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Selective Action of Erythromycin on Initiating Ribosomes†

Phang-Cheng Tai, Brian J. Wallace,‡ and Bernard D. Davis*

ABSTRACT: Polysomal ribosomes of *Escherichia coli,* engaged in chain elongation, are shown to have a much lower affinity than free ribosomes for erythromycin (Ery), and they are 1000-fold less sensitive to inhibition by the antibiotic. The difference depends on peptidyl-tRNA: "pseudopolysomes," containing mRNA but no nascent peptide, are highly sensitive, like free ribosomes. Ery blocks initiating ribosomes not in formation of the initiation complex but in some later reaction, which is neither recognition of the next aminoacyl-tRNA nor peptidyl transfer of fMet. It is not certain whether the blocked reaction is the first translocation or some step in a later round of translation. The blocked ribosomal complexes are evidently unstable, for in cells inhibited by Ery the polysomes turn over extensively without protein synthesis, and they incorporate methionine but not valine. This cyclic blockade of initiation sites can explain the dominance of sensitivity over resistance in Ery^S/Ery^R heterozygotes.

Erythromycin A (Ery), a macrolide antibiotic, blocks protein synthesis in cells and extracts of sensitive bacteria, and it has been found to bind to the 50S ribosomal subunit (Taubman *et al.,* 1966; Wilhelm and Corcoran, 1967; Mao, 1967; Mao and Putterman, 1969; Teraoka, 1970). However, despite extensive studies during the past few years the mechanism of the inhibition remains unclear.

We have approached this problem by using purified polysomes of *Escherichia coli,* which can carry out peptide chain elongation but not reinitiation (Tai *et al.,* 1973b). The results show that Ery, like several other antibiotics (Tai *et al.,* 1973a; Wallace *et al.,* 1973, 1974), specifically inhibits initiating ribosomes and not elongating ribosomes. Moreover, the ribosomes are blocked by Ery shortly after initiation, and the blocked complexes are unstable, as shown by the turnover of polysomal ribosomes in cells. This cyclic blockade of initiation sites can explain the dominance of sensitivity over resistance to Ery in heterozygotes, just as similar findings have explained the dominance of sensitivity to streptomycin (Wallace and Davis, 1973) or to spectinomycin (Wallace *et al.,* 1974).

Materials and Methods

Bacterial strains, growth conditions, the preparations used for protein synthesis (S30 extracts, supernatant factors, crude initiation factors (IF), NH_4Cl-washed ribosomes, and phage R17 RNA), and the conditions of synthesis have been described (Tai *et al.,* 1973b).

Preparation of IF-Free Polysomes. Purified polysomes were prepared by gel filtration on Sepharose 4B from lysates of *E. coli* strain MRE600 (endogenous polysomes), and also from an S30 extract of *E. coli* strain S26 incubated with phage R17 RNA (R17 polysomes). Only preparations essentially free of reinitiation (Tai *et al.,* 1973b) were used.

Binding of [^{14}C]*Erythromycin.* Reaction mixtures (in 0.2 ml) contained buffer A (10 mM Tris-HCl (pH 7.6), 50 mM NH_4Cl, 8 mM $Mg(OAc_2)$, 1 mM dithiothreitol, about 2 A_{260} units (46 pmol) of ribosomes, and 0.75 μM [^{14}C]-*N*-methylerythromycin (11.3 μCi/mg = 13.5 cpm/pmol). After incubation 3 ml of buffer A was added and samples

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[1] Abbreviations used are: Ery, erythromycin; IF, initiation factor(s).

