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Breakdown of Pulse-Labeled Ribonucleic Acid in *Bacillus megaterium*, Revealed by Exposure to the Antibiotics Mithramycin, Chromomycin, and Nogalamycin

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SUMMARY

The decay of pulse-labeled RNA in *Bacillus megaterium* in the presence of actinomycin D, mithramycin, chromomycin A_3, nogalamycin, and daunomycin was investigated. Actinomycin (10 μg/ml) was found to induce decay characterized by a half-life ($t_{1/2}$) of 50 ± 1.4 sec and a stable fraction of 45 ± 1.0% (eight determinations), comparable with estimates obtained by other workers. Concentrations of mithramycin between 5 and 100 μg/ml were found to induce decay. The values of $t_{1/2}$ and the stable fraction decreased with increasing mithramycin concentration, approaching values similar to those found with actinomycin. Chromomycin at concentrations between 2 and 50 μg/ml produced decay, and at intermediate concentrations the $t_{1/2}$ and stable fraction approximated the values given by actinomycin.

Nogalamycin at concentrations in the range 8–50 μg/ml induced decay characterized by $t_{1/2}$ values significantly higher than those found with actinomycin, though values of the stable fraction at concentrations up to 20 μg/ml were essentially the same as seen with actinomycin. RNA synthesis was inhibited by daunomycin at concentrations up to 80 μg/ml, revealing slight decays, but at 160 μg/ml inhibition of RNA synthesis without perceptible decay was observed.

It is concluded that the parameters of decay induced by mithramycin and chromomycin corroborate those observed with actinomycin, but that nogalamycin yields significantly different results and that daunomycin is not a suitable antibiotic for studying decay of pulse-labeled RNA. Possible explanations for the failure of nogalamycin and daunomycin to induce actinomycin-like decay, such as slow penetration of the antibiotics into the cells, binding to RNA, or direct interference with protein synthesis, are discussed.

INTRODUCTION

The fate of newly synthesized RNA in growing bacteria can be studied by pulse-labeling experiments (1–7). A culture is exposed to [^{3}H]uridine of high specific activity, and after a short labeling period incorporation into RNA is terminated by addition of an inhibitor of RNA synthesis. Part of the pulse-labeled RNA is metabolically unstable and is degraded during the succeeding few minutes to acid-soluble products, while the remainder is stable and persists in acid-insoluble form. The decay of the unstable fraction follows a more or less exponential time course, so that the whole process can

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be characterized (within the limits of accuracy imposed by the experimental methods) in terms of two parameters: the *stable fraction* and the *half-life* ($t_{1/2}$) of the unstable fraction.

Experiments of this nature demand prompt and complete inhibition of incorporation of the labeled precursor. In addition, the validity of any conclusions about the nature and significance of stable and unstable RNA rests on assumptions about the specificity of the inhibitor of RNA synthesis. Side effects on DNA synthesis are probably unimportant, but any interference with the metabolism of RNA other than simple inhibition of its synthesis may be expected to lead to aberrant decay of the unstable RNA. It is known, for instance, that interference with protein synthesis at the level of the ribosome (by addition of agents such as chloramphenicol or tetracyclines) "protects" much of the unstable RNA from decay, leading to a higher apparent stable fraction (7, 8). Such findings are consistent with the supposition that mRNA constitutes a sizable proportion of the unstable RNA, and that its breakdown is normally coupled to active protein synthesis (5–8).

The agent of choice as a specific inhibitor of RNA synthesis is actinomycin D [reviewed by Reich and Goldberg (9)]. Although its extreme selectivity for DNA-directed RNA synthesis is widely accepted, nevertheless it would be useful to have independent evidence that the decay of pulse-labeled RNA revealed by actinomycin treatment (1, 3–7) results simply from inhibition of synthesis, uncomplicated by any drug-dependent secondary effect on RNA metabolism. Two simple tests can be applied to study this problem. First, the inhibitor concentration can be increased from the minimum required to inhibit synthesis up to much higher doses, in which case the influence of any secondary effects should become more obvious. Second, the effects of diverse drugs which share the common property of inhibiting RNA synthesis can be compared. Confidence in the meaningfulness of decay characteristics would be greatly enhanced if the parameters of the decay process were found to be similar irrespective of the nature and concentration of the inhibitor used.

