DEGRADATION OF RIBONUCLEIC ACID IN MOUSE FIBROBLASTS TREATED WITH ACTINOMYCIN

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ABSTRACT

The cellular RNA content of mouse fibroblasts incubated with actinomycin decreases at a rate of about 1 to 1.5 per cent per hour, while DNA and protein content remain unchanged. This degradation affects nuclear and cytoplasmic RNA, ribosomal and soluble RNA. The breakdown products appear quantitatively in the acid-soluble fraction of the cells and the medium. Polynucleotides synthesized a short period (120 minutes) prior to exposure to actinomycin are degraded before those synthesized 8 to 12 hours previously.

When mammalian cells are exposed for many hours to concentrations of actinomycin which completely block RNA synthesis, much of their cytochemically demonstrable RNA disappears, the ratio cellular DNA/RNA increases, and the morphology of their nucleoli is altered (1, 2). In order to obtain further information concerning the physiology of actinomycin-treated cells, we have studied the RNA metabolism of mouse fibroblasts (strain L-929) in culture, and the changes caused by actinomycin. The results show that cells in which RNA synthesis is suppressed by the antibiotic degrade a large percentage of their RNA. The process of depolymerization affects all cellular RNA species of the nucleus and of the cytoplasm.

MATERIALS AND METHODS

Mouse fibroblasts were grown as monolayers in minimal Eagle's medium (3) supplemented with 5 per cent fetal bovine serum, or in spinner bottles as suspension cultures in Eagle's spinner medium (4) containing 10 per cent fetal bovine serum. Cells from monolayer cultures were harvested, after brief exposure to trypsin (0.4 mg/ml), by centrifugation in a refrigerated centrifuge, and washed twice with cold saline. All counts were performed in a hemocytometer. Cells were separated into nuclei and cytoplasm by the method of Harris (5), modified by extending the period of incubation in detergent to 20 minutes. The nuclei obtained by this method appeared free of cytoplasm when examined with the oil-immersion lens of a phase-contrast microscope; they contained 98 to 99 per cent of the cellular DNA as judged by their content of previously incorporated radioactive thymidine in acid-insoluble form. After separation of the nuclei by centrifugation at 300 *g* for 5 minutes, the supernatant was centrifuged at 10,000 *g* for 15 minutes to remove mitochondria, and the ribosomes were isolated by centrifugation at 150,000 *g* for 180 minutes.

Radioactive precursors were obtained from regular commercial sources. Radioactivity of cell fractions and extracts was measured in a scintillation counter.

RNA was purified from whole cells, or from cell fractions, by a phenol method (6), in the presence of polyvinyl sulfate. After removal of phenol, the RNA was precipitated by 2 volumes of ethanol, redissolved and reprecipitated twice, and analyzed chromatographically on columns of methylated serum albumin-kieselguhr (7), or in sucrose gradients (8). The absorbancy (260 mμ) and radioactivity of aliquots of fractions were measured. To measure nucleic acid and protein, cells were extracted and washed with perchloric acid and subjected to Schmidt-Thannhauser fractionation (9). RNA was then determined by the orcinol method (10), DNA by the diphenylamine technique (11), and protein by the Lowry method

TABLE I

Effect of Actinomycin on Polymer Composition of Mouse Fibroblasts

Period of incubation with actinomycin (1 μg/ml)	RNA	DNA	RNA/DNA	Protein
hrs.	*μg/10⁷ cells*	*μg/10⁷ cells*		*μg/10⁷ cells*
0	366	225	1.62	742
24	214	211	1.02	732
48	127	222	0.57	730

Experiments performed with cells growing as monolayers.

(12). In some experiments the nucleic acid content and specific radioactivity of whole cell RNA and DNA were determined as follows: the washed cells were extracted and washed with cold $HClO_4$ until the washings were free of radioactivity and UV-absorbing material. The RNA was then degraded by alkali (0.5 N KOH, 18 hours, 37°C) and measured, following precipitation of the DNA by $HClO_4$, by UV-absorption or orcinol, an aliquot being taken for estimation of radioactivity. The precipitated residue was washed with 0.25 N $HClO_4$ until the washings were free of radioactivity and UV-absorbing material, and hydrolyzed with $HClO_4$ (0.5 N, 70°, 15 minutes) to solubilize the DNA. DNA content was then measured by UV-absorption or by the diphenylamine method, an aliquot serving for measurement of radioactivity.

Nucleotide composition of alkaline hydrolysates of RNA fractions was determined by two-dimensional chromatography on paper (13).

Actinomycin was generously provided by Merck, Sharp, and Dohme, Inc., West Point, Pennsylvania. Unless otherwise specified, it was used at a concentration of 1 μg/ml. At this concentration, and with the cell population density used in our experiments (1×10^6/ml), RNA synthesis is suppressed by more than 95 per cent within 15 minutes.