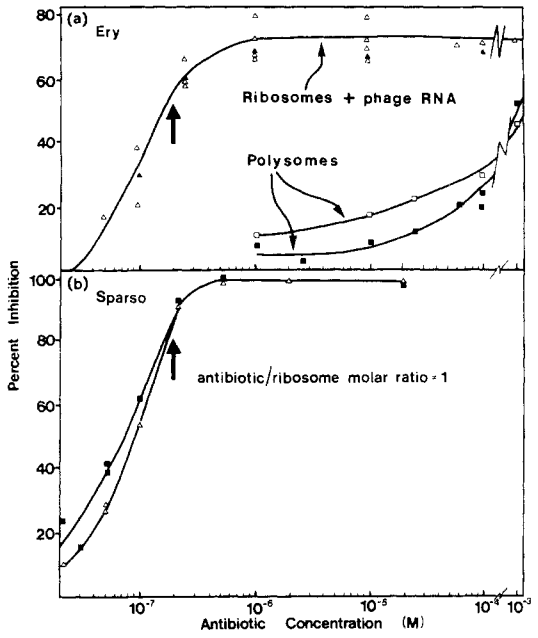

FIGURE 1: Inhibition by erythromycin and sparsomycin of polypeptide synthesis on initiating ribosomes and IF-free polysomes. Reaction mixtures (0.1 ml) were incubated at 34°, with antibiotics at the concentrations indicated. Noninitiating systems, containing either 50 μg of IF-free endogenous polysomes (■) or 25 μg of R17-polysomes (□) (Materials and Methods), were incubated for 6 min. Initiating mixtures, containing preincubated S30 extracts (▲) or NH_4Cl-washed ribosomes plus initiation factors (△), and supplemented with R17 RNA, were incubated for 30 min. The incorporation of [^{14}C]valine (in the presence of the other amino acids) was determined, and the results were expressed as per cent inhibition compared to the control without antibiotic (ranging from 3200 to 8800 cpm in different experiments).

were analyzed for radioactivity retained by Millipore filters (Teraoka, 1970).

Chemicals and Reagents. [^{3}H]Puromycin hydrochloride and radioactive amino acids were from New England Nuclear Corp. Ery and [^{14}C]-*N*-methylerythromycin were generous gifts of Dr. J. Mao of Abbott Laboratories (North Chicago, Ill.). In some experiments Ery lactobionate (Abbott Laboratories) was used, with identical results. Sparsomycin was a gift of the Cancer Chemotherapy Service Center, Bethesda, Md., and Sepharose 4B and 6B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Partially purified EFG factor was a gift of Dr. J. Bodley. Other chemicals were from commercial sources.

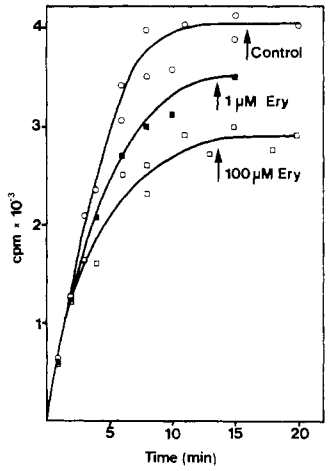

FIGURE 2: Kinetics of inhibition of peptide synthesis on endogenous polysomes. Reaction mixtures with endogenous polysomes, as in Figure 1, were incubated with Ery as indicated, and incorporation was analyzed in samples taken at intervals.

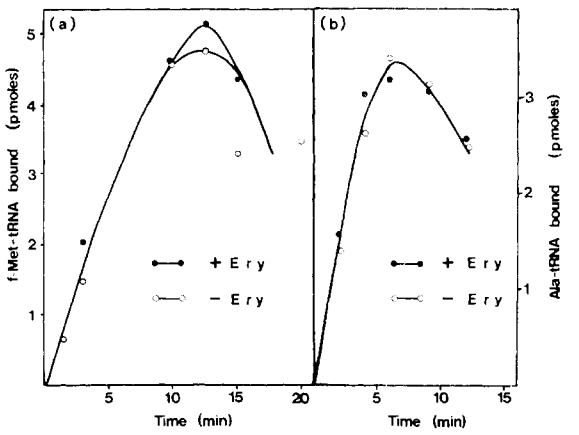

FIGURE 3: Lack of inhibition of binding of fMet-tRNA and Ala-tRNA. NH_4Cl-washed ribosomes (60 μg), with or without 2.5 μM Ery, were incubated as previously described (Wallace *et al.*, 1974) with R17 RNA, the other required components, and either (a) [^{3}H]fMet-tRNA (1350 cpm/pmol) or (b) fMet-tRNA and [^{3}H]Ala-tRNA (1950 cpm/pmol). Retention of radioactivity on Millipore filters was measured. Background retention was subtracted: 1.5 pmol in (a), without R17 RNA; 0.7 pmol in (b), without GTP. Experiments (a) and (b) were carried out on different days.

Results

Sensitivity of Initiating Ribosomes and Purified Polysomes to Inhibition by Erythromycin. Peptide synthesis by free ribosomes initiating on phage RNA, and dependent on initiation factors, is very sensitive to Ery: inhibition was 50% at 0.1 μM (Figure 1a). Similar results were seen whether the free ribosomes were obtained by washing with NH_4Cl or by runoff in crude lysates. In contrast, endogenous IF-free polysomes were about 1000-fold less sensitive (Figure 1a), and moderate concentrations of the antibiotic had little effect on their rate of incorporation or on its extent (Figure 2). Moreover, the difference was not due to the messenger, for polysomes formed *in vitro* on phage R17 RNA and then purified gave the same results as endogenous polysomes (Figure 1a).

In a control test for the responsiveness of the polysomal ribosomes they were found to be as sensitive to sparsomycin as initiating ribosomes (Figure 1b).