These approaches have been tried by Grinsted (6), who found the $t_{1/2}$ and stable fraction values to be independent of actinomycin concentration within the range 2–75 μg/ml. However, his investigations with two other inhibitors of RNA synthesis, proflavine and ethidium, revealed a strong dependence of decay parameters on the inhibitor concentration—presumably reflecting direct interference with RNA breakdown. Grinsted and McQuillen (10) also investigated the effect of rifampicin, which prevents initiation by bacterial RNA polymerase (11), and noted decay of pulse-labeled RNA, but the results were not comparable with those produced by actinomycin because of the relatively slow inhibition of [^{3}H]uridine incorporation by rifampicin.

We have pursued the problem further, using four antibiotics which, like actinomycin, bind to DNA so as to inhibit RNA synthesis but are chemically unrelated to actinomycin and hence should be unlikely to display the same secondary effects, if any. Chromomycin A_3 and mithramycin are related antibiotics which share a common chromophore (Fig. 1); their binding to DNA (12–15) results in very powerful inhibition of RNA synthesis, but the nature of their interaction with DNA is clearly different from that of actinomycin (16). Nogalamycin and daunomycin (Fig. 1) belong to a different (anthracycline) group of antibiotics, and, like actinomycin, their binding to DNA (12, 13, 17, 18) occurs by intercalation (16). Using these antibiotics, we have asked the following questions: (a) whether decay of pulse-labeled RNA induced by these inhibitors can be observed, (b) whether the parameters of such decay are related in any way to the antibiotic concentration, and (c) whether such parameters agree with those found using actinomycin.

MATERIALS AND METHODS

Antibiotics. Chromomycin A_3 was purchased from Calbiochem. The other antibiotics were gifts (to M. W.) from Dr. T. J. McBride, Chas. Pfizer & Company, Inc., Maywood, N. J. (mithramycin); Dr. P. F.