RESULTS

Mouse fibroblast monolayers incubated with actinomycin (1 μg/ml) lose RNA, but not DNA or protein, for 48 hours (Table I). In the experiment shown, approximately ⅔ of the cellular RNA present at the time of addition of actinomycin was lost after 48 hours. The proportion of RNA lost in this way varied, in different experiments, from 40 to 70 per cent of that originally present. The data of many experiments suggest that more cellular RNA was lost from younger cultures whose cells had a higher initial content of RNA, but this point was not investigated systematically.

The RNA is degraded to acid-soluble nucleotides whose structure has not been fully characterized. After 24 hours the degradation products are found almost entirely in a greatly expanded

TABLE II

Effect of Actinomycin on the Ribonucleic Acid Content in Subcellular Fractions of Mouse Fibroblasts

Period of incubation with actinomycin (1 μg/ml)	Nuclear RNA	Ribosomal RNA	Soluble RNA	DNA
hrs.		*μg/10⁷ cells*		
0	40	212	64	211
24	14	125	41	210

Experiment performed with cells growing as monolayers. Analysis for DNA was performed prior to rupture. Soluble RNA is defined as the acid-precipitable RNA remaining in the postribosomal supernatant fraction.

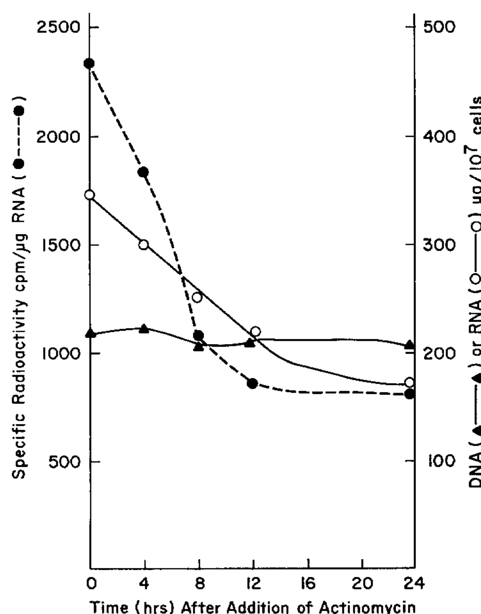

FIGURE 1 Effect of actinomycin on breakdown of RNA. After exposure to H^3-guanosine (1 μg/ml, 0.25 μc/ml) for 1 hour, a control sample was removed, and actinomycin (1 μg/ml) was added to the remainder. Cells harvested at the times shown were analyzed for DNA and RNA, and the specific activity of RNA was determined as described in Materials and Methods.

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acid-soluble intracellular pool; after 48 hours, the acid-soluble material is distributed approximately equally between the medium and the intracellular pool.

Fibroblasts in suspension cultures tolerate actinomycin less satisfactorily than do monolayers. Although the rate of loss of RNA is comparable in the two types of cultures, cells in spinner cultures begin to lose DNA and nuclear material after 18 to 24 hours, whereas no such loss is seen in monolayers before 48 hours.

The degradative process which occurs in the presence of actinomycin affects the RNA from all cellular fractions (Table II), although the loss from the nuclear fraction is slightly greater, and that from the soluble fraction slightly less than the average.

pulse-labeled many hours before actinomycin addition, should *rise* in the presence of the antibiotic; whereas the reverse (*i.e.* a drop in specific activity of RNA) would be expected for cells exposed to radioactive precursors immediately before incubation with actinomycin. The data in Table III show that this expectation is fulfilled.

It appeared possible that in the course of its degradation, RNA of high molecular weight might give rise to detectable amounts of polynucleotide species of intermediate size. To test this possibility, the total RNA of several control cultures and of cells exposed to actinomycin for 24 hours was analyzed by chromatography on methylated albumin-kieselguhr. The chromatographic profiles of the two preparations were

TABLE III

Effect of Time of Addition of Actinomycin on Loss of Previously Incorporated H^3-Guanosine from RNA

	Actinomycin added at end of labeling period			Actinomycin added 12 hrs. after labeling		
	0 hr.	24 hrs.	48 hrs.	0 hr.	24 hrs.	48 hrs.
CPM/μg RNA	1220	650	580	1280	1730	2180
RNA, $\mu g/10^7$ *cells*	312	194	136	307	187	129

A group of monolayer cultures was exposed to H^3-guanosine (1 μg/ml; 0.25 μc/ml) for an hour, washed with non-radioactive guanosine, and divided into two lots. After removal of a control sample, actinomycin (1 μg/ml) was added to the first lot. The second lot was permitted to grow for 12 hours, at which time another control culture was removed, and actinomycin (1 μg/ml) was added to the remainder. The content of RNA and residual radioactivity of RNA in cultures of each lot were determined after the indicated periods of incubation with actinomycin.