Formation of Initiation Complexes in the Presence of Erythromycin. The specific sensitivity of initiating ribosomes to Ery does not appear to depend on inhibition of the formation of initiation complexes: R17 RNA-directed binding of [^{3}H]fMet-tRNA to free ribosomes was unaffected (in kinetics or in extent; Figure 3a), just as has been noted for AUG-directed binding of fMet-tRNA (Mao and Robishaw, 1971, 1972) and for poly(U)-directed binding of *N*-Ac-Phe-tRNA (Oleinick and Corcoran, 1970). Ery must therefore block a step following initiation.

Failure of Erythromycin to Inhibit Recognition and Peptide Bond Formation. The next step in protein synthesis, after formation of initiation complexes, is recognition of the aminoacyl-tRNA coded for after fMet-tRNA. With phage R17 RNA this would be largely Ala-tRNA, in the coat protein; and the presence of Ery, during formation as well as testing of the complexes, did not affect their binding of [^{3}H]Ala-tRNA (Figure 3b).

This binding may reflect not only recognition but subsequent peptidyl transfer, resulting in dipeptide formation. Peptidyl transfer was first tested for with a model reaction:

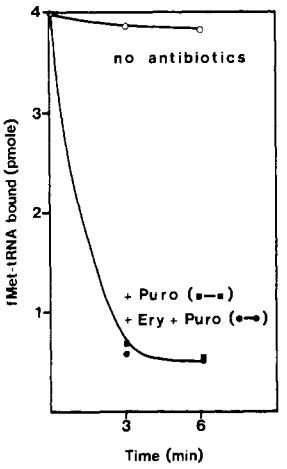

FIGURE 4: Release of [^{3}H]fMet from preformed initiation complexes by puromycin in the presence of erythromycin. Initiation complexes were prepared as described (Wallace *et al.*, 1974), and portions containing 4.8 pmol of bound [^{3}H]fMet-tRNA and 18 pmol of ribosomes, in 0.05 ml of buffer A with 1 mM dithiothreitol, were incubated with or without 30 μM puromycin and 50 μM Ery. At the times indicated samples were analyzed as in Figure 3a for bound [^{3}H]fMet-tRNA; a blank value of 0.3 pmol, obtained with 0 Mg^{2+}, was subtracted.

release by puromycin of [^{3}H]fMet from preformed initiation complexes, made with R17 RNA. As Figure 4 shows, Ery, added to isolated complexes, did not affect this reaction. Similar results were obtained when Ery was added before initiation complex formation, to ensure its binding to the ribosomes (see below); and this addition also failed to inhibit formation of fMet-Ala (unpublished observations). These findings indicate that Ery inhibits some step after the first peptidyl transfer.

Binding of Erythromycin to Free and to Polysomal Ribosomes. At 34° free ribosomes were found to bind 1 molar equiv of Ery, at moderate concentrations, as previously reported (Oleinick and Corcoran, 1969; Mao and Putterman, 1969; Otaka *et al.*, 1970), but a purified polysome preparation bound only 0.2 molar equiv (Table I). Moreover, at 0°, where there would be little runoff during the binding assay, binding by the polysome preparation was reduced to a negligible level while binding by the free ribosomes showed only a small decrease (Table I). When purified polysomes were converted to free ribosomes in the presence of [^{14}C]Ery, either gradually by runoff or more rapidly by puromycin, the release was accompanied by a proportional increase in the binding of the antibiotic (Figure 5). (Control tests showed no effect of puromycin on the binding of Ery to free ribosomes.) It seems clear that polysomal ribosomes have a low affinity for Ery, which can account for their insensitivity to inhibition by the antibiotic.

Aspects of Polysomal Ribosomes that Prevent Binding of Erythromycin. To distinguish whether the low affinity of polysomal ribosomes for Ery is due to bound mRNA or whether it also requires bound peptidyl-tRNA, purified polysomes were freed of the latter by incubation with puromycin, EFG, and GTP, in the absence of the supernatant ribosome release factor (Hirashima and Kaji, 1972; P.-C. Tai and B. D. Davis, unpublished). As Table II shows, at 10 min almost all of the ribosomes remained on the messenger, though most of the nascent peptide had been released. Moreover, following this release the resulting "pseudopolysomes" bound 1 molar equivalent of Ery per ribosome, just like the free ribosomes released in the presence of S100.