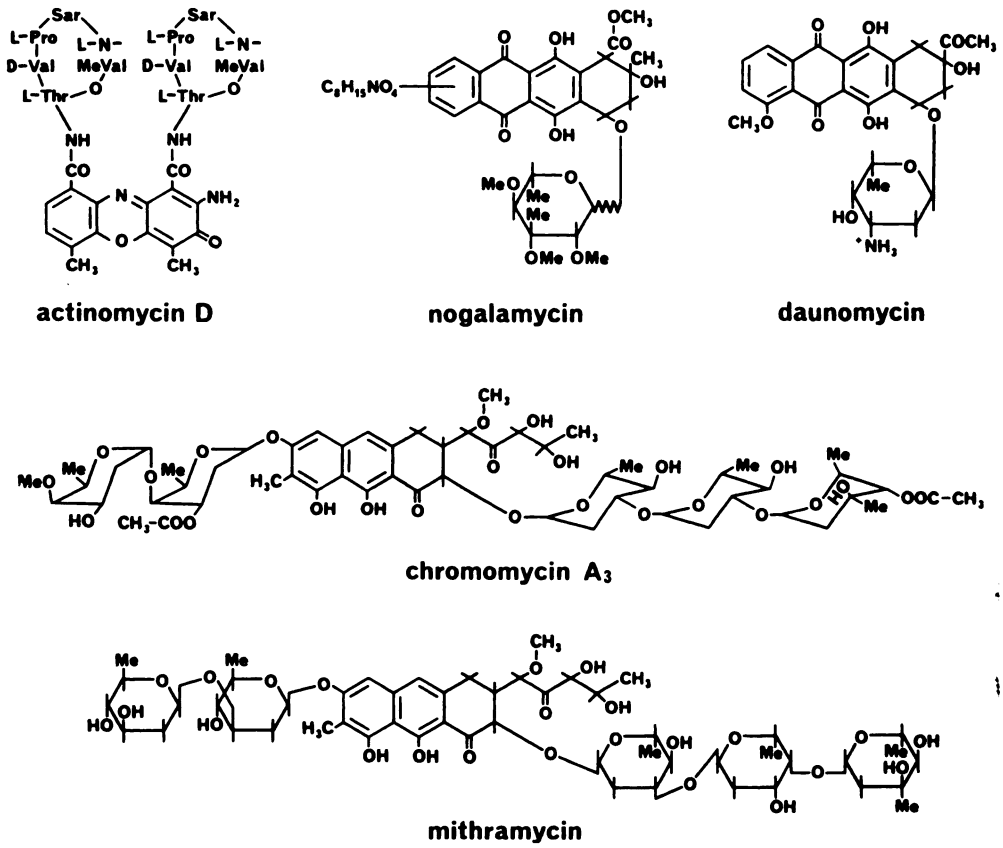

FIG. 1. *Structural formulae of antibiotics which inhibit RNA synthesis*

The chromophore shown for nogalamycin is one of several possibilities having different arrangements of the aromatic hydroxyl groups; it is substituted by the sugar nogalarose and a $C_8H_{15}NO_4$ moiety thought to represent an amino sugar (P. F. Wiley, personal communication).

Wiley, the Upjohn Company, Kalamazoo, Mich. (nogalamycin); Dr. B. Camerino, Farmitalia, Milan, Italy (daunomycin); and Professor E. F. Gale, Department of Biochemistry, University of Cambridge (actinomycin D; a product of Merck Sharp & Dohme). Stock antibiotic solutions containing 1 or 2 mg/ml were prepared in ethanol (actinomycin, chromomycin, nogalamycin) or in glass-distilled water (mithramycin, daunomycin). All solutions were protected from light and were freshly prepared whenever possible; otherwise they were stored in the dark at −23°. Controls were performed to verify that the presence of alcohol in some of the experiments did not noticeably affect decay characteristics of pulse-labeled RNA.

Organism. *Bacillus megaterium* strain KM, maintained freeze-dried in this laboratory, was used.

Growth of cultures. Stock cultures were established from the freeze-dried organism in the minimal salts medium (C medium) of McQuillen and Roberts (19) supplemented with 0.1% (w/v) glucose and incubated at 37°. The organism was subcultured daily into fresh C medium plus 0.1% glucose. Purity was checked from time to time by plating and microscopic examination.

Experimental cultures were set up by centrifuging an overnight 5-ml stock culture at 4000 rpm for 2 min, resuspending the bacterial pellet in 15 ml of C medium supplemented with 1% (w/v) glucose and 0.1% (w/v) peptone (Difco), and incubating in a 100-ml conical flask in a shaking water bath at 37°. Growth was followed turbidimetric-

ally by measuring the optical density at 600 mμ with a 1-cm light path in a Unicam SP 500 series II spectrophotometer. As soon as the optical density reached 0.2–0.25, corresponding to about 3×10^7 cells/ml, a few milliliters of the culture were transferred into another 100-ml flask containing fresh, warmed C medium, 1% glucose, and 0.1% peptone to yield a total volume of 15 ml. The flask to culture volume ratio was chosen to ensure that the liquid was spread sufficiently thinly to allow efficient aeration upon shaking, while still permitting samples to be taken in rapid succession. Under these conditions the doubling time was 21 min. Experimental cultures were grown through at least four or five generations of fully exponential growth before experiments were commenced, at which time the optical density was planned to reach 0.15–0.18, corresponding to 0.06–0.08 mg (dry weight) of cells per milliliter, or about 2×10^7 cells/ml.

Pulse labeling. At experimental time zero minus 30 sec, 0.1 ml of [5-^{3}H]uridine (The Radiochemical Centre, Amersham; specific activity, 29.8 Ci/mmole) was added to the 15-ml culture, giving a final activity of 6 μCi/ml. The antibiotic was added at zero time. Samples (nominally 0.5 ml) were withdrawn from the flask with a specially adapted syringe. Three samples were taken during the 30-sec labeling period; 12 during the 60 sec immediately following drug addition; six between 70 and 120 sec; and nine more at intervals up to 720 sec (12 min) after addition of the antibiotic. Samples were squirted into 2 × ½-inch glass vials containing 2 ml of ice-cold 7% (w/v) trichloracetic acid and immediately vortex-mixed. The vials plus trichloracetic acid had previously been weighed, so that from a second weighing at the end of the experiment the exact weights of the samples taken could be calculated. Correction could thus be made for sampling errors, which generally fell within ±10%. The samples were then kept cold until filtration, normally within 1–2 hr. The contents of each vial were filtered thrugh a Whatman No. GF/C 2.5-cm glass fiber disc and washed twice with 4 ml of cold 7% trichloracetic acid followed by 4 ml of cold 1% (v/v) acetic acid. The filters were heat-dried and counted in a Nuclear-Chicago liquid scintillation counter, using a fluid containing 0.4% (w/v) Butyl-PBD (2-(4′-t-Butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole; Ciba) in sulfur-free toluene.

