ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1982, p. 811–818
0066-4804/82/050811-08$02.00/0

Vol. 21, No. 5

# Erythromycin, Carbomycin, and Spiramycin Inhibit Protein Synthesis by Stimulating the Dissociation of Peptidyl-tRNA from Ribosomes

JOHN R. MENNINGER* AND DONALD P. OTTO
*Department of Zoology, University of Iowa, Iowa City, Iowa 52242*

Received 31 August 1981/Accepted 12 February 1982

In mutant *Escherichia coli* with temperature-sensitive peptidyl-tRNA hydrolase (aminoacyl-tRNA hydrolase; EC 3.1.1.29), peptidyl-tRNA accumulates at the nonpermissive temperature (40°C), and the cells die. These consequences of high temperature were enhanced if the cells were first treated with erythromycin, carbomycin, or spiramycin at doses sufficient to inhibit protein synthesis in wild-type cells but not sufficient to kill either mutant or wild-type cells at the permissive temperature (30°C). Since peptidyl-tRNA hydrolase in the mutant cells is inactivated rapidly and irreversibly at 40°C, the enhanced accumulation of peptidyl-tRNA and killing were the result of enhanced dissociation, stimulated by the antibiotics, of peptidyl-tRNA from ribosomes. The implications of these findings for inhibition of cell growth and protein synthesis are discussed. Certain alternative interpretations are shown to be inconsistent with the relevant data. Previous conflicting observations on the effects of macrolide antibiotics are explained in terms of our observations. We conclude that erythromycin, carbomycin, and spiramycin (and probably all macrolides) have as a primary mechanism of action the stimulation of dissociation of peptidyl-tRNA from ribosomes, probably during translocation.

The mechanism of action of the macrolide antibiotics has been a matter of controversy for some time (7, 31). A few years ago, we reported data concerning a novel effect of erythromycin on protein synthesis in vivo, namely, the enhancement of the dissociation of peptidyl-tRNA from ribosomes (J. R. Menninger, Fed. Proc. 33:1335, 1974). This phenomenon was subsequently verified in vitro (24). Our in vivo studies have now been extended to two other antibiotics of the macrolide group, carbomycin and spiramycin.

We used a strain of *Escherichia coli* (*ts*8) with a mutation in the structural gene (*pth*) for peptidyl-tRNA hydrolase (aminoacyl-tRNA hydrolase, EC 3.1.1.29) which renders that enzyme activity temperature sensitive. Peptidyl-tRNA hydrolase normally acts in the cell to catalyze the hydrolysis of intact peptides from peptidyl-tRNA that has dissociated from ribosomes during protein synthesis. A temperature-sensitive hydrolase activity allows measurement of the dissociation of peptidyl-tRNA from ribosomes at 40°C since the first step in the scavenging pathway for dissociated peptidyl-tRNA is blocked by the high temperature (18). Shortly after cultures growing at 30°C are shifted to the nonpermissive temperature (40°C), protein synthesis is inhibited (1, 18), and the cells die; i.e., they are unable to form a colony at permissive temperatures (21). All of these effects were assayed in the presence of erythromycin, carbomycin, and spiramycin at doses chosen, after the evaluation of several concentrations, so that no lethal effects of the drugs were observed at permissive temperatures.

The results presented below show that all three of these antibiotics stimulated the dissociation of peptidyl-tRNA from ribosomes of *E. coli* when given at doses that inhibited the growth of the cells. We propose, therefore, that the mechanism of action of the macrolide antibiotics is to stimulate the dissociation of peptidyl-tRNA from ribosomes, possibly during attempted translocation. We believe the data show that, in the presence of effective doses of macrolides, neither peptide bond formation nor translocation as such was inhibited. Instead, frequent dissociation of peptidyl-tRNA from ribosomes reduced to growth-inhibiting levels the probability of the completion of the synthesis of useful proteins.

## MATERIALS AND METHODS

**Bacterial strains.** *E. coli* strain *ts*8 [$F^+$ *lacZ* $trp^+$ *pth*(Ts)] was derived from strain CA244 ($F^+$ *lacZ trp* $pth^+$) by P1-mediated transduction, with strain

AA7852 [*trp*$^+$ *pth*(Ts)] as donor, and selection for tryptophan independence (22).

**Permeabilization with EDTA.** Since the cells were not normally permeable to spiramycin, they were permeabilized by the addition of 0.9 mM EDTA to aerating cultures for 5 min at 30°C. The EDTA was then titrated with an equivalent amount of $MgCl_2$, the $Mg^{2+}$, $Ca^{2+}$, and $Fe^{3+}$ of the medium were restored to their original concentrations, and the cells were allowed to equilibrate at 30°C before being treated with spiramycin. To avoid the difficulty of using EDTA with the phosphate-buffered medium M9 (22), we grew spiramycin-treated cultures in a Tris-buffered medium consisting of 0.1 M Tris-hydrochloride (pH 8.0), 8.5 mM NaCl, 100 mM KCl, 20 mM $NH_4Cl$, 0.5 mM $KH_2PO_4$, 0.16 mM $Na_2SO_4$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 83 mM D-glucose and supplemented with 5 μg of thiamine per ml and 19 of the 20 common amino acids (leucine omitted) at 40 μg/ml.