TABLE I: Binding of [^{14}C]Erythromycin to Free Ribosomes and Purified Polysomes.[a]

Conditions	[^{14}C]-Erythromycin Bound (pmoles)	Molar Ratio Ery: Ribosome
(A) Free ribosomes		
0°	34.8	0.81
34°	43.5	1.01
(B) Polysomes		
0°	2.4	0.05
34°	8.6	0.20

[a] Free ribosomes (NH_4Cl washed) or purified polysomes were incubated with 0.75 μM [^{14}C]Ery for 10 min at 0 or 34°, filtered, and counted, as described in Materials and Methods. Background retention without ribosomes (83 cpm = 6.1 pmol) has been subtracted.

Thus bound mRNA without peptidyl-tRNA does not impair the binding of Ery.

Table II further shows that in the absence of EFG and GTP puromycin caused only partial release of nascent peptides, the remainder presumably residing in the A site; and this partial release was accompanied by a proportionate increase of Ery binding from 0.19 to 0.72 molecule per ribosome. Further addition of EFG and GTP, enabling peptidyl-tRNA to move from the A site to the puromycin-reactive P site, led to complete release, and the binding value rose to 1.0. We conclude that the presence of peptidyl-tRNA on the A site, just as on the P site, prevents the high-affinity binding of Ery observed with free ribosomes.

Effect of Erythromycin on Polysome Metabolism in Cells. Cells inhibited by Ery maintain a high level of polysomes (Ennis, 1972). Indeed, with 100 μg/ml, which inhibited protein synthesis over 99% after 5 min, the polysome level remained substantial for at least 40 min (Figure 6a). However, these polysomes are not static: just as in cells inhibited by streptomycin (Wallace and Davis, 1973) or by spectinomycin (Wallace *et al.*, 1974), they consist of recycling unstable complexes blocked shortly after initiation.

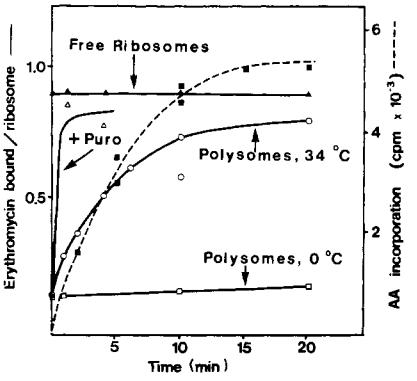

FIGURE 5: Effect of polysome runoff on erythromycin binding. Purified polysomes (39 pmol in 0.2 ml), in a complete protein-synthesizing mixture, were incubated at 34° either with unlabeled amino acids and 0.75 μM [^{14}C]Ery (○——○) or with [^{14}C]valine and unlabeled Ery (■- - -■); samples were analyzed at intervals for bound Ery or for valine incorporation. Binding of Ery was also assayed with polysomes at 0° (□——□); with polysomes at 34° in the presence of 50 μM puromycin (△——△), and with NH_4Cl-washed ribosomes at 34° under identical conditions (▲——▲). Background retention of [^{14}C]Ery without ribosomes (5 pmol) has been subtracted.

TABLE II: Binding of [^{14}C]Erythromycin to Ribosomes in Various States.[a]

Conditions	(A) Polysome Content	(B) Peptidyl-[^{3}H]-puromycin Formed (pmoles)	(C) [^{14}C]Ery Bound (molecules per ribosome)
(1) Control: Polysomes + Ery in buffer	90%		0.19
(2) Partial pseudopolysomes = (1) + puromycin	90%	11.9	0.72
(3) Complete pseudopolysomes = (1) + puromycin, EFG, and GTP-generating system	90%	18.3	0.98
(4) Free ribosomes = (1) + puromycin, S100, and GTP-generating system	10%		1.00

[a] Purified polysomes were incubated at 34° for 10 min with Ery (0.75 μM), as in Table I, together with other components as noted. The polysomes were converted to "pseudopolysomes" by treatment with puromycin in a purified system lacking the ribosome release factor of Hirashima and Kaji (1972). Conversion was incomplete (condition 2) unless the components required for translocation were also added (condition 3). (The GTP-generating system consisted of 1 mM ATP-Tris, 0.02 mM GTP, 5 mM potassium phosphoenolpyruvate, and 30 μg/ml of pyruvate kinase; EFG was used at 100 μg/ml.) As a control (condition 4) polysomes were converted to free ribosomes by incubation with puromycin plus all factors, provided as S100 at 1/10 volume. The proportion of polysomes (and of pseudopolysomes) was determined in a sucrose gradient as previously described (Tai *et al.*, 1973b). In tubes of similar composition the labeling was varied for different purposes. (A) For measurement of survival of polysomes or pseudopolysomes (Tai *et al.*, 1973b) the incubation mixtures contained unlabeled Ery and unlabeled puromycin (50 μM). (B) To determine the extent of pseudopolysome formation [^{3}H]puromycin (4 μM; 935 Ci/mol) was used, with 23 pmol of ribosomes in 0.1 ml, and Cl_3CCOOH precipitable peptidyl-puromycin was assayed (Pestka, 1972a); background retention by the filter (1.2 pmol), determined in the presence of free ribosomes which carry no nascent peptide, was subtracted. (C) To determine Ery binding, as in Table I, [^{14}C]Ery (0.75 μM) was used and the puromycin (50 μM) was unlabeled; background retention without ribosomes (5.6 pmol) was subtracted.