Several types of experiments have been performed to determine whether there might be any preferential breakdown of RNA based on the length of time intervening between the period of synthesis and the onset of exposure to actinomycin. The results of one experiment are shown in Fig. 1. It can be seen that the specific activity of RNA decreased at a more rapid rate than did the total cellular RNA content, particularly during the earliest times following addition of actinomycin. This finding suggests that the RNA molecules synthesized immediately prior to the exposure to actinomycin are degraded preferentially. Another type of experiment was performed to test this possibility. It is assumed that "young" polynucleotides are relatively unstable in the presence of actinomycin, and that "old" polynucleotides are relatively stable. If this were so, the specific radioactivity of RNA,

qualitatively very similar, the only difference being the presence of a slightly higher proportion of soluble RNA in the actinomycin-treated sample. No accumulation of RNA species was observed in the size range between soluble and ribosomal RNA, and the width of the peak corresponding to soluble RNA was the same for RNA from both types of cultures.

In an attempt to increase the resolving power of the method, several analyses were performed on RNA extracted from cells grown in the presence of H^3-guanosine. Here again no evidence could be obtained for the existence of degradation products of intermediate size. Finally, after incubation at 37°, pH 8.5, for 30 minutes to hydrolyze amino acyl esters already present, RNA from the peaks corresponding to the soluble fraction of both preparations was assayed for the ability to accept amino acids. (The activating

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TABLE IV

Acceptor Activity of s-RNA After Treatment of L Cells with Actinomycin

Precursor	Time of incubation with actinomycin (1 μg/ml)	CPM/1 OD 260
	hrs.	
A C^{14} algae hydrolysate	0	4559
	24	4799
C^{14} leucine	0	934
	24	1010
B C^{14} leucine	0	1320
	24	965
C C^{14} algae hydrolysate	0	4760
	24	4490
	0	4220
	24	3950

Experiment A: s-RNA was isolated from a methylated albumin-kieselguhr column after phenol extraction of the 105,000 *g* supernatant. Experiments B and C: s-RNA was isolated from a methylated albumin-kieselguhr column after hot phenol extraction of the whole cells. The activating enzyme preparation consisted of the 100,000 *g* supernatant of a rat liver homogenate. Each reaction mixture contained 1 ml: 50 μmoles Tris-HCl buffer pH 7.2, 10 μmoles $MgCl_2$, 50 μmoles KCl, 2 μmoles ATP (adenosine triphosphate), 5 μmoles PEP (phosphoenolpyruvate), 2 μg PEPkinase, 2 μc of C^{14} algae hydrolysate or C^{14} leucine 10 μc/μmole.

enzyme preparation in this case consisted of pH 5 enzymes from rat liver and from *Escherichia coli.*) The specific acceptor activity for amino acids of the two preparations was identical (Table IV). We conclude that the breakdown of high molecular weight RNA in actinomycin-treated cells gives rise to no detectable accumulation of polynucleotides whose size would permit retention by columns of methylated albumin.

Nucleotide analyses of total cell RNA and RNA from cellular fractions have also been performed with the aim of detecting any preferential deggradative activity. As seen in Table V, the overall nucleotide composition of total RNA of actinomycin-treated cells did not differ from the controls; the same was true of the nucleotide composition of the individual fractions obtained by differential centrifugation of cell extracts prepared by the method of Harris (see Materials and Methods).

In view of the extensive degradation of ribosomal RNA, it appeared possible that changes in the sedimenting behavior of ribosomes from actinomycin-treated cells might be observed. This would be expected particularly if the breakdown of one of the species of ribosomal RNA initiated ribosomal disintegration. To test this possibility, a culture of fibroblasts was exposed to a pulse (1 hour) of H^3-guanosine and permitted to grow for 24 hours prior to the addition of actinomycin. After a further period of 24 hours in the presence of actinomycin the cells were harvested; a large excess of non-radioactive cells was added as carrier, and ribosomes were prepared and then analyzed by centrifugation in sucrose density gradients. The results (Fig. 2) show that the profile of radioactivity corresponds closely to that of UV-absorption. There is no evidence for the existence of an appreciable quantity of partially degraded ribosomes sedimenting at densities lower than those corresponding to the major peak obtained from normal cells. When the same experiment was repeated using RNA extracted from ribosomes, the peaks of radioactivity coincided with those of UV-absorption.