**Assay of accumulated peptidyl-tRNA.** The methods described previously (18) for isolating tRNA and assaying for peptidyl-tRNA were used, except that the cells were grown in media supplemented with 19 of the 20 common amino acids (leucine omitted) at 40 μg/ml. The peptidyl-tRNA fraction, expressed as a percentage, of various isoaccepting families of tRNA was assayed as the increase in amino acid-accepting activity, measured in the presence of all 20 amino acids, that was stimulated by the addition of active peptidyl-tRNA hydrolase to the assay.

**Protein synthesis inhibition.** Cultures of *ts*8 or CA244, aerated by bubbling, were brought to exponential growth at 30°C. Portions of the cultures were incubated for 12 min, with or without a drug, and then transferred to a 40°C bath. At various times afterward, 0.5-ml samples were pulse-labeled by incubation at the appropriate temperature with [$^{14}$C]leucine for 1 min, until incorporation was stopped by the addition of trichloroacetic acid [18].

**Cell survival.** Exponentially growing cultures of *ts*8 or CA244 at 30°C were shifted abruptly to a higher temperature by a $10^{-3}$ dilution in aerated growth medium of the same type at the new temperature. At various times after the temperature change, samples of each culture were diluted by a factor of $10^{-2}$ in room temperature growth medium and spread on room temperature nutrient agar plates. The number of colonies formed after overnight incubation at 30°C (permissive temperature) was divided by the number found in a sample taken at the time of the temperature shift to give the fractional survival (21).

**Antibiotics.** Erythromycin base was a gift from Abbott Laboratories, North Chicago, Ill., and carbomycin was a gift from Pfizer Inc., New York, N.Y. Spiramycin was a gift from Rhodia, Inc., Hamburg, W. Germany, and was a mixture of spiramycins I (63%), II (24%), and III (13%).

## RESULTS

**Inhibition of protein synthesis.** The rates of incorporation of labeled leucine into hot acid-precipitable form were measured for the wild-type and mutant (*pth*) cells in the presence of erythromycin (Fig. 1), carbomycin (data not shown), and spiramycin (data not shown). Because spiramycin did not readily penetrate our strains of *E. coli*, the cells were made more permeable by brief treatment with EDTA before that drug was added (see above). The doses of drug chosen did have inhibitory effects on the rate of [$^{14}$C]leucine incorporation by both the mutant (*pth*) and the wild-type strains (Fig. 1). The inhibition was slight in some cases, because doses were chosen to avoid a cytotoxic effect on the *pth* cells at permissive temperatures. Generally, we found that strains containing the *pth*(Ts) allele tended to be hypersensitive to erythromycin, even at low temperatures (data not shown). In the absence of any drug, the stimulation of the rate of leucine incorporation after the shift to 40°C is to be expected because of the temperature dependence of the protein synthesis step. The abrupt drop in the rate of leucine incorporation by *pth* cells in the absence of drugs is always observed (1, 18); its explanation is still not complete (see reference 20), but we believe that accumulation of peptidyl-tRNA is responsible.

**Cell survival.** The correlation between the cytotoxic effect on *pth* cells raised to nonpermissive temperatures and the accumulation of peptidyl-tRNA (21) allows measurements of the former to assay the latter. Figure 2 shows the survival at the nonpermissive temperature of colony-forming cells from both mutant (*pth*) and wild-type strains treated with erythromycin or carbomycin. It can be seen that the chosen doses blocked the growth of the wild-type cells and enhanced the killing of *pth* cells raised to 40°C, relative to controls without antibiotics. The killing of *pth* cells was also enhanced by treatment with spiramycin (data not shown). The *pth* cells were so susceptible to spiramycin that a 50-fold higher dose was needed to demonstrate growth inhibition of the wild-type cells. The permeabilization treatment needed to ensure access of the drug to the ribosomes in the cells had a negligible effect on cell survival.

**Accumulation of peptidyl-tRNA.** Direct measures of the accumulation of peptidyl-tRNA in mutant (*pth*) and wild-type cells are shown in Fig. 3 and 4. At various times after the shift to the nonpermissive temperature (40°C), cells in 200-ml cultures were arrested by the addition of ice-cold trichloroacetic acid and collected by centrifugation, and their tRNA was extracted with phenol and ultrasonication (18). After partial purification of the tRNA and the removal of its amino acids (but not peptides), its leucine-accepting activity was assayed in the absence and presence of active peptidyl-tRNA hydrolase (18). In the latter case, peptides are removed (18, 20), and the additional leucine-accepting activity thus revealed, expressed as a percentage of the total, is shown in Fig. 3 and 4.

