Polysome Metabolism in *Escherichia coli*: Amicetin, an Antibiotic That Stabilizes Polysomes¹

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Amicetin, an aminohexosepyrimidine nucleoside antibiotic, inhibited protein synthesis in intact bacterial cells and in cell-free extracts derived from these cells. At concentrations of antibiotic which rapidly and completely inhibited protein synthesis, the polysomes in the cell were stabilized. Amicetin also inhibited the breakdown of polysomes induced by the streptogramin A antibiotics, rifampin, or puromycin, antibiotics that require peptide bond formation for expression of their action on polysomes. Thus, amicetin is another addition to the list of drugs which may be useful in studying polysome and messenger ribonucleic acid metabolism in vivo. An in vitro system is described which may be generally useful in studying polysome metabolism when antibiotic-sensitive strains are not available.

Aside from the inherent interest in finding out how antibiotics inhibit cell growth, antibiotics can be useful in vivo: (i) as reagents to inhibit protein synthesis at defined steps in the biosynthetic pathway; (ii) to dissociate protein from nucleic acid synthesis, thus facilitating studies on the relationships between nucleic acid and protein synthesis; and (iii) in studies of various aspects of the ribosome cycle. This investigation on the mode and site of action of amicetin was directed toward these ends.

Amicetin, an aminohexosepyrimidine nucleoside antibiotic, inhibits protein synthesis in sensitive cells, and in cell-free extracts derived from these cells (1). Previous studies have shown that in vitro the antibiotic interacts with the 50S ribosome subunit (3); inhibits the puromycin reaction (17); inhibits di- and oligophenylalanine synthesis dependent on guanosine triphosphate and factor G (18); and inhibits the binding of the phenylalanyl-transfer ribonucleic acid (RNA) oligonucleotide C-A-C-C-A(Phe) to ribosomes (S. Pestka, Symp. on Inhibitors of Protein Synthesis and Membrane Function, Granada, Spain, *in press*). Little is known concerning the in vivo action of the drug. In the present investigation, I have studied the effects of amicetin on in vivo protein synthesis, focusing on the effects of the antibiotic on polysome metabolism.

MATERIALS AND METHODS

The methods used in this investigation are outlined in the Materials and Methods section of the

preceding paper (8) or are indicated in the legends to the figures and table.

RESULTS AND DISCUSSION

Effect of amicetin on protein synthesis in bacterial cells. Wild-type *Escherichia coli* is relatively insensitive to the action of the antibiotic; consequently, strains of *E. coli* were used which were more sensitive to amicetin than was the wild type. A description of the isolation and properties of these strains is given elsewhere (7). Figure 1 shows the effect of amicetin on protein synthesis in intact cells of the wild type W1895 and the sensitive mutant H135. As can be seen, the mutant is much more sensitive to the antibiotic than the parent.

Effect of amicetin on RNA synthesis. Amicetin at concentrations that completely inhibit protein synthesis had relatively little effect on RNA synthesis (Fig. 2). These results confirm those of Bloch and Coutsogeorgopoulos (2), who have shown that RNA and deoxyribonucleic acid synthesis continue in the presence of the antibiotic.

Reversibility of amicetin inhibition of protein synthesis. The action of amicetin on protein synthesis was readily reversed by washing the cells free from the drug. In this experiment (Fig. 3), cells of strain H135 were treated with amicetin (0.4 mM) for 45 min. The cells were then washed free from drug, and the ability of these cells to synthesize protein was compared with that of untreated growing cells at the same density.

Effect of amicetin on polysome content of cells and entrance of pulse-labeled RNA into polysomes. As was indicated in the preceding paper

¹ A preliminary report of this work was presented at the Annual Meeting of the American Society for Microbiology in Minneapolis, Minn., 1971.

FIG. 1. Effect of ampicillin on protein synthesis in bacterial and animal cells. Amounts of 5 ml of a bacterial culture growing in mineral salts-glucose medium containing methionine (100 $\mu\text{g}/\text{ml}$) and phenylalanine (10 $\mu\text{g}/\text{ml}$) were added at 15-sec intervals to flasks containing the indicated concentrations of ampicillin. Then ^{14}C -phenylalanine (0.1 $\mu\text{Ci}/\text{ml}$) was added at 15-sec intervals to each flask, and samples were taken at 15 min. The radioactivity incorporated into protein was determined. The same results were obtained when 30-min points were used. The results are given as per cent incorporation compared to a control culture incubated in the absence of ampicillin. Incorporation of radioactivity into protein was determined as previously described (4).