Under these conditions of labeling it has been shown that tritiated uridine acts as a specific precursor of RNA (6, 8). It was also verified that varying the concentration of trichloracetic acid used in the precipitation and washing procedures between 2 and 10% did not affect the results, nor did allowing samples to stand at 0–2° for several hours longer than the 1–2 hr routinely allowed.

Treatment of results. Counts per minute recorded for each sample were corrected to counts per minute per 0.5-g sample and then plotted against the sampling time. From this decay curve the peak incorporation and stable counts per minute were estimated, yielding the stable fraction (a percentage).

The half-life of breakdown of the unstable pulse-labeled RNA ($t_{1/2}$) was computed on a PDP/8L computer, using a program written by Dr. R. W. King. Given the counts per minute in RNA at a given time, it subtracted the counts which ultimately remained stable, calculated the $\log_{10}$ of the unstable counts, and printed the values in a table. When given a value less than 30% larger than the stable counts per minute, it was programmed to reject any further data and to compute a least-squares plot of $\log_{10}$ (unstable counts) with respect to time, weighting each point by a factor proportional to the calculated unstable counts per minute. (This method of weighting gives greatest weight to high points, and least to low points, which are small differences due to the subtraction of two large numbers and therefore inherently less accurate.) The computer specified the coordinates of its least-squares fitted line, together with the rate constant of decay and exponential half-life of the unstable RNA.

RESULTS

Actinomycin D. Figure 2 shows the effect of actinomycin at a final concentration of 10 μg/ml. In this experiment 46% of the pulse-labeled RNA remained stable and the half-life of decay of the unstable RNA was 51 sec. These parameters are representative of a

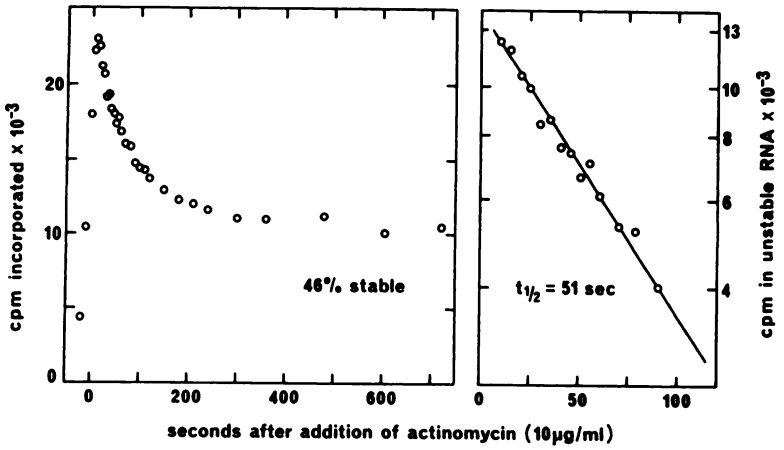

FIG. 2. *Breakdown of pulse-labeled RNA in the presence of actinomycin*
The culture was pulse-labeled with [³H]uridine for 30 sec as described under MATERIALS AND METHODS. At left, the total trichloroacetic acid-insoluble radioactivity is plotted. At right, the same data after subtraction of the counts per minute in stable RNA (10,700 cpm) are plotted on a semilogarithmic scale. The line, corresponding to the half-life shown, is computer-drawn (see the text).

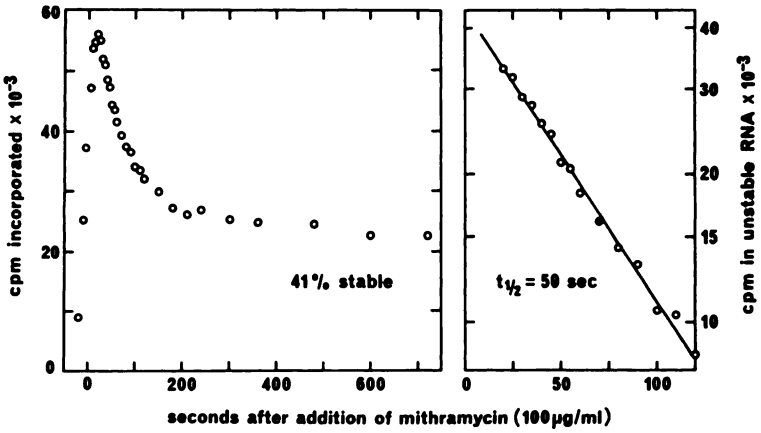

FIG. 3. *Breakdown of pulse-labeled RNA in the presence of mithramycin*
Details are described in the legend to Fig. 2.