The dissociation from ribosomes of peptidyl-tRNA capable of accepting leucine (peptidyl-

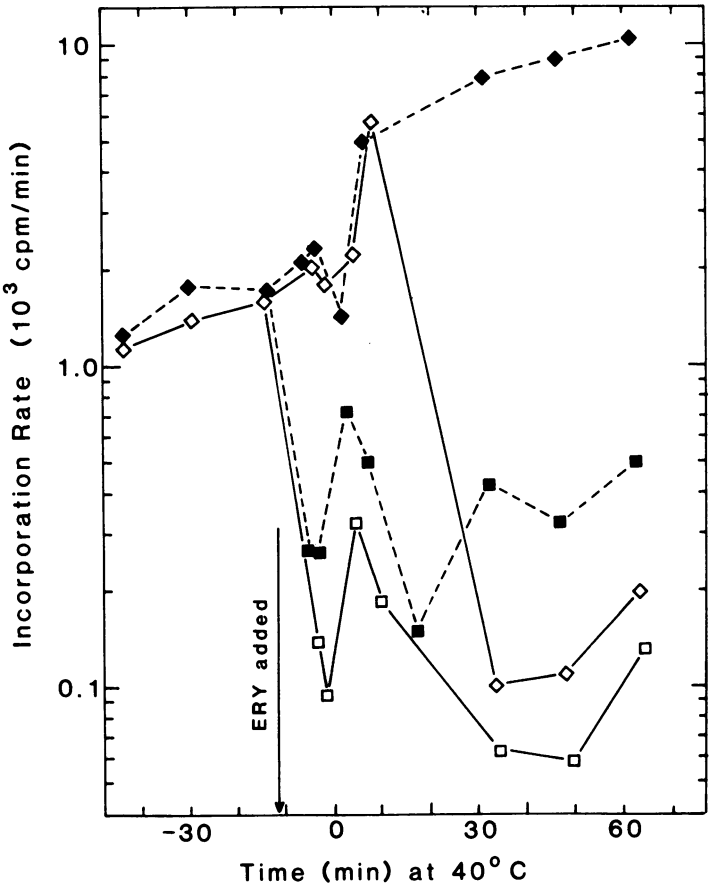


FIG. 1. Time course of hot trichloroacetic acid-precipitable leucine incorporation rates with (□, ■) and without (◇, ◆) erythromycin (ERY). Cultures (10 ml) of *pth*-containing mutant *ts*8 (◇, □) and wild-type strain CA244 (◆, ■) were grown with bubbling aeration at 30°C in phosphate-buffered medium M9 until the tubes were placed in a 40°C bath at time zero. Erythromycin at 80 μg/ml was added to the cultures 12 min before the temperature shift and maintained at that concentration during the 40°C incubation.

$tRNA^{Leu}$), once the peptides were removed, was significantly enhanced in *pth* cells by erythromycin and carbomycin (Fig. 3). In the case of spiramycin, Fig. 4 shows that the rate of accumulation of peptidyl-$tRNA^{Leu}$ in *pth* cells was enhanced, relative to untreated controls, after two doses. The permeabilization treatment alone tended instead to reduce the rate of accumulation of peptidyl-$tRNA^{Leu}$ in *pth* cells was enhanced, relative to untreated controls, after treatment with two doses. The permeabilization treatment alone tended instead to reduce the rate of accumulation of peptidyl-tRNA, relative to completely untreated cells. Neither the antibiotics nor the permeabilization affected the accumulation of peptidyl-$tRNA^{Leu}$ in wild-type cells; its accumulation was negligible.

## DISCUSSION

Peptidyl-tRNA normally dissociates with a low probability from the ribosomes of *E. coli* during protein synthesis (18, 20). In mutant [*pth*(Ts)] cells at nonpermissive temperatures, peptidyl-tRNA accumulates (18, 20) and the cells die (21) because the enzyme peptidyl-tRNA hydrolase, which normally scavenges peptidyl-tRNA, is inactivated rapidly and irreversibly at high temperatures (22).

Each of the three macrolide antibiotics tested, erythromycin, carbomycin, and spiramycin, stimulated the accumulation of peptidyl-$tRNA^{Leu}$ in *pth*(Ts) cells at the nonpermissive temperature (40°C) (Fig. 3 and 4). The cytotoxic effect of accumulating peptidyl-tRNA was also enhanced under those conditions (Fig. 2). The enhanced accumulation must have been due to an enhanced dissociation induced by the antibiotics tested, of peptidyl-tRNA from ribosomes. These results are in agreement with previously reported data for the action of erythromycin on *E. coli* cells (18, 21; J. R. Menninger, Fed. Proc. 33:1334, 1974) and on cell-free extracts (24).