Thus when renewal of mRNA was prevented, by addition of rifampicin, Ery-inhibited cells soon lost their polysomes (Figure 6a). Moreover, these polysomes could be shown to carry blocked initiation complexes, rather than the usual nascent peptides: Ery-treated cells, no longer synthesizing protein, could incorporate pulsed [^{3}H]methionine into their polysomes, but not [^{14}C]valine (Figure 7).

The breakdown of these Ery-blocked polyinitiation complexes is not as rapid as the runoff of the polysomes in growing cells. Thus, the extent of pulse-labeling with [^{3}H]methionine was lower in cells inhibited by Ery than in growing cells (Figure 7), and Ery decreased the rate of polysome loss after addition of rifampicin (Figure 6a).

The inhibition of polysomes by high concentrations of Ery, observed in extracts (Figure 1a), also results in a slowing of polysome runoff in cells, tested in the presence of rifampicin to prevent messenger renewal (compare Ery + Rif in Figure 6b with Rif alone in Figure 6a). In addition, with Ery alone the higher concentration tested (1000 μg/ml), despite its slowing of runoff, caused a faster drop in the polysome level than the lower concentration, 100 μg/ml (Figure 6b *vs.* 6a). This result suggests that the high concentration inhibits reinitiation. This conclusion was confirmed by the virtual absence of any effect of rifampicin on the rate of polysome loss in the presence of 1000 μg/ml of Ery (Figure 6b), contrasted with its marked effect in the presence of 100 μg/ml of Ery (Figure 6a).

Discussion

Difference in Interaction of Erythromycin with Free Ribosomes and with Polysomal Ribosomes. Earlier studies have already suggested that Ery fails to inhibit elongating ribosomes, for it had only a slight effect on amino acid incorporation by S30 extracts of *Staphylococcus aureus* (Mao and Putterman, 1968), *Bacillus subtilis* (Oleinick and Corcoran, 1970), or *Escherichia coli* (Cannon and Burns, 1971). However, the fragmented polysomes in such preparations have low overall endogenous activity, and so a significant fraction of the incorporation might reflect processes other than protein synthesis. Moreover, the partial inhibition that was observed might reflect either a slight effect on elongating ribosomes or a greater effect on a few reinitiating ribosomes. We have therefore tested more directly the effects of Ery on initiating ribosomes (*i.e.*, free ribosomes of *E. coli* in the presence of phage R17 RNA and IF) and on elongating ribosomes (*i.e.*, purified initiation-free polysomes; Tai *et al.*, 1973b).

Initiating ribosomes were found to be very sensitive to Ery, inhibition of amino acid incorporation being nearly maximal at 0.2 μM (Figure 1), which provides 1 molecule/ribosome. In contrast, with purified polysomes on the same messenger (or on bacterial mRNA) this concentration of Ery had very little effect, either on the extent (Figure 1) or on the kinetics (Figure 2) of incorporation. It is thus clear that at the concentrations encountered in chemotherapy Ery inhibits only initiating ribosomes. It evidently blocks these ribosomes at some step following initiation, as will be discussed below, for it does not impair formation of initiation complexes (Figure 3).

The low sensitivity of elongating ribosomes to Ery is evidently due to low affinity for the antibiotic. This possibility was first suggested by observations with a crude lysate (Oleinick and Corcoran, 1970), in which release of the peptidyl-tRNA by puromycin increased the binding of Ery from 0.8 to 1.1 molecules per ribosomes. With purified polysomes we have been able to demonstrate a much larger difference: a preparation of elongating ribosomes bound less than one-fifth as much Ery (at 0.75 μM) as did free ribosomes (Table I); similar results have been reported by Pestka (1974). Much of this binding may have been on free ri-

