MOLECULAR PHARMACOLOGY, 10, 214-224

Breakdown of Pulse-Labeled Ribonucleic Acid and Polysomes in *Bacillus megaterium*: Actions of Streptolydigin, Echinomycin, and Triostins

MICHAEL WARING[1] AND ANDREW MAKOFF[2]

Department of Pharmacology, Medical School, Cambridge, England

(Received September 25, 1973)

SUMMARY

WARING, MICHAEL, AND MAKOFF, ANDREW: Breakdown of pulse-labeled ribonucleic acid and polysomes in *Bacillus megaterium*: actions of streptolydigin, echinomycin, and triostins. *Mol. Pharmacol.* **10**, 214-224 (1974).

When newly synthesized RNA was pulse-labeled by addition of [5-^{3}H]uridine, the incorporation being terminated after 30 sec by addition of an antibiotic inhibitor of RNA synthesis, significant incorporation of label into acid-insoluble material other than RNA occurred. When correction for this effect was made, the parameters of decay of pulse-labeled RNA revealed with actinomycin D at 10 μg/ml were significantly lower than those previously reported: a mean half-life of 36 $\pm$ 3 sec and a mean stable fraction of 39 $\pm$ 4% were found. Streptolydigin, echinomycin, and triostins A and C yielded (corrected) curves for decay of pulse-labeled RNA similar to those seen using actinomycin. With streptolydigin and echinomycin the parameters of decay were independent of antibiotic concentration over a wide range and were comparable with the parameters quoted for actinomycin. Similar values were also given by the triostin antibiotics tested at 5 μg/ml. Echinomycin appeared to be approximately 4-5 times more potent than actinomycin D, judged by the relative concentrations required to halt incorporation of the precursor and reveal decay of pulse-labeled RNA. Direct measurements, using incorporation of [*methyl*-^{3}H]thymidine, showed that both actinomycin and echinomycin inhibit DNA synthesis in *Bacillus megaterium*. Streptolydigin had no inhibitory action. Actinomycin, echinomycin, and streptolydigin all caused degradation of polysomes in protoplasts of *B. megaterium*. With each antibiotic the time to 50% decay was approximately 5 min, in agreement with earlier estimates using actinomycin. A large discrepancy between the apparent half-lives of decay of pulse-labeled RNA and of polysomes therefore was seen with all antibiotics tested. The results attest to the usefulness of studies on the decay of pulse-labeled RNA as a means of investigating the specificity, rapidity of action, and relative potency of antibiotics which inhibit RNA synthesis.

INTRODUCTION

Pulse-labeling experiments have shown that in bacteria newly synthesized RNA consists of two distinct fractions, one being stable and the other breaking down to acid-

This work was supported by grants from the Royal Society and the Medical Research Council.

[1] Recipient of a grant from Jesus College, Cambridge; to whom correspondence should be addressed.

[2] Recipient of a grant from Trinity College, Cambridge.

soluble products within a few minutes of termination of the pulse (1–8). In the past many such experiments have been carried out using actinomycin D to prevent further incorporation of label into RNA (1–8). Meaningful interpretation of these experiments must rest on the assumption that actinomycin is a specific inhibitor of RNA synthesis and has no other relevant actions.

In a previous paper (8) we sought to examine this question of specificity, via the argument that if the observed breakdown of pulse-labeled RNA is genuine it should occur in the same fashion irrespective of the nature and concentration of the inhibitor employed, provided only that sufficient inhibitor is present to terminate the precursor pulse promptly and completely (6, 8). The breakdown of the labile fraction of RNA follows exponential kinetics; thus the whole process may be characterized in terms of the percentage of labeled RNA which remains stable and the half-life of decay of the unstable fraction. Four antibiotics were tested, of which two (chromomycin A_3 and mithramycin) were found to yield the same parameters of decay as actinomycin over a substantial range of concentrations (8). This was taken as evidence that the characteristics of breakdown of pulse-labeled RNA are not peculiar to the use of actinomycin D as an inhibitor.

However, in an earlier study Grinsted (6) found that the parameters of decay induced by proflavine and ethidium were not the same as those seen with actinomycin and showed a strong dependence on the drug concentration. We obtained similar results with nogalamycin and daunomycin, and found the action of daunomycin to be much slower and/or weaker than the other antibiotics (8). These anomalies can be attributed to secondary interference with the metabolism of RNA and point to a serious lack of specificity in the action of the drugs as inhibitors of RNA synthesis (6, 8).

We have pursued the problem further using streptolydigin, echinomycin, and triostins. Our purpose has been twofold. First, we wished to verify that the parameters of decay of pulse-labeled RNA appear the same when the inhibitor has a radically different mode of action. All the drugs used to date share a common basis of action, in that they inhibit RNA synthesis by binding to and blocking the template function of DNA, albeit by different mechanisms (8–11). Streptolydigin acts quite differently, by binding directly to RNA polymerase, most probably to the β-subunit of the enzyme (12–17).