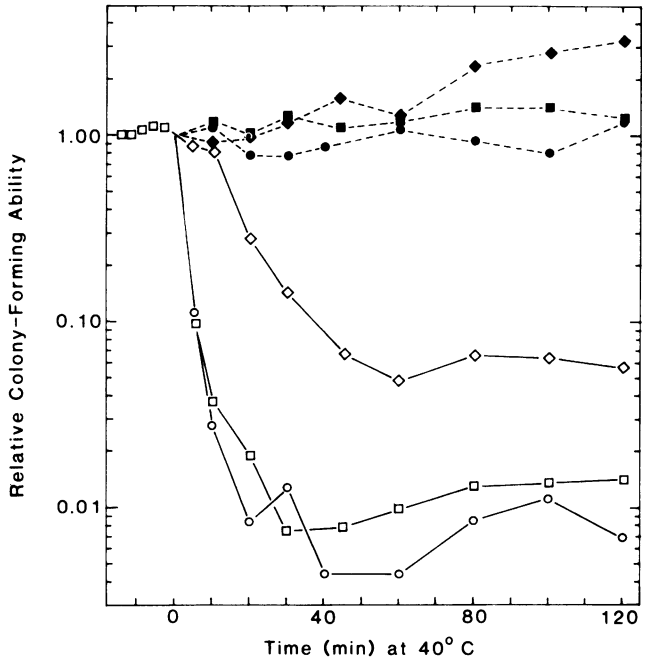


FIG. 2. Time course of survival of *pth*-containing mutant *ts*8 (◇, □, ○) and wild-type strain CA244 (◆, ■, ●) at 40°C in phosphate-buffered medium M9. Part of each culture was treated with either erythromycin at 80 μg/ml (□, ■) or carbomycin at 500 μg/ml (○, ●); the remainder was not exposed to any drug (◇, ◆). Drugs were added 15 min before the temperature shift and maintained at constant concentration during the 40°C incubation.

We think it likely, therefore, that all macrolide antibiotics stimulate the dissociation of peptidyl-tRNA from ribosomes.

Enhanced dissociation of peptidyl-tRNA from ribosomes should affect both cell growth and measurements of protein synthesis. To see how this might happen, one should consider the difficulty of completing the synthesis of a measurable or active protein. If $P_D$ is the probability, per elongation, of dissociation of any peptidyl-tRNA from a ribosome, then $1 - P_D$ is the probability of nondissociation, and $P_C = (1 - P_D)^L$ is the probability that the synthesis of a protein of length = $L$ amino acids will be completed. $P_D$ can be as high as 0.004 for peptidyl-tRNA capable of accepting lysine when measured by our assays in the absence of drugs (20). If some agent set $P_D$ to that level for all peptidyl-tRNAs, it would have the effect of reducing $P_C$ to 0.30, thus wasting 70% of attempted syntheses for a typical protein of 300 amino acids. If macrolides raised $P_D$ to 0.010 for all peptidyl-tRNAs, $P_C$ would be reduced to 0.05 for a protein of 300 amino acids and to $1.6 \times 10^{-5}$ for a protein as long as β-galactosidase ($L$ = 1,100). It is clear that this response would distort the metabolism of any cell or organelle for which continual protein synthesis is necessary. Stimulation of the dissociation of peptidyl-tRNA from ribosomes is thus, we believe, a sufficient mechanism to explain the inhibition of cell growth by macrolide antibiotics.

As the probability of dissociation of peptidyl-tRNA rises, the synthesis of peptides measurable by trichloroacetic acid precipitation should eventually become inhibited. Assuming that peptides must be at least 20 amino acids long to be acid precipitable, the average $P_D$ must have been at least 0.060 to account for the 70% reduction in protein synthesis seen in Fig. 1 for *pth*$^+$-containing strain CA244 cells in the presence of erythromycin.

This $P_D$ value could be an overestimate of the effect of the antibiotic alone. Since not all peptidyl-tRNAs are attacked by hydrolase at equal rates (13), it seems likely that the accumulation of some peptidyl-tRNAs, undetected in our present experiments, might occur in even wild type cells treated with macrolide antibiotics as the hydrolase becomes overloaded (no conditions have been found that induce additional hydrolase activity [P.-F. Tan, M.S. thesis, University of Oregon, Eugene, 1972]). The accumulation of peptidyl-tRNAs is equivalent to starvation for the amino acid normally acylated to the tRNAs that are so sequestered. In *pth*(Ts)-containing strain ts8, it has been shown that starvation for an amino acid enhances the dissociation of peptidyl-tRNA from ribosomes (4). By this means, a weaker stimulation of dissociation of

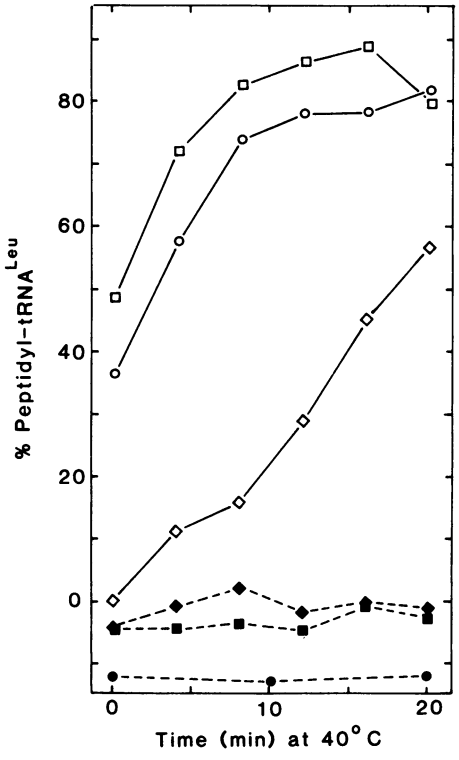


FIG. 3. Time course of accumulation of peptidyl-tRNA$^{Leu}$ at 40°C in cells of *pth*-containing mutant *ts*8 (□, ○, ◇) and wild-type strain CA244 (■, ●, ◆) in phosphate-buffered medium M9. The effect of erythromycin (□, ■) and carbomycin (○, ●) as well as the accumulation of peptidyl-tRNA$^{Leu}$ in cells not treated with antibiotics (◇, ◆), are shown. Carbomycin at 500 μg/ml (○, ●) or erythromycin at 80 μg/ml (□, ■) was added 15 min before the temperature shift and maintained at those concentrations during the 40°C incubation. The fraction of leucine-accepting tRNA that was able to be labeled with radioactive leucine after peptides were removed is plotted as a percentage.