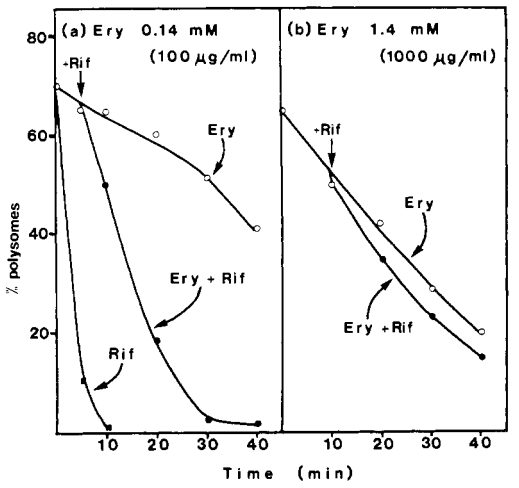

FIGURE 6: Effect of erythromycin on polysome metabolism in cells. To growing cultures of *E. coli* MRE600, at 2×10^8 cells/ml, Ery as indicated, or rifampicin (200 μg/ml), was added at 0 time; rifampicin was also added, after several minutes (vertical arrow), to part of each culture treated with Ery. Samples taken at intervals were analyzed for polysomes as described (Wallace and Davis, 1973).

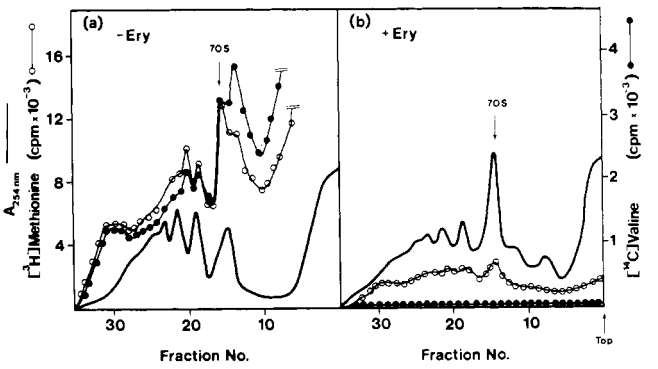

FIGURE 7: Differential labeling of polysomes with [^{3}H]methionine and [^{14}C]valine. (a) A growing culture of strain MRE600, at 2×10^8 cells/ml, was pulsed for 1.0 min with both [^{3}H]methionine (1 μCi and 0.048 μg/ml) and [^{14}C]valine (0.1 μCi and 0.044 μg/ml with isoleucine added), and then was rapidly poured onto ice. (b) A portion of the growing culture was incubated with Ery (100 μg/ml) for 20 min before being similarly pulsed. Cell lysates were prepared and analyzed for polysomes, ^{3}H, and ^{14}C as described (Wallace and Davis, 1973).

bosomes released during the assay, for at 0° binding by the polysomal preparation was negligible while the free ribosomes showed only a slight percentage decrease (Table I). Polysomal ribosomes thus appear to have a very low affinity for Ery. However, this affinity is difficult to determine directly with precision because of the high affinity of any contaminating free ribosomes; the midpoint of the concentration–inhibition curves probably provides the best index.

The low affinity of polysomal ribosomes for Ery evidently depends on the presence of peptidyl-tRNA, and not on mRNA alone: "pseudopolysomes" (containing mRNA but not peptidyl-tRNA), formed by treatment of polysomes with puromycin in the absence of ribosome-release factor, exhibited high affinity, like free ribosomes (Table II). With pactamycin, in contrast, mRNA appears to be responsible for the poor binding to polysomes (Goldberg *et al.*, 1973).

With the purified polysomes puromycin alone caused release of only part of the polypeptides, presumably from those ribosomes with peptidyl-tRNA in the P site: the addition of EFG and GTP, to permit translocation, resulted in further release (Table II). Since these two different degrees of release were accompanied by proportionate increases in the binding of Ery, it appears that peptidyl-tRNA on either the A site or the P site impairs that binding. This result is consistent with the failure of Ery to inhibit chain elongation on polysomal ribosomes, for during this process peptidyl-tRNA occupies the two sites alternately. Whether the peptidyl-tRNA in either position covers the Ery-binding site directly, or whether it has a more indirect conformational effect, remains to be answered.

Elongating polysomes can be inhibited by Ery at sufficiently high concentrations, above 100 μM (Figure 1). However, this action is clearly nonspecific, for such concentrations inhibit synthesis in resistant as well as in sensitive S30 preparations (Oleinick and Corcoran, 1969). This nonspecific interaction appears to interfere in addition with reinitiation, since high concentrations of Ery inhibit the cyclic renewal of polysomes in cells (Figure 6b).