Second, we wished to develop the system as a means of investigating the specificity, rapidity of action, and relative potency of antibiotics which inhibit RNA synthesis. Its usefulness is illustrated by the results with echinomycin (identical with quinomycin A) and the structurally related triostins (18, 19). The molecular basis of action of these antibiotics is akin to that of actinomycin (9), involving intercalation, though the detailed mechanism of their interaction with DNA has not yet been formulated.[3] No other system is capable of assessing the promptness with which RNA synthesis is totally blocked, or the relative potency of different inhibitors during the critical initial period of exposure of the cells. As an indication of specificity we adopted the criterion proposed previously (8), i.e., that the parameters of decay of pulse-labeled RNA should be independent of concentration of the antibiotic over as wide a range as possible.

In addition, we made a few direct measurements of the action of the antibiotics on DNA synthesis. We also compared the stability of polysomes in protoplasts of *Bacillus megaterium* exposed to streptolydigin, echinomycin, and actinomycin D at appropriate concentrations. It is well known that following inhibition of RNA synthesis by actinomycin the bulk of the polysomes are degraded, ostensibly reflecting the degradation of bulk messenger RNA engaged in protein synthesis (7, 20, 21).

MATERIALS AND METHODS

Antibiotics. Actinomycin D was a product of Merck Sharp & Dohme; echinomycin, of Ciba-Geigy; and streptolydigin, of the Upjohn Company; triostins A and C were gifts from Dr. H. Otsuka. All the antibiotics were kept in stock solution in the dark and below 0°. Echinomycin was dissolved in absolute ethanol; actinomycin D and streptolydigin,

[3] M. Waring, unpublished experiments.

in 96% ethanol; and triostins A and C, in methanol.

Bacterial cultures. B. megaterium strain KM, maintained freeze-dried in this laboratory, was cultured and maintained in glucose-salts medium as described by Fok and Waring (8).

Pulse-labeling with [5-^{3}H]uridine. The following modifications were applied to the method of Fok and Waring (8). Samples were ejected into 1 ml of 7% (w/v) trichloracetic acid instead of 2 ml, to achieve more efficient Vortex mixing after the addition. Except during the preliminary experiments, six additional samples were taken at approximately 2-min intervals after the addition of the antibiotic. These were squirted into 1 ml of 1 N NaOH, mixed using a Vortex mixer, and then incubated overnight at 37° to digest RNA. They were then neutralized, and trichloracetic acid was added to a final concentration of about 7%. These samples could now be filtered and treated as the others. The use of alkali digestion to estimate the incorporation of label into DNA and other alkali-resistant material has been described previously (4, 22). The results were computed as previously described (8).

Identification of labeled material on filters. The use of ribonuclease to digest RNA on filters containing trichloracetic acid-insoluble material has been described by Cundliffe (7). The filters were washed in toluene, dried, and incubated in a Tris-potassium buffer (pH 7.5), containing 20 μg/ml of pancreatic ribonuclease (Worthington), for several hours at 37°. Trichloracetic acid was then added to give a final concentration of 7%. Acid-insoluble radioactivity was estimated in the usual way. Control incubations were also performed with Tris-potassium buffer alone.

Pulse-labeling with [methyl-^{3}H]thymidine. At experimental time zero minus 30 sec, 0.1 ml of [*methyl*-^{3}H]thymidine (Radiochemical Centre, Amersham; specific activity, 22 Ci/mmole) was added to 15 ml of exponentially growing cells, giving a final activity of about 3 μCi/ml. Samples were taken into 1 N NaOH and treated as described above. The first two samples were taken at experimental times −20 and −10 sec. The third sample was taken at zero time, while simultaneously 7 ml of the culture was quickly transferred to a flask containing the antibiotic. Alternate samples were taken from the antibiotic-containing and control cultures at intervals over the next 12 min, as indicated under RESULTS.

Breakdown of polysomes in protoplasts. ^{32}P-Labeled protoplasts of *B. megaterium* were prepared as described by Cundliffe (20, 21). Samples (0.5 ml) were taken into glass vials containing Triton X-100 (Rohm & Haas; final concentration, 0.1%, v/v), deoxyribonuclease (Worthington; final concentration, 5 μg/ml), and pancreatic ribonuclease (final concentration, 2 μg/ml). The vials were immediately frozen and stored at −30°. Six samples were taken in all: one before addition of the antibiotic, and the others 1, 3, 5, 10, and 15 min later.

Samples (0.2 ml) were layered on linear sucrose density gradients [total volume, 5 ml; 15–40% (w/v) sucrose in 10 mM Tris–5 mM magnesium acetate–100 mM KCl, pH 7.6, at 20°, adjusted with HCl] (20, 21). These were centrifuged at 40,000 rpm for 180 min at 2° in an MSE 65 ultracentrifuge, using a 3 × 5 ml rotor. Sixty 6-drop fractions were collected and counted in a Nuclear-Chicago liquid scintillation counter. The scintillant fluid contained 6 g of 2-(4′-*tert*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole (Ciba), 125 ml of water, and 500 ml of Triton X-100 in 1 liter of sulphur-free toluene. Assayed in this way, polysomes present in the cells at the time of sampling are converted to particles sedimenting at approximately 70 S while the products of polysome breakdown *in vivo* are ribosomal subunits sedimenting at 50 S and 30 S (20, 21). Thus the polysome fraction can be calculated from the ratio (^{32}P radioactivity sedimenting at 70 S)/(total ^{32}P radioactivity sedimenting at 70 S + 50 S + 30 S).