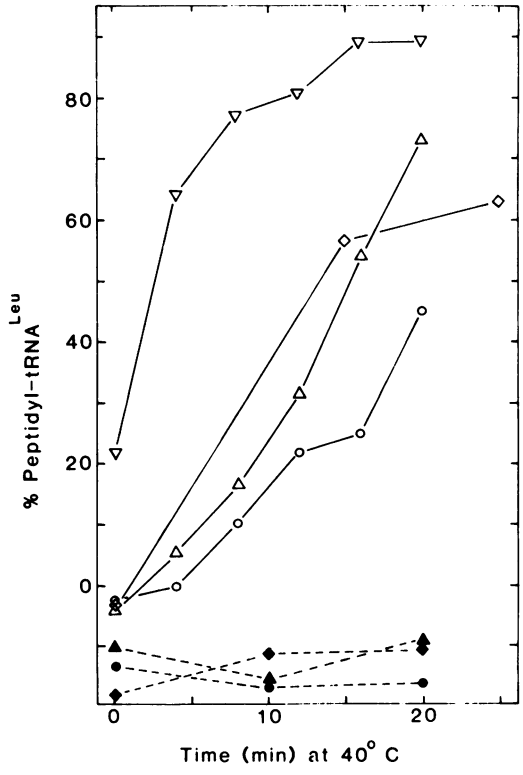


FIG. 4. Time course accumulation of peptidyl-tRNA$^{Leu}$ at 40°C in cells of *pth*-containing mutant *ts*8 (◇, △, ○, ▽) and in wild-type strain CA244 (◆, ▲, ●) in a Tris-buffered medium (see text). The effects on strain *ts*8 of spiramycin at 0.5 μg/ml (△) and at 2.0 μg/ml (▽) and on strain CA244 of spiramycin at 25 μg/ml (▲) are shown. Spiramycin was added 15 min before the temperature shift and maintained at constant concentration during the 40°C incubation. Cells were treated with spiramycin after being permeabilized with 0.9 mM EDTA (see text). For comparison, peptidyl-tRNA accumulation with neither permeabilization nor drug treatment (◇, ◆) and with permeabilization alone (○, ●) is shown.

peptidyl-tRNA by macrolide antibiotics could become amplified and lead nevertheless to the observed inhibition of acid-precipitable amino acid incorporation and cell growth.

It is important to realize the quantitative implications of the data Fig. 3 and 4. The number of tRNAs in an *E. coli* cell growing in a glucose medium is approximately nine times the number of ribosomes (12). When peptidyl-tRNA accumulates to over 80% of total tRNA, it means that the average ribosome must have participated in more than seven rounds of peptidyl-tRNA synthesis and dissociation. If leucine-accepting tRNAs behaved the same as other tRNAs, which we have every reason to believe is the case (20), and if all of the ribosomes were blocked, their total peptidyl-tRNA content could account for less than one-seventh of the observed dissociated and accumulated peptidyl-tRNA$^{Leu}$. This finding rules out mechanisms in which the ribosome is permanently blocked by a macrolide during protein synthesis. Since all of the tested drugs enhanced the dissociation of peptidyl-tRNA from ribosomes, and since that dissociation was sufficient to explain the observed effects of the drugs on cell growth and protein synthesis, we favor a model in which stimulated dissociation of peptidyl-tRNA from ribosomes is the primary mechanism of action of macrolide antibiotics.

It might be argued that a block at one or another step of peptide chain elongation could itself lead to the dissociation of peptidyl-tRNA. We do not favor this interpretation because it

appears inconsistent with our own data and with those of others. First, a block would be expected to slow overall protein synthesis and, thus, the synthesis and dissociation of peptidyl-tRNA. This can be seen by considering the time necessary to synthesize and dissociate a peptidyl-tRNA of length = $L$ amino acids. If $R_A$ is the rate of addition of amino acids to a growing peptide, then $1/(R_A P_D)$ is the average time necessary to add the last amino acid and dissociate a peptidyl-tRNA. The time necessary to add the previous amino acids, without dissociating peptidyl-tRNA, is $(L - 1)/[R_A(1 - P_D)^{(L-1)}]$. These times are greater than $1/R_A$ and $(L - 1)/R_A$ because of the requirements for dissociation and nondissociation, respectively, that are imposed. The total time necessary to synthesize and dissociate a peptidyl-tRNA of length = $L$ amino acids is the sum of the above times, $(1/R_A)\{1/P_D + [(L - 1)/(1 - P_D)^{(L-1)}]\}$, and the observed rate of dissociation of peptidyl-tRNAs is simply the reciprocal of this total time. It is clear that if macrolides act to reduce $R_A$ without modifying $P_D$, then only a reduction in the rate of dissociation of peptidyl-tRNAs would be expected. Macrolides instead stimulated the dissociation of peptidyl-tRNAs (Fig. 3 and 4). They must, therefore, have had a direct enhancing effect on $P_D$. It is simpler to assume that they had no effect on $R_A$, although if they had, the increase in $P_D$ would have had to have been even greater to explain our results.