Cyclic Blockade and the Dominance of Erythromycin Sensitivity. The insensitivity of polysomes to inhibition by Ery seemed initially to contradict the observation that Ery "stabilizes" polysomes in cells (Ennis, 1972). However, the paradox was resolved by the finding that these are unusual polysomes, which recycle even though they are not synthesizing protein: in Ery-inhibited cells they can be pulse-labeled with methionine but not with valine (Figure 7), and their rapid breakdown is revealed when mRNA renewal is blocked by rifampicin (Figure 6a). This selective labeling further shows that Ery specifically blocks ribosomes shortly after initiation in cells, just as in extracts. The spontaneous release of complexes blocked by Ery is slower than normal runoff (Figures 6a and 7), whereas less difference was observed with complexes blocked by streptomycin (Wallace and Davis, 1973) or by spectinomycin (Wallace *et al.*, 1974).

The finding that cells treated with Ery accumulate blocked polyinitiation complexes, and that these turn over, explains the dominance of sensitivity to Ery over resistance, observed in heterozygous cells (Nomura and Engbaek, 1972). Thus just as with streptomycin (Wallace and Davis, 1973) and spectinomycin (Wallace *et al.*, 1974), each cycle of release and reinitiation by an inhibited sensitive ribosome blocks an initiation site for several minutes, and in heterozygotes this blockade would effectively exclude the otherwise active resistant ribosomes from practically all the initiation sites.

The instability of the blocked complexes may be the reason that even at saturating concentrations Ery (Figure 1), like spectinomycin (Wallace *et al.*, 1974), causes only incomplete inhibition (70–80%) of translation by initiating ribosomes in extracts, whereas streptomycin causes virtually complete inhibition (Modolell and Davis, 1968; Wallace *et al.*, 1973). Ery and spectinomycin interact with free ribosomes reversibly, and they have low affinity for elongating ribosomes; hence if the initiating ribosomes blocked by these antibiotics should occasionally "read through" under the conditions obtaining in extracts, instead of falling off the messenger, they might then assume the conformation of elongating ribosomes and release the antibiotic. Streptomycin, in contrast, interacts irreversibly with initiating ribosomes and thus might be expected to prevent them from occasionally resuming elongation.

The Reaction Inhibited by Erythromycin. While it is clear that Ery blocks ribosomes shortly after initiation, the precise step that it blocks is not certain. Its presence (during formation as well as testing of the initiation complexes)

does not impair recognition of the first aminoacyl-tRNA (Figure 3), peptidyl transfer of fMet to puromycin, or dipeptide formation. The transfer to puromycin, with natural messenger, confirms earlier findings, made with trinucleotide AUG (Mao and Robishaw, 1971; Vogel *et al.*, 1971) or with the "fragment" reaction without messenger (Monro and Vazquez, 1967).

These results suggest a block in the first translocation following initiation. However, they are also compatible with inhibition of a step in a later round of translation, as has been observed with fusidic acid (Bodley *et al.*, 1970). This possibility is, in fact, supported by the finding that with ribosomes translating poly(A) Ery causes the accumulation of trilysine (Tanaka and Teraoka, 1966, 1968; Mao and Robishaw, 1971). Whether the blocked reaction is a translocation or a peptidyl transfer is not clear. The considerable literature on the action of Ery (reviewed in Pestka, 1971; Cundliffe, 1972) provides contradictory evidence for each; and the interpretation of the experiments is complicated by the present finding that low concentrations of Ery selectively inhibit and bind to initiating ribosomes, while high concentrations inhibit elongating ribosomes as well.

Particularly cogent evidence for a block in translocation was provided by Cundliffe and McQuillen (1967; see also Cannon and Burns, 1971). They observed that the release of nascent polypeptide from polysomes by puromycin, in protoplasts of *Bacillus megaterium*, was inhibited by Ery alone but not by Ery following pretreatment with a tetracycline. Since the latter blocks the A site, and hence should fix the peptidyl-tRNA in the P site, it seemed clear that Ery does not affect the reaction of peptidyl-tRNA already in the P site; hence the inhibition by Ery alone would imply a block in translocation of peptidyl-tRNA in the A site. However, this interpretation no longer seems certain. Thus we have seen that polysomal ribosomes (of *E. coli*) exhibit 1/1000 as high a sensitivity (and presumably affinity) as free ribosomes. Hence if the Ery (at 250 μg/ml) in the *B. megaterium* experiments acted only on free ribosomes it would allow polysome runoff and would then block the subsequently formed complexes after initiation, in a puromycin-unreactive position. The effect of tetracycline would be readily explained (see also Oleinick, 1974): by fixing the polysomes it would prevent them from being converted into initiating ribosomes. Alternatively, if the concentration of Ery were high enough to block the polysomal ribosomes the interpretation of these experiments might also be uncertain. Thus, since Ery must bind to initiating ribosomes at one stage in order to block another, the same may be true of its interaction with elongating ribosomes. Tetracycline could be preventing them from reaching a conformation suitable for the binding of Ery. It is not clear whether the Ery in these experiments was in the higher or lower concentration range: the persistence of labeling of the polysomes with a mixture of amino acids after addition of Ery (Cundliffe and McQuillen, 1967) suggests the higher range, but the label might have been in short peptides, turning over on blocked "polyinitiation" complexes.