number of determinations; in eight similar experiments the stable fraction was 45 ± 1.0% and the half-life of decay was 50 ± 1.4 sec (values are means ± standard errors). During the 30-sec pulse in Fig. 2 approximately 20,000 cpm were incorporated into RNA per 0.5-g sample, representing 1–2% utilization of the [³H]uridine added. The peak incorporation was found to be somewhat variable among different experiments (cf. Figs. 2–7), but, as the low percentage utilization would suggest, this was not related in any way to exhaustion of the added precursor, since in untreated cultures incorporation continued at a linear rate for at least 60 sec. Stable fraction percentage values were always referred to the incorporation observed at the peak. One factor which contributes to determination of the actual peak value is the rapidity of inhibition of [³H]uridine incorporation. With actinomycin the onset of decay was usually apparent by 5 sec after addition of the drug, but this was not always the case in experiments with other antibiotics.

Experiments using actinomycin at final

concentrations of 5 and 20 μg/ml yielded results comparable with those quoted above; thus 10 μg of actinomycin per milliliter is in the "plateau" region, where the parameters of pulse-labeled RNA behavior are independent of actinomycin concentration (6).

Mithramycin. A range of mithramycin concentrations between 5 and 100 μg/ml was tested, and one example at the highest concentration is shown in Fig. 3. In this case the stable fraction was 41% and $t_{1/2}$ was 50 sec. The general similarity to the results with actinomycin (Fig. 2) is evident. At all the concentrations of mithramycin tested, decay of pulse-labeled RNA could be demonstrated, but the stable fraction and $t_{1/2}$ values were not constant. Both decreased as the antibiotic concentration was increased (Fig. 4), tending toward values similar to those found with actinomycin, which were effectively reached at 80–100 μg of mithramycin per milliliter.

The onset of decay was generally slower than seen with actinomycin, commencing 15–30 sec after addition of mithramycin. The time to onset of decay was inversely related to the antibiotic concentration, which would suggest that slow penetration into the cells must be taken into account in interpreting the mithramycin results (see DISCUSSION).

Chromomycin A_3. The effects of this antibiotic were investigated at concentrations from 2 to 50 μg/ml. Figure 5 shows the breakdown of pulse-labeled RNA in the presence of chromomycin at 5 μg/ml. Here $t_{1/2}$ = 51 sec and 41% of the labeled RNA is stable, again mimicking the effects of actinomycin. The time to onset of decay was 10–15 sec after addition of the antibiotic (Fig. 5), and with other concentrations of chromomycin the same phenomenon seen with mithramycin was noted; i.e., the higher the antibiotic concentration, the shorter the

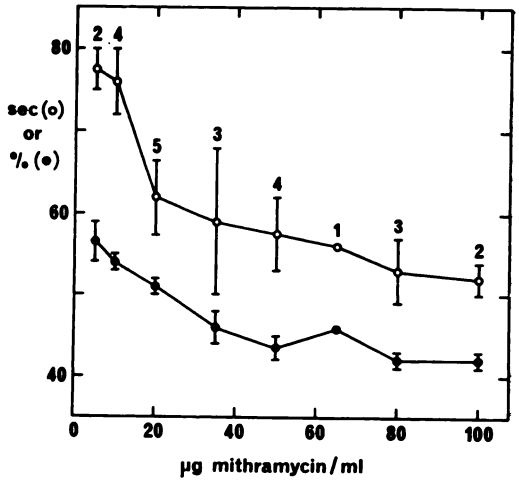

FIG. 4. *Dependence of parameters of decay of pulse-labeled RNA on mithramycin concentration*
○, $t_{1/2}$; ●, stable fraction. The same numerical ordinate is used for both parameters. Bars represent standard errors, and figures show the number of experiments at each antibiotic concentration.

