasmic ribosomal RNA. Thus, at 180 min after orotic acid when 28 S and 18 S RNA were already labeled in the cytoplasm, the administration of actinomycin did not reduce the radioactivity of these ribosomal moieties. It did, of course, prevent the further accumulation of label which occurred in the animals which had been treated with saline at 180 min (see Table 1 and Fig. 4).

In other experiments (not shown here) liver polysomes were collected from rats killed 180 min after receiving actinomycin D (2 mg/kg) and tested in the gradients described by Staehelin (17) and Revel (18) and their co-workers. The results confirmed those of the earlier workers: there was only a small shift from the polymeric (8 or more) units to dimers and monomers during the 3-hr period. Ribosomal monomers and dimers were also isolated from nuclear fractions of livers of rats according to the methods described by Howell *et al.* (19). Such preparations are presumably contaminated with endoplasmic reticulum. At 180 min after actinomycin (2 mg/kg) there was no evidence of altered sedimentation patterns of ribosomal material in these crude preparations. Similar results were obtained with ribosomes from nuclear fractions given 3 extra centrifugations in the same medium described by Howell *et al.* (19) but to which 1% carboxymethylcellulose was added. Finally, we obtained no evidence in preliminary experiments that treatment with actinomycin increased ribonuclease activity in either liver nuclei or cytoplasm. This is consistent with the reported finding that nuclear ribonuclease is active in liver even in the absence of the drug (13) and suggests that there is a normal degradation of nuclear RNA which

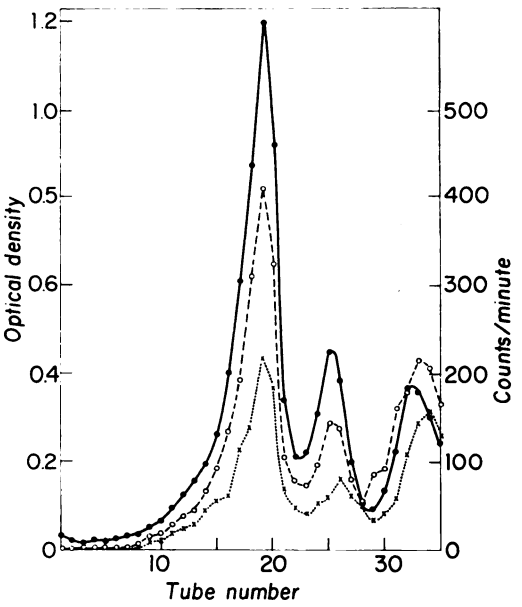

FIG. 4. *Composite sedimentation profiles of cytoplasmic RNA prelabeled 180 min*

Filled circles show optical density of RNA isolated from control rats. Open circles and crosses indicate radioactivity of RNA from liver of saline and actinomycin-treated rats, respectively, chased for 140 min after prelabeling for 180 min. The composite was fitted only at the 28 S maxima of each optical density curve (shown here as tube 19). Radioactivity of the 180-min control, which is not shown, was almost identical with that from rats given actinomycin.

is uncovered when agents such as actinomycin D, ethionine (13) or aflatoxin (20) inhibit RNA synthesis. However, the evidence presented here indicated that there could not have been extensive degradation of older RNA in nucleus or cytoplasm after the drug was given.

In recent studies we noted evidence of stimulated adrenal corticoid secretions in animals treated with actinomycin (21). It seemed possible that an increased rate of turnover of nuclear RNA might occur

TABLE 2

Effects of actinomycin D on 40 min prelabeled RNA in liver of adrenalectomized rats

There were six rats in each group; A_D (2 mg/kg); saline and orotic acid were given intravenously.

Conditions	Time of sacrifice	Specific activity ± S.D.	RNA:DNA ± S.D.
Control, 6-^{14}C-orotic acid	40 min	1980 ± 340[a]	20.6 ± 1.23[b]
Prelabel 40 min:			
Act. D for 140 min	3 hr	1100 ± 140[a]	18.5 ± 1.21[b]
Saline for 140 min	3 hr	3390 ± 480[a]	19.8 ± 3.30[b]

[a] Differences between groups are significant at $P < 0.001$.

[b] Differences between groups are not significant: $P > 0.1$.

secondary to such stimulation, and for this reason we tested adrenalectomized rats. However, Table 2 indicates that there was extensive loss of newly synthesized RNA even though the bulk of the RNA was conserved after treatment with the antibiotic. Other adrenalectomized rats were killed 40 min after labeled orotic acid was given, and nuclear and cytoplasmic RNA from their livers were extracted with phenol and analyzed in sucrose gradients. The results were similar to those obtained with intact rats (Fig. 1A and D) and indicated that, in the adrenalectomized animals reported in Table 2, newly synthesized RNA was localized in nuclear fractions when actinomycin was given 40 min after orotic acid.