RESULTS

Preliminary experiments. Experiments were first carried out with actinomycin D at 10 μg/ml. The values so obtained for percentage of stable fraction and half-life of decay of RNA were comparable with the results of Fok and Waring (8). Three such

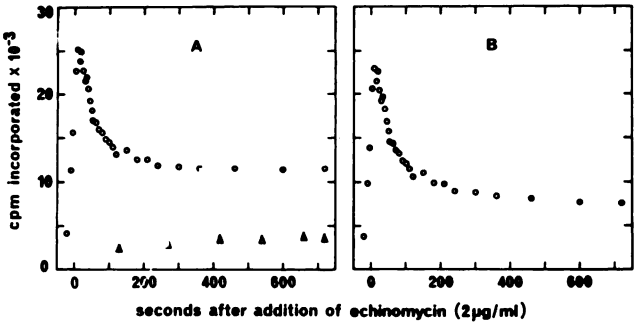

FIG. 1. *Breakdown of pulse-labeled RNA in the presence of streptolydigin*
The culture was pulse-labeled with [5-³H]uridine for 30 sec as described under MATERIALS AND METHODS A. O, total trichloracetic acid-insoluble radioactivity; △, acid-insoluble, alkali-resistant radioactivity. B. Trichloracetic acid-insoluble, alkali-labile radioactivity obtained by subtraction of the two curves in A.

experiments gave mean values of 46% stable fraction and 46 sec half-life (see subsequent experiments with actinomycin D below).

Streptolydigin. The upper curve of Fig. 1A (circles) shows a decay profile obtained with streptolydigin at 10 μg/ml. This type of curve is substantially different from the type obtained with actinomycin D and other inhibitors of RNA synthesis so far tested (1–8), in that a continued incorporation of radioactivity into trichloracetic acid-insoluble material occurs after, and presumably during, the initial phase of decay. This phenomenon was reproducible, and many similar curves were obtained with streptolydigin in the range 5–100 μg/ml.

Ribonuclease treatment of filters from such experiments revealed that this increase in trichloracetic acid-insoluble radioactivity was not due to synthesis of labeled RNA, for a steady increase in ribonuclease-resistant, acid-insoluble counts was found, suggestive of incorporation into DNA. The ribonuclease-resistant radioactivity was also insensitive to digestion by alkali, but a substantial proportion (over half) was rendered acid-soluble by treatment with deoxyribonuclease. This confirmed that it represented largely, if not entirely, incorporation into DNA. An attempt to suppress it by adding a "chase" of unlabeled uridine in 10^5-fold excess after the 30-sec pulse failed, presumably because of difficulties in saturating the intracellular nucleotide pools. Another possible palliative procedure, that of adding unlabeled cytosine before the pulse, was abandoned because exogenous cytosine is very poorly utilized for nucleic acid synthesis by this organism.

For these reasons the modified experimental procedure involving the taking of additional samples into NaOH was adopted (see MATERIALS AND METHODS). The lower curve in Fig. 1A (triangles) shows the alkali-resistant radioactivity. As can be seen, this is parallel to the increase in trichloracetic acid-insoluble counts in the upper curve. On the assumption that the lower curve represents the incorporation of label into material other than RNA, it was subtracted from the upper curve to generate Fig. 1B. This decay curve is similar in shape to curves obtained using actinomycin D, but yields slightly lower parameters (stable fraction and half-life of decay of unstable RNA).

Using this procedure, half-lives and stable fractions were determined for streptolydigin in the range 1–100 μg/ml. The experiment at 1 μg/ml did not yield a convincing decay, but those at 2 μg/ml and above gave parameters which are presented in Fig. 2. From this graph it can be seen that the parameters remain essentially constant over the concentration range 10–100 μg/ml.

Actinomycin D. The modified method was applied to actinomycin at 10 μg/ml. The results from three such experiments are shown in Table 1. It is evident that the correction for labeling of DNA and any other alkali-resistant, acid-insoluble material low-

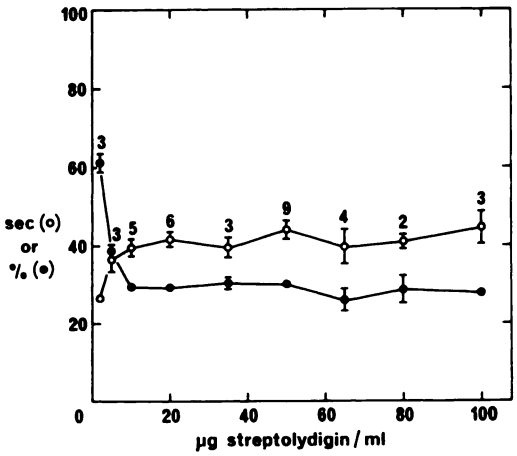

FIG. 2. *Dependence of parameters of decay of pulse-labeled RNA on streptolydigin concentration*

O, half-life; ●, 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. Where no error bar appears the standard error is smaller than the symbol drawn. All values have been corrected for incorporation into trichloracetic acid-insoluble, alkali-resistant material, determined separately in each experiment in the manner illustrated in Fig. 1.