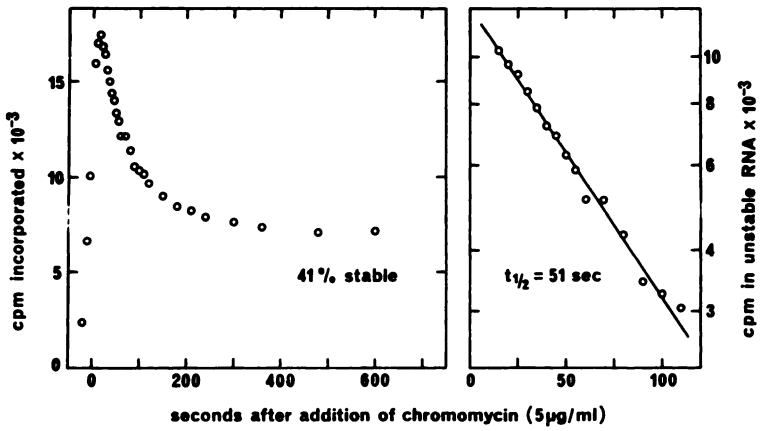

FIG. 5. *Breakdown of pulse-labeled RNA in the presence of chromomycin*
Details are described in the legend to Fig. 2.

time to onset of decay (25 sec at 2 μg/ml, decreasing to less than 5 sec at 50 μg/ml).

In Fig. 6 the relationship between the parameters of decay and chromomycin concentration is plotted. There is a plateau region between 5 and 20 μg/ml, where the $t_{1/2}$ and stable fraction values are constant and approximately equal to those found with actinomycin. Above and below this concentration range the stable fraction remains much the same but there is a significant increase in the observed half-life of decay.

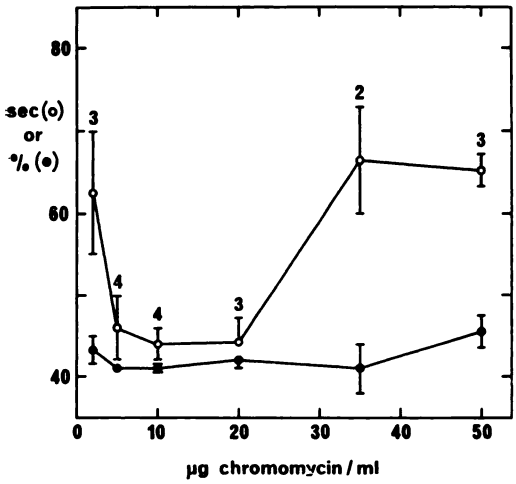

FIG. 6. *Dependence of parameters of decay of pulse-labeled RNA on chromomycin concentration*
○, $t_{1/2}$; ●, stable fraction. The same numerical ordinate is used for both parameters. Bars represent standard errors, and figures show the number of experiments at each antibiotic concentration.

Nogalamycin. Breakdown of pulse-labeled RNA was observed in the presence of nogalamycin at concentrations between 8 and 50 μg/ml (Fig. 7). At 3 μg/ml the action of the antibiotic proved too slow to be useful: [^{3}H]uridine incorporation was progressively slowed but was not halted until 60 sec after addition of the nogalamycin. Thereafter there was a small but definite decay, with an apparently stable fraction in the region of 70%.

Table 1 summarizes the characteristics of decay at four concentrations of nogalamycin. It can be seen that with the exception of 50 μg of nogalamycin per milliliter, the stable percentage values were of the same magnitude as seen with actinomycin, mithramycin, and chromomycin, but the rates of breakdown were considerably slower.

Daunomycin. Four concentrations of this antibiotic were studied: 35, 50, 80, and 160 μg/ml. A result at 50 μg/ml is shown in Fig. 8. The curve shows in exaggerated form the same features observed with 3 μg/ml of nogalamycin, i.e., a long time to onset of decay (100 sec) and a high stable fraction (63% at 720 sec). Results at 35 and 80 μg/ml were similar. It appears that daunomycin is a much less prompt and/or effective inhibitor of [^{3}H]uridine incorporation than the other antibiotics studied, but the experience with nogalamycin suggested