(8), ampicillin stabilized polysomes. Ampicillin was added to a culture of strain H101 growing at 37 C, and incubation was continued for an additional 15 min. Although protein synthesis was completely inhibited, the polysome content of the cells was preserved (Fig. 4). These polysomes can be degraded *in vitro* by the addition of ribonuclease (0.1 $\mu\text{g}/\text{ml}$) in the cold before centrifugation. In this experiment, the cultures were also pulse-labeled with ^{14}C -uracil 30 sec before harvesting. The dashed lines in Fig. 4 indicate the radioactivity associated with the polysomes. It is clear that pulse-labeled RNA can associate with polysomes in drug-treated cells as well as in growing cells. This finding is not unique, since it has already been shown that pulse-labeled RNA enters polysomes in chloramphenicol-treated cells (21). In another experiment, not shown here, it was also shown that nascent peptide remained attached to ribosomes during ampicillin inhibition.

Consequently, ampicillin is another addition to the list of drugs which may be useful in studying polysome and messenger RNA metabolism *in vivo*.

Effect of ampicillin on the action of puromycin,

synergistin A, and rifampin. The effect of ampicillin on the action of a number of antibiotics of known action was also studied.

Puromycin, which inhibits protein synthesis by forming a peptide linkage with the growing peptide chain on the polysomes (11, 14, 15, 20), causes a rapid breakdown of polysomes (9, 19). The effect of ampicillin on the puromycin-induced breakdown of polysomes *in vivo* is shown in Fig. 5. Cells of strain H135 were treated with puromycin (0.2 mM), and samples were taken at intervals and analyzed for polysome content. As can be seen in Fig. 5, the breakdown of polysomes induced by puromycin was rapid, being complete after 15 sec (which was the earliest sample taken). In contrast to this finding, cells treated with ampicillin 5 min before addition of puromycin retained the bulk of their polysomes, although there was a slight continual loss with increasing time of incubation.

Synergistin A and vernamycin A are antibiotics which apparently inhibit protein synthesis by interfering with initiation of protein synthesis (H. L. Ennis and K. E. Duffy, *Fed. Proc.* 30:1235, 1971), but do not affect elongation (5; Pestka, *in press*). Experiments similar to the one described in Fig. 5 were done except that

FIG. 2. Effect of ampicillin on RNA synthesis. Cells of strain H101 were grown in mineral salts-glucose medium containing methionine (100 $\mu\text{g}/\text{ml}$) and uracil (20 $\mu\text{g}/\text{ml}$). Ampicillin (0.4 mM) was added to one culture (●), and the other culture was incubated in the absence of the drug (○). ^{14}C -uracil (0.1 $\mu\text{Ci}/\text{ml}$) was added, and samples were taken at the indicated intervals. Incorporation of radioactivity into RNA was determined as previously described (4).

FIG. 3. Reversibility of ampicillin inhibition of protein synthesis. Growing cells of strain H135 were incubated with ampicillin (0.4 mM) for 45 min in a mineral-salts glucose medium. The cells were then centrifuged and washed free from the drug by two more cycles of suspension in mineral salts buffer and centrifugation. Finally, the cells were suspended in mineral salts-glucose growth medium, and the incorporation of ^{14}C -leucine into protein was determined at the indicated intervals (●). This is compared to a culture of growing cells at the same cell density manipulated in the same way as the inhibited culture except that the cells were not previously incubated in the presence of ampicillin (○).

synergistin A was used instead of puromycin. Synergistin A (0.2 mM) treatment also resulted in the loss of the majority of intracellular polysomes (Fig. 6). Since in the presence of synergistin A new proteins are not initiated, the loss of polysomes under this condition is a reflection of the time it takes to finish protein chains already in the process of being synthesized. It is shown in Fig. 6 that ampicillin also markedly inhibited the synergistin A-induced breakdown of polysomes. The results obtained with vernamycin A were identical.

Rifampin is an antibiotic that inhibits initiation of new RNA synthesis (12). Therefore, rifampin can be used to study the loss of intracellular polysomes as a consequence of the normal decay of messenger RNA, uncomplicated by new RNA synthesis. An experiment with rifampin (0.03 mM) was performed in the same way as those described above for puromycin or synergistin A. As has been described by Schlessinger and co-workers (21), rifampin treatment resulted in a rapid loss of polysomes, with a half-life of approximately 2.5 min (Fig. 7). Pre-treatment of cells with ampicillin 5 min before exposure to rifampin resulted in a marked although not complete stabilization of polysomes. The polysomes so treated broke down with a

FIG. 4. Sucrose density gradient analysis of extracts derived from *E. coli* H101 during growth and during inhibition of protein synthesis by ampicillin. The extracts were prepared from growing cells (A) and from cells incubated for 15 min in the presence of ampicillin, 0.4 mM (B), and were analyzed as previously described (8). The extract was centrifuged in a Spinco SW 25.1 rotor at 1°C and 25,000 rev/min for 3 hr and 15 min. At 30 sec before harvesting, ^{14}C -uracil (0.5 $\mu\text{Ci}/\text{ml}$, 0.1 $\mu\text{g}/\text{ml}$) was added to each culture. Solid line: absorbance at 254 nm. Dashed line: radioactivity.

half-life of approximately 9 min. Since the polysomes were eventually degraded in the presence of both rifampin and ampicillin, the results also indicate that simultaneous RNA synthesis may be necessary for complete stabilization of polysomes. Perhaps nucleases can degrade the messenger RNA bound to the ribosomes, and continued entrance of new messenger maintains the polysome configuration.