DISCUSSION

After actinomycin D is administered to rats, there is a loss of newly formed nuclear RNA in the liver. The loss may be the consequence of changes in nucleolar function that occurs in the presence of this agent (22, 23). Nucleolar morphology is altered by actinomycin within 1 hr in intact liver (24, 25) and within 3 hr in regenerating liver (26, 27). Since nucleoli seem to be instrumental in the formation of ribosomal RNA from 18 S, 28 S, and 45 S precursors (11), it is reasonable to speculate that damage to the organelle could result in loss of labeled ribosomal precursors due to the activity of nucleolar ribonuclease (28). However, it is not yet known whether nucleolar damage results from a proximal action by actinomycin D on this organelle or whether the change in its morphology is a secondary consequence of inhibition of RNA synthesis.

There were two forms of RNA which were not degraded after treatment with actinomycin: RNA already present in ribosomes and nuclear RNA labeled for only 5 min prior to administration of the drug. Ribosomal RNA appears to be stable because it is relatively resistant to ribonuclease, but stability of 5-min labeled RNA is not so readily explained. It is known that RNA synthesis is not completely inhibited in rat liver even after high doses of actinomycin. For example, in our previous studies of this agent (21), lethal doses ($2 \times LD_{50}$ or 1 mg/kg) were only partially and reversibly effective in inhibiting the incorporation of labeled orotic acid: 84–95% inhibition at 30 and 60 min after treatment, 75% or less inhibition at 2 and 4 hr, and recovery by 16 hr. In the current experiments, even with the higher dose used (2 mg/kg), small amounts of incorporation may have occurred. If so, the apparent stability of the 5-min labeled RNA could have been due to continuing turnover of RNA on limited portions of the DNA template or to a more general synthesis of RNA that had been slowed by the presence of bound actinomycin. However, it is also possible that the actinomycin-resistant radioactivity was present in incomplete chains of DNA-associated RNA, the growth of which was blocked by the presence of the inhibitor. Although DNA–RNA hybrids from liver are not notably resistant to ribonuclease when tested *in vitro* (29), they might be stable in chromatin in cells if

nuclear ribonuclease is confined to the nucleolus (11, 12).

RNA that was labeled 40 min or less did not appear in cytoplasm after actinomycin was administered. This observation differs from those of Darnell (6, 7) and Perry (11) and their co-workers with HeLa and L cells, respectively. They observed transfer of ribosomes or ribosomal precursors (8) into the cytoplasm in the presence of high concentrations of the inhibitor. However, Harris (9, 10) found degradation of nuclear RNA in HeLa cells treated with actinomycin and suggested that the transfer observed by others (6, 7) may have been due to incomplete inhibition of RNA synthesis in the presence of large pools of labeled RNA precursors. Even though inhibition of synthesis is presumably incomplete in liver of treated rats, we found little or no evidence of migration of labeled 18 S and 28 S RNA to the cytoplasm in liver. Newly synthesized RNA seems to be degraded within the nucleus and, therefore, never reaches the cytoplasm.

It is also possible, however, that newly synthesized RNA is destroyed in the cytoplasm, upon emerging from the nucleus but before acquiring a more stable ribosomal configuration. In HeLa cells, for example, the initial event in ribosomal synthesis is the formation of 45 S RNA in nuclei. This RNA then forms two components, 16 S and 35 S (7, 30). The 16 S component appears rapidly in the cytoplasm while the 35 S unit remains in the nucleus. Subsequently 35 S is transformed into 28 S and is then found in the cytoplasm. In these cells, actinomycin leads to a loss of 45 S, but with the accompanying formation of cytoplasmic 16 S and nuclear 35 S RNA. In liver (see Fig. 1B) approximately the same amounts of activity are found in the regions of 45 S, 28 S, and 18 S RNA after treatment with the agent. It is likely, of course, that the timetable for the emergence of RNA from nuclei is different for HeLa cells than it is for liver, but we have been so far unable to find conditions which would indicate separate entry of 18 S and 28 S into liver cytoplasm from the nucleus. If such 18 S and 28 S particles do enter the cytoplasm separately but in constant ratios, it is possible that cytoplasmic degradation might occur before the component RNA units are stabilized as ribosomes. This question is currently under investigation. However, the point remains that we are not able to demonstrate migration of labeled RNA into liver cytoplasm, even when RNA synthesis is probably only partially inhibited by actinomycin. Without compelling evidence to the contrary, it seems preferable at the present time to assume that ribosomes are formed in the nucleus (presumably under control of the nucleolus) and that destruction of ribosomal precursors occurs here rather than in the cytoplasm.

The response to actinomycin in liver includes not only production of cytotoxicity after inhibition of nucleic acid synthesis (21, 31), but also degradation of newly synthesized RNA and concurrent interference with the formation of ribosomes. Indeed, degradative loss of RNA seems more closely related to actinomycin's tumoricidal action than does inhibition of RNA synthesis. This relationship has been demonstrated in current studies with Ridgway osteogenic sarcoma in mice treated with a single, sublethal, curative dose of actinomycin (32). Within 24 hr, this tumor loses almost half of its total RNA but with only partial inhibition of RNA synthesis and little or no net loss of DNA or protein (33). Thus the effects described in liver may be a model that will contribute to our understanding of the selective cytotoxicity of this agent in sensitive tumors.

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