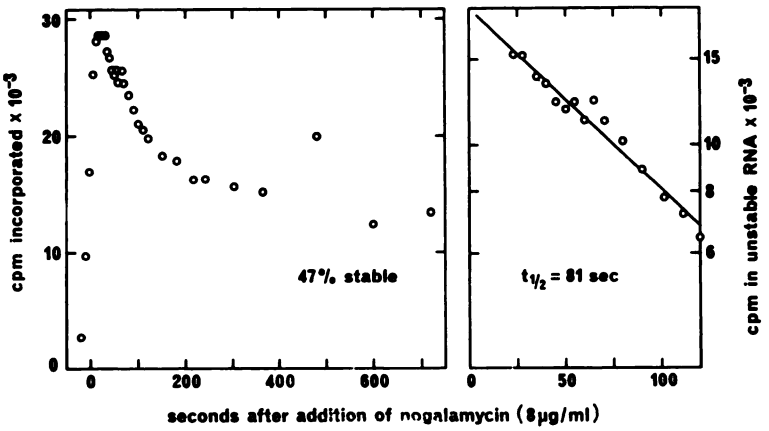

FIG. 7. *Breakdown of pulse-labeled RNA in the presence of nogalamycin*
Details are described in the legend to Fig. 2.

TABLE 1

Characteristics of breakdown of pulse-labeled RNA in the presence of nogalamycin

Antibiotic concentration	Time to onset of decay	$t_{1/2}$	Stable fraction
μg/ml	*sec*	*sec*	%
8	23	81	47
10	11	83	47
20	<5	82	43
50	<5	92	61

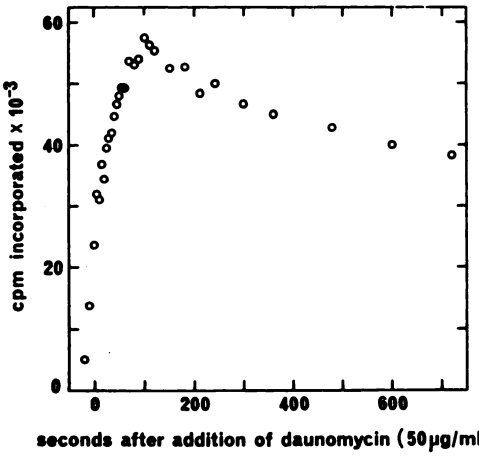

FIG. 8. *Effect of daunomycin on incorporation of* [^{3}H]*uridine*

The labeled precursor was added 30 sec prior to addition of daunomycin. Values plotted are total radioactivity incorporated into acid-insoluble material.

that actinomycin-like decays might still be observable at even higher daunomycin concentrations. This proved not to be the case, for although at 160 μg/ml the incorporation was halted within 10–15 sec, none of the labeled RNA broke down at all.

DISCUSSION

The major technical limitation in determining accurate curves for the breakdown of pulse-labeled RNA lies in sampling errors which stem from the need to take successive samples from a rapidly shaking culture at intervals of 5 sec or less. By the simple expedient of weighing to correct for sampling errors we have minimized this problem and eliminated much of the scatter seen in the data of earlier workers. It is therefore gratifying to note that all our curves could be fitted quite well by a single exponential decay (cf. Figs. 2–7).

In terms of the three questions posed in the INTRODUCTION, our results may be summarized as follows. (a) Breakdown of pulse-labeled RNA can be observed when RNA synthesis is inhibited by mithramycin, chromomycin, and nogalamycin, and, to a limited extent, in the presence of daunomycin. (b) With mithramycin, chromomycin, and nogalamycin, concentration ranges exist within which the parameters of decay are essentially independent of the antibiotic concentration. (c) At the "plateau" antibiotic concentrations the $t_{1/2}$ and stable fraction are similar to those found with actinomycin, except for the longer half-lives produced by nogalamycin. Thus the criteria which an antibiotic must satisfy if it is to provide useful data on the metabolism of pulse-labeled RNA (see INTRODUCTION) are best met by mithramycin and chromomycin. The fact that they reproduce the parameters of decay seen with actinomycin adds weight to the belief that these parameters are a true reflection of events resulting solely from inhibition of RNA synthesis. Mithramycin and chromomycin are the first antibiotics which have been shown to reproduce exactly the effects of actinomycin on pulse-labeled RNA under comparable conditions. The actual parameters measured here are in agreement with those reported by Schaechter and McQuillen (3), using actinomycin with the same organism and culture conditions.