TABLE 1

Characteristics of breakdown of pulse-labeled RNA in the presence of actinomycin D

In each experiment the incorporation of label into alkali-resistant material has been subtracted.

Antibiotic concentration	Stable fraction		Half-life	
μg/ml	*%*		*sec*	
10	41.1	36 ± 3	37.9	39 ± 4
10	29.3		32.1	
10	36.6		46.8	
1	65.4		40.1	
0.23	—[a]			

[a] No decay curve obtained (Fig. 5).

ered the apparent half-life and stable fraction by approximately 10 sec and 10%, respectively. (These values should be compared with those for actinomycin reported at the beginning of this section.) The significance of this is discussed later.

Echinomycin. Figure 3A shows the uncorrected decay curve obtained from an experiment using echinomycin at 2 μg/ml. The corrected curve (Fig. 3B) was obtained in exactly the same way as with streptolydigin. Decay curves in the concentration range 0.2–20 μg/ml were determined, and their parameters are expressed in Fig. 4. Parameters at concentrations exceeding 20 μg/ml were inaccessible because of the low solubility of the antibiotic. At 0.1 μg/ml no decay was revealed, presumably because insufficient echinomycin was present to stop RNA synthesis completely. The "plateau region" for echinomycin, where the parameters of decay are substantially independent of concentration, lies in the range 1–20 μg/ml.

That echinomycin is much more potent than streptolydigin in its inhibition of RNA synthesis is immediately evident. A few additional experiments were performed in order to compare its potency with that of actinomycin D. At 0.2 μg/ml (approximately 0.2 μM) the concentration of echinomycin is in the "threshold region," where there is just sufficient inhibitory action to reveal a decay (Fig. 5A), albeit with parameters which are significantly higher than those found at concentrations within the plateau region. At an equimolar concentration of actinomycin D (0.23 μg/ml) only a decrease in the rate of labeling of RNA occurred (Fig. 5B), showing that this concentration was clearly below the corresponding threshold value. Only when the actinomycin concentration was raised 4–5-fold was a comparable decay observed, again characterized by parameters significantly higher than those seen with "plateau" concentrations of this antibiotic (Fig. 5C). In the latter curve there is still evidence of substantial continued synthesis of RNA, whereas with 0.2 μg/ml of echinomycin there is not, suggesting that strict comparability of effect might require an even higher concentration of actinomycin D. At all events, these experiments show that echinomycin is at least 4–5 times more potent as an inhibitor of RNA synthesis than actinomycin D.

The triostins. Echinomycin is a member of the quinoxaline group of antibiotics, which also includes the triostins (23). They are characterized by the possession of the qui-

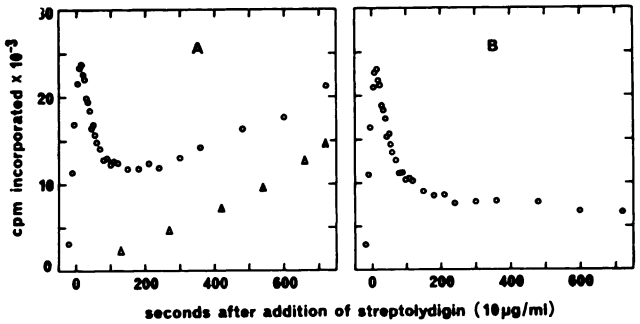

FIG. 3. *Breakdown of pulse-labeled RNA in the presence of echinomycin*
Details are described in the legend to Fig. 1.

noxaline 2-carboxamide chromophore. Triostin A is structurally homologous to echinomycin but lacks the dithian ring, which is replaced by a disulphide bridge. In triostin C, the *N*-methylvaline residues of triostin A are replaced by dimethylalloisoleucine (24). In view of these structural similarities it was of interest to investigate the actions of the triostins on pulse-labeled RNA.

Both triostins A and C were tested at 5 μg/ml. Owing to their low solubility in ethanol, methanol was used as solvent. Figure 6 shows that each antibiotic yields a similar decay profile. Averaging the results of three experiments, the stable fraction was 35 ± 2% and the half-life 53 ± 2 sec. Thus the stable fraction is in good agreement with the values produced by the other antibiotics, although the half-life may be somewhat higher. More experiments with a range of antibiotic concentrations would be required to establish whether $t_{1/2}$ is significantly different with triostins.