Protein Synthesis in Actinomycin-Treated Cells

At the concentration of actinomycin employed in these experiments, protein synthesis falls rap-

TABLE V

Nucleotide Composition of Total RNA of Mouse Fibroblasts

In moles per 100 moles nucleotide

	Normal L cells	After 24 hrs. in the presence of actinomycin	48 hrs. in the presence of actinomycin
Adenylic acid	20.1	18.7	19.6
Guanylic acid	33.1	34.2	33.6
Cytidylic acid	28.8	29.2	29.1
Uridylic acid	18.0	17.8	17.6
$\frac{A + U^*}{G + C}$	0.62	0.58	0.59
$\frac{6 - Am}{6 - K}$	0.96	0.92	0.95

* Abbreviations: A, adenylic acid; U, uridylic acid; G, guanylic acid; C, cytidylic acid; 6–Am, 6-amino nucleotides; 6–K, 6-keto nucleotides.

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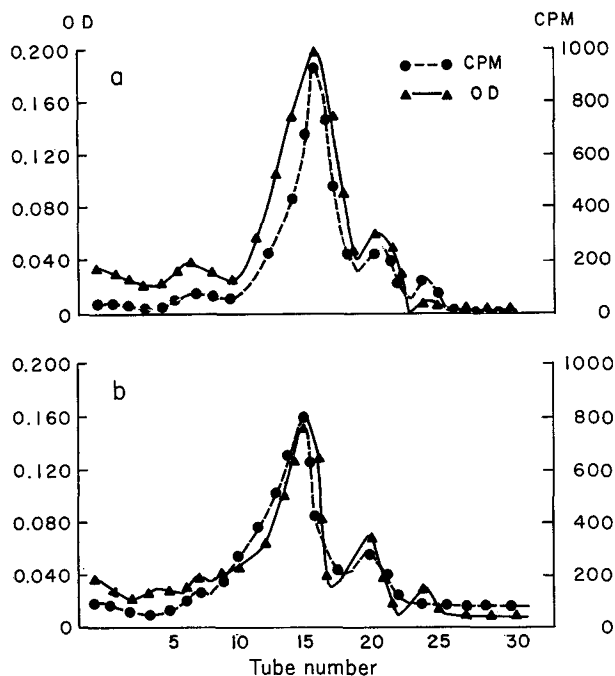

FIGURE 2 Sucrose gradient analysis (linear gradient 5 to 20 per cent, 25,000 RPM, 12 hours, in SW 25 rotor of Spinco centrifuge Model L) of radioactive ribosomes (*a*) from normal cells, and (*b*) from cells treated with actinomycin (1 μg/ml) for 24 hours. Radioactivity (●—●), A260, O.D. 260 mμ (▲—▲).

idly, especially after 4 hours (14). Using the incorporation of radioactive lysine into hot acid-insoluble material as a measure of the rate of protein synthesis, we have found that protein formation in cells exposed to actinomycin for 24 and 48 hours is reduced to 7 to 15 and 4 to 7 per cent, respectively, of that in the exponentially growing control culture. The site of lysine incorporation in these cells was examined by radioautography to establish whether nuclear or cytoplasmic incorporation had been suppressed preferentially; there was no significant change in the grain distribution pattern although the total level of incorporation was greatly reduced in comparison with controls.

DISCUSSION

The results presented above show that the arrest of RNA synthesis caused by actinomycin is associated with the progressive loss of a major proportion of the cell's RNA. This loss, which leads to the corresponding accumulation of acid-soluble nucleotide material, affects the RNA of the nucleus and of all size classes in the cytoplasm. The present data do not permit any conclusion to be drawn concerning the nature of the breakdown process, which could be accounted for by alternate interpretations: (*a*) all species of cellular RNA are in constant metabolic turnover, and actinomycin, by preventing resynthesis, unmasks and does not impede the normal breakdown; or (*b*) actinomycin in some unknown way activates a depolymerizing activity, not normally present, in the manner previously observed in bacteria (15). Further work will be required to distinguish between these alternatives.

It can be recalled that actinomycin-treated, RNA-depleted cells effectively support the growth of Mengovirus (14). Such cells may, therefore, prove useful for the investigation of some aspects of cellular physiology.

It is of interest to compare the present findings with those reported previously by workers in other laboratories (16–20). These investigators observed the behavior of cellular RNA metabolism, principally during a few hours' exposure to actinomycin. Their findings revealed (*a*) that the flow of RNA radioactivity from nucleus to cytoplasm was interrupted by actinomycin, and (*b*) that nuclear RNA, or at least its most recently labeled molecules, was rapidly degraded in the presence of actinomycin, which we have confirmed. In contrast, our experiments show that during long periods (24 to 48 hours) of incubation with actinomycin, all species of cellular RNA, nuclear as well as cytoplasmic, are degraded in the ab-

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sence of RNA synthesis. When actinomycin is added following a brief exposure to radioactive precursors, the initial rapid drop in specific radioactivity of RNA (Fig. 1) probably corresponds to the breakdown of the rapidly labeled fraction. The succeeding depolymerization is associated with a proportional decrease of radioactivity, suggesting random loss of the remaining polynucleotides. The basis of this difference in rate of degradation of the two classes of RNA molecules remains obscure.

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