Second, we have already published data showing that interference with elongation does not necessarily lead to enhanced dissociation of peptidyl-tRNA. When chloramphenicol is added at low doses to *pth*(Ts)-containing strains, they accumulate peptidyl-tRNA more slowly (18) and die less rapidly (21) than in the absence of drugs. This is a specific example of an agent that lowers $R_A$, with the expected result that the dissociation of peptidyl-tRNA is slowed. Other antibiotics behave similarly. Fusidic acid was tested because it has been suggested that blocking translocation might lead to the dissociation of peptidyl-tRNA (blocking translocation has also been suggested as a mechanism of action for the macrolides [7]). Even with the permeabilization treatment described above, we never measured a large growth-inhibiting effect of fusidic acid on the *E. coli* strain that we used. Nevertheless, the small effect on the cytotoxicity assay that was detected was in the direction of inhibiting the accumulation of peptidyl-tRNA (data not shown).

There is no experimental basis for deciding from which site on the ribosome peptidyl-tRNA dissociates during protein synthesis in the absence of drugs. The ribosome editor hypothesis (19) proposes that peptidyl-tRNA dissociates because its structure is inappropriate for the mRNA codon. However, decoding interactions can occur at both the A and the P sites (10, 14, 16, 17, 28, 32). Cabañas and Modolell (2) have recently shown that inappropriate peptidyl-tRNA can dissociate, with high probability, from the A site, but in their experiments, translocation was blocked, and a less common route of dissociation may thereby have been revealed. Erythromycin has been shown by Pestka (26) to bind to isolated polyribosomes only after they have been treated with puromycin. Furthermore, he showed that puromycin treatment also allows erythromycin, carbomycin, and spiramycin to inhibit the binding of labeled chloramphenicol to the treated polyribosomes. These experiments and others (27) suggest that these three macrolides bind to the 50S ribosomal subunit so as to block the P site. If peptidyl-tRNA already occupies the P site, binding of macrolides doesn't occur; treatment with puromycin allows transfer of the peptide to puromycin and unblocking of the P site. Since the antibiotic must presumably be bound to exert its effect, these findings suggest that the dissociation of peptidyl-tRNA that is stimulated by macrolides occurs either from the ribosomal A site or during translocation. Since erythromycin has been shown not to stimulate the dissociation of a peptidyl-tRNA analog (*N*-acetyl-phenylalanyl-tRNA) from the A site of isolated ribosomes under conditions in which bottromycin $A_2$ does stimulate such dissociation (25), we currently prefer the hypothesis that macrolides stimulate the dissociation of peptidyl-tRNA during attempted translocation from the A site to the P site.

The "breakdown" of polysomes upon treatment with spiramycin (5, 9) and carbomycin (9) has been interpreted as occurring via normal runoff, followed by an antibiotic-induced block at or shortly after initiation of a new peptide. We suggest instead that macrolide-induced polysome breakdown results from stimulation of the dissociation of peptidyl-tRNA, folowed by ribosome dissociation from the message. If $P_D$ is sufficiently large, few polysomes will reform, and few acid-precipitable peptides will be synthesized, even though peptide bond formation may be continuing at near-normal rates. Pretreatment of cells or ribosomes with peptide chain elongation-blocking agents, like chloramphenicol, blocks the dissociation of peptidyl-tRNA (18, 21) and thus should, based on this argument, also block the macrolide-induced loss of polysomes.

Several laboratories have reported that the addition of erythromycin does not lead to the conversion of polysomes to single ribosomes (6, 8, 9). The resulting polysomes are not normal,

however; they apparently engage in peptide bond formation since they can be pulse-labeled with methionine, although not with valine (29). This has been interpreted by Tai et al. (29) as being due to an inhibition by erythromycin of a step after the initiation of protein synthesis, but exactly which step it is has not been specified. Since erythromycin stimulates the dissociation of peptidyl-tRNA, one would expect to find only short peptidyl-tRNAs in association with such polysomes, and whereas each peptidyl-tRNA would be expected to be initiated and labeled with formyl-methionine, the detection of other amino acid labels might be difficult. This is in fact what Tai et al. observed (29). We suggest that the peculiar behavior of erythromycin is due to the stimulation of the dissociation of peptidyl-tRNA but, unlike other macrolides, without the stimulation of the dissociation of deacylated tRNA from the P site. The latter seems reasonable because of the smaller size of erythromycin relative to other macrolides. Carbomycin and spiramycin are antibiotics whose bulk may, upon binding of these antibiotics near the ribosomal P site, destabilize the deacylated tRNA and thus lead to polysome breakdown. If deacylated tRNA remains, perhaps even stabilized by erythromycin, it could act to bind the ribosome to the mRNA, resulting in the lack of polysome breakdown, despite the loss of peptidyl-tRNA. The inhibition by erythromycin of dissociation of deacylated tRNA from isolated ribosomes was reported some years ago (11).