Actions of actinomycin D, echinomycin, and streptolydigin on DNA synthesis. Direct measurements of DNA synthesis were performed by following the incorporation of [*methyl*-^{3}H]thymidine into acid-insoluble, alkali-resistant material. The antibiotics were used at concentrations well within the plateau region for breakdown of pulse-labeled RNA; 10 μg/ml of actinomycin D, 5 μg/ml of echinomycin, and 20 μg/ml of streptolydigin. The results are presented in Fig. 7, with each plot normalized with respect to one control.

Streptolydigin appeared to have no effect on DNA synthesis. Actinomycin halted the

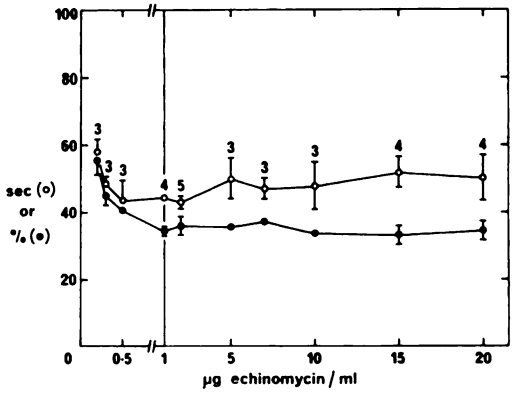

FIG. 4. *Dependence of parameters of decay of pulse-labeled RNA on echinomycin concentration*
Details are described in the legend to Fig. 2. For clarity the scale of the abscissa has been expanded 5-fold below 1 μg/ml.

labeling of DNA within 20 sec; echinomycin acted more slowly, within 60 sec. This explains why the correction for labeling of sodium hydroxide-resistant material in the [H^3]uridine incorporation experiments with the latter two antibiotics is relatively small compared to that for streptolydigin (cf. Figs. 1A and 3A), and confirms that that labeling occurs primarily in DNA. It is interesting that echinomycin acts more slowly than actinomycin to halt the labeling of DNA, particularly since it is more potent than actinomycin in inhibiting [^{3}H]uridine incorporation. At present we are at a loss to account for this difference. Perhaps echinomycin is not only more potent than actinomycin as an inhibitor of RNA synthesis but also more genuinely selective.

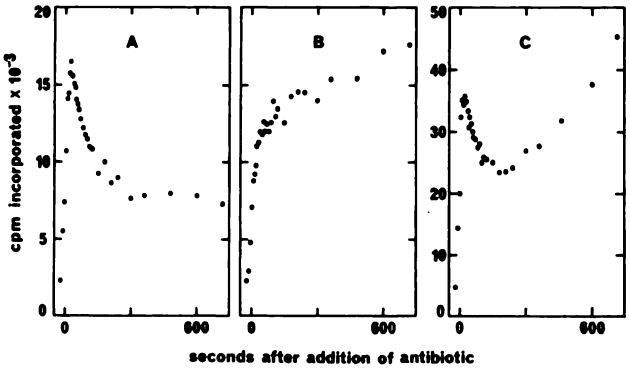

FIG. 5. *Breakdown of pulse-labeled RNA in the presence of antibiotics at approximately threshold concentrations*

All three plots have been corrected for incorporationof label into alkali-resistant material. A. Echinomycin at 0.2 μg/ml. B. Actinomycin D at 0.23 μg/ml. C. Actinomycin D at 1 μg/ml.

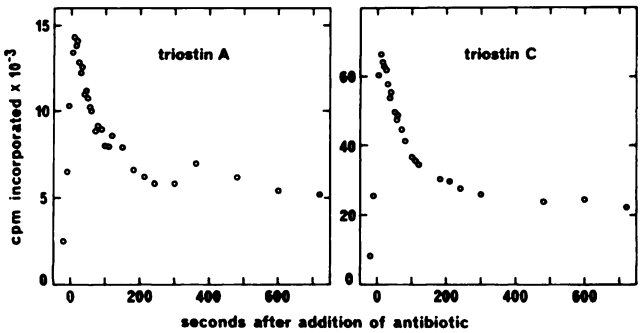

FIG. 6. *Breakdown of pulse-labeled RNA in the presence of triostins*

Both plots have been corrected for incorporation of label into alkali-resistant material.