With all the antibiotics, concentrations below the plateau range produce much the same pattern of effects: a long time to onset of decay and higher values for $t_{1/2}$ and the stable fraction. Two possible explanations may be considered. First, the antibiotic might fail to stop incorporation completely, so that a low rate of continuing RNA synthesis is superimposed on the over-all decay. This would have the effect of slowing the observed decay and yield a higher apparent stable fraction, although a net increase in trichloracetic acid-insoluble radioactivity might be expected after longer intervals. Effects of this type were observed by Grinsted (6) with 1 μg/ml of actinomycin, and are to

be expected with any antibiotic at sufficiently low concentration. Second, the antibiotic might stop incorporation completely, but not sufficiently rapidly, so that the effective pulse-labeling time is greater than 30 sec. It is known that increasing the pulse time results in a slower decay and a rise in the stable fraction (3, 8). Although both explanations might apply, we favor a dominant role for the latter: it accords well with our findings (a) that the time to onset of decay is inversely related to the antibiotic concentration, suggestive of slow permeation into the cell, and (b) that the effects are much more marked with mithramycin and daunomycin than with their less water-soluble analogues chromomycin and nogalamycin, whose more lipophilic character may facilitate their uptake by the cell. We attempted to investigate the possibility that RNA synthesis might continue after addition of antibiotics by adding the antibiotic and [^{3}H]uridine simultaneously. The results were inconclusive (miniature decays were usually observed) and would in any event have been difficult to interpret because the specific activities of nucleotide pools under these conditions could hardly have been comparable to specific activities after a 30-sec pulse.

The rise in $t_{1/2}$ seen at higher chromomycin concentrations, and the higher $t_{1/2}$ values consistently produced by nogalamycin, are more difficult to explain. It is possible that they reflect interaction of the antibiotics with RNA or direct interference with protein synthesis, which probably explains the similar effects reported with proflavine and ethidium (6), for the concentrations used here are much higher than those which have been studied by other workers (15, 18). At these high concentrations a more generalized effect on cell metabolism is also possible. In any event it is likely that the same phenomena, perhaps in more acute form, are responsible for the failure of daunomycin to reveal clear decay of RNA; the curve in Fig. 8 could well be interpreted as incipient decay of a sizable fraction of the RNA, with a grossly extended half-life. Since daunomycin is known to be capable of interacting with RNA (12, 17), it is easy to imagine that significant binding to mRNA might occur and hence offer protection against degradation. If so, a substantial effect on protein synthesis would be expected. Investigations into the effects of daunomycin, and of the other antibiotics, on amino acid incorporation and polyribosome stability would clearly be of interest.

Although not the primary concern of this paper, some mention should be made of the significance of the stable and unstable RNA fractions. It is generally agreed that newly synthesized (mature) rRNAs and tRNA contribute to the stable fraction and that mRNA accounts for a substantial portion of the unstable fraction, but there is reason to doubt the simplest interpretation that these species of RNA are neatly divided between one fraction or the other (3–6, 20). Zimmerman and Levinthal (4) showed that in *Bacillus subtilis* the pulse radioactivity in the stable RNA was entirely in rRNA and tRNA, and that the unstable fraction exhibited the heterodisperse sedimentation characteristics expected of mRNA, but that no incomplete rRNA remained after actinomycin treatment. It is probable, therefore, that breakdown of incomplete rRNA and tRNA molecules contributes importantly to the unstable fraction of pulse-labeled RNA (4, 6). The recent discovery of precursor rRNAs (21) and tRNA (22) adds a new dimension to the problem, however, for the "extra" sequences present in these RNAs are cleaved off during the maturation process and must presumably contribute to the unstable fraction. It is of interest that mithramycin has been reported to inhibit selectively the synthesis of rRNA (15), but at concentrations orders of magnitude lower than are required to demonstrate decay of pulse-labeled RNA.

Our experiments extend previous studies of mithramycin, chromomycin, nogalamycin, and daunomycin as potentially useful tools in the investigation of macromolecular synthesis (23). Some of these antibiotics should prove to be useful alternatives to actinomycin as powerful inhibitors of RNA synthesis in biological systems where, for one reason or another, the use of actinomycin is not possible, or where it is necessary to seek confirmation of actinomycin-dependent phenomena.

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