Similar considerations apply to experiments in which recognition was blocked by fusidic acid (Burns and Cundliffe, 1973). The nascent peptides in these cells were initially reactive with puromycin, but after addition of Ery the reactivity slowly decreased. This result suggested slow "leakage" past the fusidic block, allowing a subsequent block in translocation by Ery. However, these findings would also be explained if the slow leakage yielded the conformation required for binding of Ery, which might then block either translocation or peptidyl transfer.

These ingenious experiments with mixtures of antibiotics thus do not appear to establish translocation as the step blocked by Ery. However, this mechanism is favored by a more direct study of translocation, in extracts: Ery inhibits the translocation-dependent release of deacylated tRNA after peptidyl transfer (Igarashi *et al.*, 1969; Tanaka *et al.*, 1973). It fails to inhibit EFG-dependent GTP hydrolysis (Tanaka *et al.*, 1969; Mao and Robishaw, 1971); but this hydrolysis might be uncoupled from other aspects of translocation.

The evidence on peptidyl transfer is also indecisive, especially in the light of the present findings. Thus Ery can block the reaction of puromycin with certain synthetic oligopeptides but not with others (reviewed in Pestka, 1971), and so it has been thought to act on peptidyl transferase when the peptide has the proper length and perhaps composition (Mao and Robishaw, 1971, 1972; Cerna *et al.*, 1971; Tanaka *et al.*, 1971). However, the required peptide length would be restricted to certain intermediate values, for Ery fails to inhibit transfer to puromycin either of fMet or of the long peptides on endogenous polysomes, as noted above (see also Pestka, 1972b). The present findings suggest an additional possibility: the sensitivity of initiating ribosomes may depend not on chain length *per se* but on reaching some critical step in the ribosome cycle, after free ribosomes have bound Ery. Some of the synthetic systems may resemble initiating ribosomes in binding Ery and then reaching this Ery-sensitive step, while others may fail to reach that step or may fail to bind Ery. The step that Ery blocks after initiation thus remains unsettled.

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Histidyl-tRNAs and Histidyl-tRNA Synthetases in Wild Type and Cytoplasmic Petite Mutants of *Saccharomyces cerevisiae*†

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ABSTRACT: *Saccharomyces cerevisiae* has two distinguishable species of $tRNA^{His}$. They can be separated from each other by sequential chromatography on DEAE-Sephadex and a modified reversed phase column. One of these tRNAs seems to be coded by a mitochondrial gene because it is absent in a mutant lacking mtDNA and it hybridizes specifically to mtDNA. This mitochondrial $tRNA^{His}$ can be charged by the histidyl-tRNA synthetase isolated from *Salmonella typhimurium,* whereas the cytoplasmic $tRNA^{His}$ cannot be acylated under the same conditions. There are also two different histidyl-tRNA synthetases in yeast. One of these accounts for 10% of the total histidyl-tRNA synthetase activity in the cell and cosediments with mitochondria in sucrose gradients; the rest of the activity is in the soluble fraction. Although both enzymes behave identically on a DEAE-cellulose column and show similar molecular weights (100,000), they can be distinguished on the basis of their affinities for tRNA. The mitochondrial synthetase fails to charge bulk *Escherichia coli* tRNA, whereas the cytoplasmic enzyme charges it efficiently. The cytoplasmic enzyme has a 10–20-fold greater affinity for both cytoplasmic and mitochondrial $tRNA^{His}$. The mitochondrial synthetase seems to be under the control of a nuclear gene, because it is found in mutants lacking mtDNA.

Histidyl-tRNA synthetase and $tRNA^{His}$ have been implicated in the repression control of the histidine pathway in *Salmonella typhimurium* (Brenner and Ames, 1971; Goldberger, 1974). For this reason it seemed worthwhile to investigate the properties of the activating enzyme and $tRNA^{His}$ in a eucaryote, *Saccharomyces cerevisiae,* as a preliminary step toward elucidation of regulatory processes in this organism.

Yeast cells contain two distinct systems which can synthesize protein, one in the cytoplasm and one in the mitochondrion. Mitochondria of several organisms have been shown to contain their own activating enzymes (Barnett *et al.,* 1967; Buck and Nass, 1969; Kislev and Eisenstadt, 1972) and tRNA species (Barnett *et al.,* 1967; Buck and Nass, 1969; Casey *et al.* 1972). Hybridization experiments

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