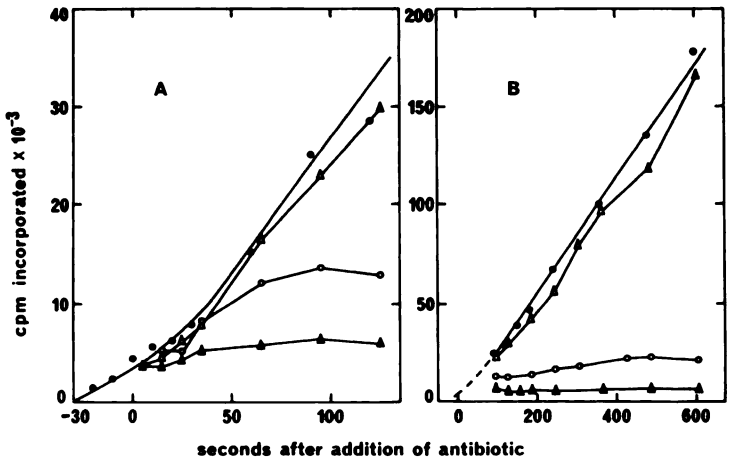

FIG. 7. *Effects of antibiotics on DNA synthesis*

The ordinate shows radioactivity incorporated from [*methyl*-³H]thymidine into alkali-resistant, acid-insoluble material. ▲, actinomycin D; ○, echinomycin; △, streptolydigin; ●, control. All plots were normalized with respect to a single control. A. Incorporation during the first 2 min. B. Same data omitted.

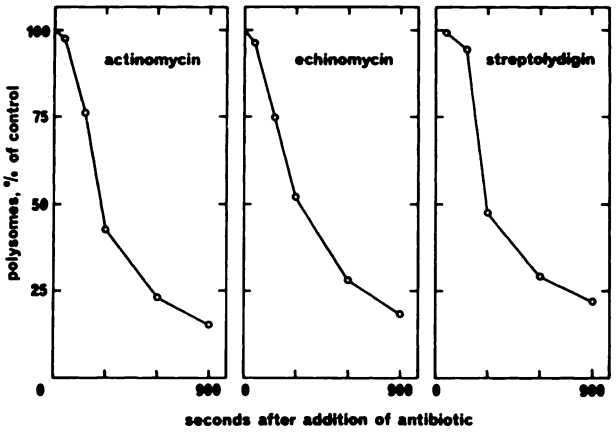

FIG. 8. *Breakdown of polysomes following addition of antibiotics*

Protoplasts of *B. megaterium* were labeled in the steady state with ^{32}P as described under MATERIALS AND METHODS. The ordinate represents the fraction of ribosomal material present as polysomes, as a percentage of the control value determined immediately prior to addition of the antibiotic. In these controls the fraction of the total ribosomal material present as polysomes was 70–90%. Antibiotics were added to the following final concentrations: actinomycin, 10 μg/ml; echinomycin, 5 μg/ml; streptolydigin, 20 μg/ml.

Breakdown of polysomes induced by antibiotics in protoplasts. Sedimentation profiles were determined at known times after addition of each antibiotic. As outlined in MATERIALS AND METHODS, the fraction of ribosomal material present as polysomes at the time of sampling was calculated from the size of the 70 S peak relative to the total size of the 70 S, 50 S, and 30 S peaks. Figure 8 shows for each antibiotic a plot of this fraction, expressed as a percentage of the control fraction (no antibiotic present), against time after addition of the inhibitor. The antibiotics were used at the same concentrations as for the DNA synthesis experiments.

Unlike the exponential decay of pulse-labeled RNA, these decay profiles are apparently sigmoidal, and so the time course could not sensibly be expressed as a half-life. The times to 50% breakdown were 280, 320, and 290 sec for actinomycin, echinomycin, and streptolydigin, respectively.

DISCUSSION

In the pulse-labeling experiments with streptolydigin, the increase in trichloracetic acid-insoluble radioactivity at late times can be accounted for by a parallel increase in ribonuclease-resistant, alkali-resistant radioactivity. Much if not all of this radioactivity must represent incorporation into DNA. The possibility that it originates from contamination of the [^3H]uridine with other labeled precursors of nucleic acid can be dismissed, because the relative incorporation did not vary from batch to batch, and levels of contamination of at least 5–10% would have been required to account for some of the observations. It must be concluded that some of the label from [5-^3H]uridine can enter DNA, and there is precedent for this view. Schaechter and McQuillen (4) reported the incorporation of label from [5-^3H]uridine into DNA in bacteriophage-infected cells of *B. megaterium*. They showed by chromatography that the DNA was labeled in its cytosine residues. (Labeling of thymine residues would not be expected, since methylation of [5-^3H]uridine should lead to loss of the ^3H.) Adams (25), using [5-^3H]uridine in mouse fibroblasts, reported a similar finding.

Only with streptolydigin was a large increase in "late" labeling found in the present work. As mentioned in the INTRODUCTION, only streptolydigin, of the antibiotics investigated with this system, inhibits RNA synthesis without direct attack on the DNA template (16). (The antibiotic rifampicin does not act directly on the template either,

but use of this drug introduces another complication, as will become apparent later.) The implication is that those antibiotics previously tested with this system, including actinomycin and echinomycin, inhibit DNA synthesis as well as RNA synthesis. This was confirmed by the experiments directly investigating the labeling of DNA by [*methyl*-^{3}H]thymidine. Both actinomycin D (26) and echinomycin (18) have previously been shown to inhibit DNA synthesis in cell-free systems.