Effect of ampicillin on protein synthesis and polysome stability in an in vitro system. One of the problems encountered in studying the effects of antibiotics on intact bacterial cells is that many of the strains used are resistant to the drugs. In many cases, resistance is due to the inability of the antibiotics to enter the cell, since, once in the cell, the drugs can act. Methods have been developed to render cells permeable to a number of compounds that normally do not penetrate the cell wall or membrane. Treatment of cells

FIG. 5. Effect of amicetin on puromycin-induced breakdown of polysomes. Cells of strain H135 were treated with puromycin (0.2 mM), and samples were taken at intervals and analyzed for polysome content as previously described (8). A 100% polysome content equals an actual polysome content of 57%. The other set of samples was incubated in the presence of amicetin (0.4 mM) for 5 min before the addition of puromycin.

FIG. 6. Effect of amicetin on synergistin-induced breakdown of polysomes. This experiment was performed as outlined in the legend to Fig. 6, except that synergistin A (0.2 mM) was added in place of puromycin. Strain H101 was used, and 100% polysome content equals an actual polysome content of 68%.

with ethylenediaminetetraacetate (13) and isolation of sensitive mutants (1, 22) are two available methods. However, ethylenediaminetetraacetate may affect other components of the cell, and it is not always a simple procedure to isolate

FIG. 7. Effect of amicetin on rifampin-induced breakdown of polysomes. This experiment was performed as outlined in the legend to Fig. 6, except that rifampin (0.03 mM) was added in place of synergistin A.

TABLE 1. Effect of amicetin on polysome stability *in vitro*^a

Expt no.	Conditions of expt	Percentage of total radioactivity in			
		30S	50S	70S	Poly-somes
1	Growing cells	7	11	31	51
2	Amicetin (0.4 mM), 2 min	7	15	31	47
3	Puromycin (0.02 mM), 1 min	8	13	65	14
4	Amicetin (0.4 mM), 1 min Puromycin (0.02 mM), 1 min	7	12	33	48

^a Polysomes were isolated from growing cells of strain H101 as described in the legend to Fig. 8. The polysomes, in this case, were labeled with ¹⁴C-uracil during the growth period. The polysomes were incubated in the *in vitro* system described in the legend to Fig. 8 in the presence of the indicated concentrations of antibiotics. The incubation was terminated by rapidly chilling the reaction mixture. The content of polysomes, ribosomes, and subunits was determined as described in the legend to Fig. 4. Average of two separate experiments.

bacterial mutants sensitive to all of the antibiotics one might want to study.

Consequently, I will now describe an *in vitro* system which may be generally useful in studying the action of drugs on protein synthesis and especially on polysome stability. The isolation of active polysomes (10) is easy to perform and has given highly reproducible results. No messenger RNA is added to this system since the polysomes are already programmed.

Amicetin immediately and completely inhibits incorporation of [^{14}C] phenylalanine into peptide in a system using polysomes isolated from growing cells of strain H101 (Fig. 8). The effect of amicetin on polysome stability in this *in vitro* system is given in Table 1. It is evident that amicetin can stabilize polysomes

FIG. 8. Effect of amicetin on *in vitro* protein synthesis. Polysomes were prepared from growing cells of strain H101 by use of Brij 58 but no deoxycholate, as described by Godson and Sinsheimer (10). The reaction mixture contained (per ml of final volume): polysomes and supernatant fluid from 5×10^8 cells (equal to an optical density of about 7 at 260 nm); tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.8, 50 mM; mercaptoethanol, 6 mM; magnesium acetate, 10 mM; phosphoenolpyruvate, 50 mM; phosphoenolpyruvate kinase, 30 μg ; adenosine triphosphate, 1 mM; guanosine triphosphate, 0.02 mM; ammonium chloride, 100 mM; mixture of 19 cold amino acids minus phenylalanine, 0.05 mM each; *E. coli* B stripped transfer RNA, 100 μg ; and [^{14}C]-phenylalanine, 0.5 μCi , 0.002 μM . The reaction was started by the addition of [^{14}C]-phenylalanine and was incubated at 37 C. Amicetin (0.4 mM) was added to the mixture 30 sec before the addition of the [^{14}C]-phenylalanine. Samples (100 μliters) taken at the indicated intervals were mixed with 0.5 ml of trichloroacetic acid, heated at 100 C for 10 min, filtered through membrane filters (0.45 μm pore size; Millipore Corp.) and counted. Control reactions, no amicetin (○). Reactions in the presence of amicetin (●).

in vitro as well as *in vivo*. In addition, amicetin can also inhibit the puromycin-induced breakdown of polysomes *in vitro* (Table 1) as well as *in vivo* (Fig. 5). I have also used this system to study the effects of other antibiotics, and in all instances the *in vitro* results are the same as those obtained with intact sensitive bacterial cells (*in preparation*).

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