We believe that our proposed mechanism of action for macrolide antibiotics can explain other apparently conflicting data on these drugs. In cell-free reactions programmed with polyadenylate, the observed stimulation of synthesis of shorter oligolysines coupled with inhibition of synthesis of longer oligolysines in the presence of erythromycin (15, 23, 30) and spiramycin (15) is consistent with our proposed mechanism. Active peptidyl-tRNA hydrolase in the cell-free extracts generally used will convert dissociated peptidyl-tRNA to free peptides which can accumulate. Dissociation of short peptidyl-tRNAs prevents their being used as substrates for further chain growth. Our model thus provides a mechanism for the suggestion by Mao and Robishaw (15) that transfer of large peptides is inhibited.

There are previous reports that have been interpreted (7) as failing to demonstrate the dissociation of peptidyl-tRNA from ribosomes. We suggest that those experimental designs would have minimized the chances of observing the phenomenon. Cundliffe and McQuillen (8) and Cundliffe (6) measured the effects of various drugs on the ability of ribosomes to transfer peptides to puromycin. After labeling protoplasts of *Bacillus megaterium* with radioactive amino acids and then adding erythromycin at 250 to 300 μg/ml for 3 or 5 min, they found little or no loss of nascent radioactivity from ribosomes after adding puromycin. If the protoplasts were treated with chlortetracycline instead of erythromycin, a great deal of nascent radioactivity loss occurred. During the treatment with erythromycin, however, the labeled amino acids were still present; ribosomes that had lost peptidyl-tRNA could have synthesized more. Depending on the details of amino acid pool equilibration and lengths of peptides, resynthesis of peptidyl-tRNA could have produced ribosomes with labeling equivalent to ribosomes treated with drugs that halt peptide chain elongation. In any case, Cundliffe and McQuillen (8) showed sucrose density gradient profiles that suggested a significant reduction in the specific labeling of ribosomes treated with erythromycin, relative to those not treated.

The results of Cannon and Burns (3) also cannot be used to assess the loss of peptidyl-tRNA induced by erythromycin. When they added 250 μg of erythromycin per ml to ribosomes actively synthesizing protein in cell-free extracts, they detected an inhibition of the ability of the reisolated ribosomes to transfer peptides to puromycin. We interpret this result as showing that peptidyl-tRNA was located largely in the A site after such treatment and was unable to be donated to puromycin. After the erythromycin treatment, the ribosomes were reisolated by sedimentation, and any dissociated peptidyl-tRNA would have been removed. The authors did not give data on the amount of radioactivity in the peptidyl-tRNA remaining on reisolated ribosomes, which our model predicts should be less than that on untreated ribosomes.

Differential effects of various macrolides on the cell-free synthesis of different peptides (reviewed in reference 7) may be explained by differential effects on the rates of dissociation of different peptidyl-tRNAs from the ribosomes. We have reported that different peptidyl-tRNA families can dissociate from ribosomes with a 30-fold range of rates (20). The final resolution of this point would require a systematic analysis of the response to the various macrolide antibiotics of the dissociation as peptidyl-tRNA of the 20 tRNA families.

In summary, we have shown that representatives of three functional groups of macrolide antibiotics (carbomycin and niddamycin; spiramycin and tylosin; erythromycin and oleandomycin) all enhanced the dissociation of peptidyl-tRNA from ribosomes of treated cells. Many confusing past observations on these drugs can be explained by this reponse. It seems likely that stimulated dissociation of peptidyl-tRNA from

ribosomes is the major mechanism of action of macrolide antibiotics.

## ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM 20626 from the National Institute of General Medical Sciences.

## LITERATURE CITED

1. **Atherly, A. G., and J. R. Menninger.** 1972. Mutant *E. coli* strain with temperature sensitive peptidyl-transfer RNA hydrolase. Nature (London) New Biol. **240**:245–246.
2. **Cabañas, M. J., and J. Modolell.** 1981. Nonenzymatic translocation and spontaneous release of noncognate peptidyl-tRNA from *Escherichia coli* ribosomes. Biochemistry **20**:5411–5416.
3. **Cannon, M., and K. Burns.** 1971. Modes of action of erythromycin and thiostrepton as inhibitors of protein synthesis. FEBS Lett. **18**:1–5.
4. **Caplan, A. B., and J. R. Menninger.** 1979. Tests of the ribosome editing hypothesis: amino acid starvation differentially enhances the dissociation of peptidyl-tRNA from the ribosome. J. Mol. Biol. **134**:621–637.
5. **Cundliffe, E.** 1969. Antibiotics and polyribosomes. II. Some effects of lincomycin, spiramycin and streptogrammin A *in vivo*. Biochemistry **8**:2063–2066.
6. **Cundliffe, E.** 1972. Bacterial protein synthesis: the effects of antibiotics upon the puromycin reaction *in vivo*, p. 242–261. *In* E. Muñoz, F. Garcia-Ferrandiz, and D. Vazquez (ed.), Molecular mechanisms of antibiotic action on protein biosynthesis and membranes. Elsevier/North-Holland Publishing Co., Amsterdam.
7. **Cundliffe, E.** 1981. Antibiotic inhibitors of ribosome function, p. 402–547. *In* E. F. Gale, E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. S. Waring (ed.), Molecular basis of antibiotic action. John Wiley & Sons, Inc., New York.
8. **Cundliffe, E., and K. McQuillen.** 1967. Bacterial protein synthesis: the effects of antibiotics. J. Mol. Biol. **30**:137–146.
9. **Ennis, H. L.** 1972. Polysome metabolism in *Escherichia coli*: effect of antibiotics on polysome stability. Antimicrob. Agents Chemother. **1**:197–203.
10. **Fairclough, R. H., and C. R. Cantor.** 1979. The distance between the anticodon loops of two tRNAs bound to the 70S *Escherichia coli* ribosome. J. Mol. Biol. **132**:575–586.
11. **Igarashi, K., H. Ishitsuka, and A. Kaji.** 1969. Comparative studies on the mechanism of action of lincomycin, streptomycin and erythromycin. Biochem. Biophys. Res. Commun. **37**:499–504.
12. **Kjeldgaard, N. O., and K. Gausing.** 1974. Regulation of biosynthesis of ribosomes, p. 369–392. *In* M. Nomura, A. Tissières, and P. Lengyel (ed.), Ribosomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Kössel, H.** 1970. Purification and properties of peptidyl-tRNA hydrolase from *Escherichia coli*. Biochim. Biophys. Acta **204**:191–202.
14. **Kuechler, E., and J. Ofengand.** 1979. Affinity labeling of tRNA binding sites on ribosomes, p. 413–444. *In* P. Schimmel, D. Söll, and J. Abelson (ed.), Transfer RNA, part 1: structure, properties and recognition. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Mao, J. C.-H., and E. E. Robishaw.** 1971. Effects of macrolides on peptide-bond formation and translocation. Biochemistry **10**:2054–2061.
16. **Matzke, A. J. M., A. Barta, and E. Kuechler.** 1980. Mechanism of translocation: relative arrangement of tRNA and mRNA on the ribosome. Proc. Natl. Acad. Sci. U.S.A. **77**:5110–5114.
17. **Matzke, A. J. M., A. Barta, and E. Kuechler.** 1980. Photo-induced crosslinking between phenylalanine transfer RNA and messenger RNA on the *Escherichia coli* ribosome. Eur. J. Biochem. **112**:169–178.
18. **Menninger, J. R.** 1976. Peptidyl transfer RNA dissociates during protein synthesis from ribosomes of *Escherichia coli*. J. Biol. Chem. **251**:3392–3398.
19. **Menninger, J. R.** 1977. Ribosome editing and the error catastrophe hypothesis of cellular aging. Mech. Aging Dev. **6**:131–142.
20. **Menninger, J. R.** 1978. The accumulation as peptidyl-transfer RNA of isoaccepting transfer RNA families in *Escherichia coli* with temperature-sensitive peptidyl-transfer RNA hydrolase. J. Biol. Chem. **253**:6808–6813.
21. **Menninger, J. R.** 1979. Accumulation of peptidyl tRNA is lethal to *Escherichia coli*. J. Bacteriol. **137**:694–696.
22. **Menninger, J. R., C. Walker, P.-F. Tan, and A. G. Atherly.** 1973. Studies on the metabolic role of peptidyl-tRNA hydrolase. I. Properties of a mutant *E. coli* with temperature-sensitive peptidyl-tRNA hydrolase. Mol. Gen. Genet. **121**:307–324.
23. **Oleinick, N. L., and J. M. Corcoran.** 1970. Evidence of a limited access of erythromycin A to functional polysomes and its action on bacterial translocation, p. 202–208. *In* H. Umezawa (ed.), Progress in antimicrobial and anticancer chemotherapy, vol. 1. University Park Press, Baltimore, Md.
24. **Otaka, T., and A. Kaji.** 1975. Release of (oligo)peptidyl-tRNA from ribosomes by erythromycin A. Proc. Natl. Acad. Sci. U.S.A. **72**:2649–2652.
25. **Otaka, T., and A. Kaji.** 1976. Mode of action of bottromycin $A_2$. Release of aminoacyl- or peptidyl-tRNA from ribosomes. J. Biol. Chem. **251**:2299–2306.
26. **Pestka, S.** 1974. Antibiotics as probes of ribosome structure: binding of chloramphenicol and erythromycin to polyribosomes; effect of other antibiotics. Antimicrob. Agents Chemother. **5**:255–267.
27. **Pestka, S., A. Nakagawa, and S. Ōmura.** 1974. Effect of leucomycins and analogues on binding [$^{14}$C]erythromycin to *Escherichia coli* ribosomes. Antimicrob. Agents Chemother. **6**:606–612.
28. **Peters, M., and M. Yarus.** 1979. Transfer RNA selection at the ribosomal A and P sites. J. Mol. Biol. **134**:471–491.
29. **Tai, P. C., B. J. Wallace, and B. D. Davis.** 1974. Selective action of erythromycin on initiating ribosomes. Biochemistry **13**:4653–4659.
30. **Tanaka, T., and H. Teraoka.** 1966. Binding of erythromycin to *Escherichia coli* ribosomes. Biochim. Biophys. Acta **114**:204–206.
31. **Vazquez, D.** 1974. Inhibitors of protein synthesis. FEBS Lett. **40**(Suppl.):563–584.
32. **Wurmbach, P., and K. H. Nierhaus.** 1979. Codon-anticodon interaction at the ribosomal P (peptidyl-tRNA) site. Proc. Natl. Acad. Sci. U.S.A. **76**:2143–2147.