The lower stable fractions and half-lives of decay obtained with actinomycin after correction for incorporation of label into alkali-resistant material are probably due mainly to the uptake of label by DNA in the time before complete inhibition of DNA synthesis. This conclusion is illustrated in the case of echinomycin by the correction curve in Fig. 3A (triangles).

Both streptolydigin and echinomycin fulfill the criterion of concentration independence over a wide range for both parameters. Above a threshold concentration (10 μg/ml for streptolydigin and 1 μg/ml for echinomycin) both the half-life and stable fraction of RNA remain constant for all concentrations tested. Below this concentration, interpretation of the parameters is difficult, since a background synthesis of RNA continues throughout the decay (8). Above it, the "plateau" values yielded by the two antibiotics compare well with the parameters determined using actinomycin (corrected for incorporation into alkali-resistant material). Both the stable fraction and half-life of decay of the unstable fraction are in fair agreement among all three inhibitors, although those for streptolydigin appear marginally lower. The limited data obtained with triostins indicate that they too yield comparable parameters, perhaps with a somewhat higher $t_{1/2}$, although no firm conclusion can be drawn while insufficient data are available. In any event, the possible significance of such relatively small differences as we have found is questionable. The overriding impression is of a consistent pattern of results common to the whole group of antibiotics, and shared with chromomycin and mithramycin (8), leading to the conclusion that all behave as model inhibitors in this system.

Particularly impressive is the rapidity with which the antibiotics act. In the plateau region of concentrations, incorporation of [^{3}H]uridine is halted and decay commences within 10 sec (Figs. 1, 3, and 6). In the threshold region, where decay profiles are observable but the parameters begin to vary with the antibiotic concentration, the time to onset of decay is longer (e.g., Fig. 5). Presumably this reflects in part the time required for the cells to accumulate the antibiotic from the medium, for the lag before the onset of decay becomes longer as the antibiotic concentration is lowered. A longer lag means an effectively longer pulse-labeling period, which may explain why the parameters of decay differ from the "plateau" values. (8). Events occurring during the extended lag period probably account for the one clear difference between the actions of streptolydigin and the DNA-binding antibiotics which we have detected, i.e., that the half-life with streptolydigin falls, rather than rises, in the threshold region (cf. Figs. 2 and 4). As yet we can only speculate on the mechanism of this effect, but doubtless it originates from the fundamentally different mode of action of streptolydigin.

Comparison of decay profiles at concentrations within the threshold region shows that echinomycin has a 4–5-fold higher potency than actinomycin D in inhibiting RNA synthesis in *B. megaterium*. It would therefore appear to be the most potent inhibitor of RNA synthesis known for this organism, and quite probably for other biological systems too. In cell-free systems rifampicin [another inhibitor of RNA synthesis, which, like streptolydigin, acts on RNA polymerase rather than the DNA template (11)] has been shown to be more potent than actinomycin D (27). However, in *B. megaterium* 20 μg/ml of rifampicin exhibited only very slight decay of pulse-labeled RNA, and the time to onset of decay was 50 sec.[4] This is consistent with the belief that rifampicin only prevents initiation of new chains of RNA and has no effect on

[4] M. Waring and A. Makoff, unpublished observations.

growing chains (27). Thus, unlike rifampicin, echinomycin has been shown to be as rapidly acting as, and more potent than, actinomycin D *in vivo*.

It is not our purpose to elucidate the nature and metabolic significance of the stable and unstable fractions of RNA. In a previous paper (8) the likely constitution of the two RNA fractions was considered, the salient point being that messenger RNA appears to contribute to the unstable fraction but is unlikely to account for all of it. The central paradox is that breakdown of pulse-labeled RNA in the presence of actinomycin can be prevented by inhibitors of protein synthesis acting at the level of the ribosome (2, 7); yet, after addition of actinomycin, both the rate of loss of protein-synthesizing ability and the rate of breakdown of polysomes follow a much slower time course than the rate of decay of pulse-labeled RNA (1, 3–7). Our few experiments on the decay of polysomes show that this discrepancy is not only observed with actinomycin. In fact, the time to 50% decay is strikingly similar for actinomycin D, echinomycin, and streptolydigin, being approximately 5 min, which is comparable with earlier estimates for actinomycin (3, 7, 20, 21). This is about 7 times the half-life of decay of pulse-labeled RNA, using the same antibiotics.

It could be that all these inhibitors have a secondary action on protein synthesis, slowing the movement of ribosomes along messenger RNA. We feel that this possibility is unlikely, in view of the practically identical time courses of polysome breakdown (Fig. 8). Assuming normal dose-response relationships, it would be fortuitous if we had arbitrarily chosen antibiotic concentrations which slowed ribosome movement to the same extent. More telling is the fact that we find much the same discrepancy between half-lives with streptolydigin, which attacks RNA polymerase, as with the DNA-binding antibiotics. This suggests that, whatever phenomena are responsible for the discrepancy, they occur irrespective of the chemical nature and mode of action of the antibiotic, and that while the decay of pulse-labeled RNA observed in the presence of antibiotics may be quite unphysiological, it is a consequence of inhibition of RNA synthesis rather than the presence of the antibiotic itself.

In conclusion, the present study has served to establish experiments on the decay of pulse-labeled RNA in *B. megaterium* as a means of critically evaluating the action of antibiotics on RNA synthesis. Wherever such antibiotics are used as tools to inhibit RNA synthesis, the events immediately consequent upon their addition must be considered of prime importance. One such immediate consequence is the breakdown of pre-existing RNA; thus any thorough study must of necessity pay attention to this phenomenon. We have shown that the *B. megaterium* system is well suited to investigations of this kind, provided that due correction is made for incorporation of the [^{3}H]-uridine precursor into trichloracetic acid-precipitable, alkali-insensitive material. As well as enabling comparison of the rapidity of action and relative potency of inhibitors, it has permitted routinely used drugs to be sorted essentially into two categories: those which act rapidly and yield a consistent set of parameters, suggesting that their action on RNA synthesis is for practical purposes "clean" (actinomycin, chromomycin, mithramycin, streptolydigin, echinomycin, and probably the triostins), and those which act more slowly and/or show signs of interfering side effects (proflavine, ethidium, rifampicin, daunomycin, and nogalamycin).

ACKNOWLEDGMENTS

We thank Mr. N. F. Totty for technical assistance, and Drs. E. Cundliffe and D. J. W. Burns for their advice and help in the experiments on breakdown of polysomes. We are especially grateful to Drs. G. B. Whitfield, Jr., H. Bickel, K. Scheibli, and H. Otsuka for generous supplies of the antibiotics.

REFERENCES

1. Levinthal, C., Keynan, A. & Higa, A. (1962) *Proc. Natl. Acad. Sci. U. S. A.*, **48**, 1631–1638.
2. Fan, D. P., Higa, A. & Levinthal, C. (1964) *J. Mol. Biol.*, **8**, 210–222.
3. Schaechter, M., Previc, E. P. & Gillespie, M. E. (1965) *J. Mol. Biol.*, **12**, 119–129.
4. Schaechter, M. & McQuillen, K. (1966) *J. Mol. Biol.*, **22**, 223–233.

5. Zimmerman, R. A. & Levinthal, C. (1967) *J. Mol. Biol.*, **30**, 349–370.
6. Grinsted, J. (1969) *Biochim. Biophys. Acta*, **179**, 268–279.
7. Cundliffe, E. (1967) Protein Synthesis and Antibiotics: Studies of Protoplasts of *Bacillus megaterium*, Ph.D. thesis, Cambridge University.
8. Fok, J. & Waring, M. (1972) *Mol. Pharmacol.*, **8**, 65–74.
9. Sobell, H. M., Jain, S. C., Sakore, T. D. & Nordman, C. E. (1971) *Nat. New Biol.*, **231**, 200–205.
10. Waring, M. J. (1970) *J. Mol. Biol.*, **54**, 247–279.
11. Waring, M. J. (1972) in *The Molecular Basis of Antibiotic Action* (Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H. & Waring, M. J., eds.), pp. 173–277, Wiley, London.
12. Siddikhol, C., Erbstoeszer, J. & Weisblum, B. (1969) *J. Bacteriol.*, **99**, 151–155.
13. Schleif, R. (1969) *Nature*, **223**, 1068–1069.
14. Von der Helm, K. & Krakow, J. S. (1972) *Nat. New Biol.*, **235**, 82–83.
15. Von der Helm, K. & Krakow, J. S. (1972) *Nat. New Biol.*, **240**, 96.
16. Cassani, G., Burgess, R. R., Goodman, H. M. & Gold, L. (1971) *Nat. New Biol.*, **230**, 197–200.
17. Lark, K. G. (1972) *J. Mol. Biol.*, **64**, 47–60.
18. Ward, D. C., Reich, E. & Goldberg, I. H. (1965) *Science*, **149**, 1259–1263.
19. Keller-Schierlein, W., Mihailovic, M. L. & Prelog, V. (1959) *Helv. Chim. Acta*, **42**, 305–322.
20. Cundliffe, E. (1968) *J. Gen. Microbiol.*, **53**, 425–430.
21. Cundliffe, E. (1969) *Biochemistry*, **8**, 2063–2066.
22. Vickers, T. G. & Midgeley, J. E. M. (1971) *Nat. New Biol.*, **233**, 210–212.
23. Otsuka, H. & Shoji, J. (1967) *Tetrahedron*, **23**, 1535–1542.
24. Otsuka, H. & Shoji, J. (1965) *Tetrahedron*, **21**, 2931–2938.
25. Adams, R. L. P. (1968) *FEBS Lett.*, **2**, 91–92.
26. Hurwitz, J., Furth, J. J., Malamy, M. & Alexander, M. (1962) *Proc. Natl. Acad. Sci. U. S. A.*, **48**, 1222–1230.
27. Sippel, A. E. & Hartmann, G. R. (1970) *Eur. J. Biochem.*, **16**, 152–157.