Rubradirin, a Selective Inhibitor of Initiation Factor Dependent Peptide-Chain Initiation†

Fritz Reusser

ABSTRACT: The antibiotic rubradirin selectivity inhibits the A-U-G directed binding of fMet-tRNA$_f$ to 30S ribosomal subunits and 70S ribosomes if the binding is initiation factor dependent (enzymatic initiation). Nonenzymatic binding of fMet-tRNA$_f$ effected in the presence of high Mg^{2+} concentrations only is not inhibited by rubradirin. Preformed 30S and 70S initiation complexes formed with the participation of initiation factors are rapidly degraded upon addition of rubradirin. A subsequent increase of the Mg^{2+} concentration in the system results in the reassociation of the complexes into a form now resistant to dissociation by rubradirin. These observations indicate that rubradirin acts as a selective inhibitor of the initiation factor dependent chain initiation processes occurring at the 30S ribosomal subunit. The results also demonstrate that the 30S and 70S initiation complexes formed with the participation of initiation factors, differ in some significant aspect from the initiation complexes induced by Mg^{2+} only.

The antibiotic rubradirin, prepared as described by Meyer (1965), inhibits protein synthesis in cell-free systems. Previous studies indicated that the antibiotic acts as a potent inhibitor of polypeptide biosynthesis directed by synthetic messenger RNA (Reusser, 1973). Phage f_2 RNA mediated protein synthesis is not susceptible to rubradirin inhibition unless the assay mixtures contain an fMet-tRNA$_f$[1] generating system. *N*-Acetylphenylalanyl-tRNA binding to salt-washed 70S ribosomes is inhibited by rubradirin if the assay system contains low amounts of Mg^{2+} and initiation factors. *N*-Acetylphenylalanyl-tRNA binding to 70S ribosomes is not impaired if the system contains high amounts of Mg^{2+}. This led us to conclude that rubradirin specifically interferes with the ribosomal process of chain initiation.

In the present communication we report studies on the effect of rubradirin on the enzymatic (initiation factor dependent) and nonenzymatic (Mg^{2+} induced) binding of fMet-tRNA$_f$ to ribosomes and on the T- and G-factor-associated ribosomal functions.

Methods

Salt-washed ribosomes and 30S and 50S ribosomal subunits were prepared as described previously (Reusser, 1973).

Escherichia coli tRNA$_f^{Met}$ was obtained from the Oak Ridge National Laboratories. Synthetase–transformylase enzyme required for the synthesis of fMet-tRNA$_f$ was isolated following the procedure of Dubnoff and Maitra (1971). [^{3}H]fMet-tRNA$_f$ was prepared as described by Caskey *et al.* (1971). Crude initiation factors were isolated from the first ribosomal salt wash fluid (Dubnoff and Maitra, 1971). The trinucleotide A-U-G was obtained from Dr. P. Leder, NIH.

Binding of fMet-tRNA$_f$ to 70S ribosomes or ribosomal subunits was assessed by the filtration technique (Nirenberg and Leder, 1964).

Purified preparations of *E. coli* T and G chain elongation factors were obtained from Dr. F. Lipmann, Rockefeller University. They had been purified through the DEAE-cellulose chromatography step of Gordon (1969). The exact compositions of the assay mixtures are given in the legends of Tables VI and VII.

Methanol induced binding of fMet-tRNA$_f$ to 50S ribosomal subunits was studied by the method of Miskin *et al.* (1970).

Results

Effect on Formation of the 70S Initiation Complex. In the presence of low Mg^{2+} concentrations (4 mM), A-U-G mediated binding of fMet-tRNA$_f$ to 70S ribosomes was completely dependent on the presence of initiation factors in our test system. This type of initiation is usually referred to as enzymatic initiation. Under these conditions rubradirin inhibited the attachment of fMet-tRNA$_f$ to 70S ribosomes to an extent of 87% (Table I). In the presence of relative high Mg^{2+} concentrations (15 mM) in the assay system, the initiation complex will form without the participation of initiation factors (nonenzymatic initiation). This type of initiation remained insensitive to rubradirin inhibition as shown in Table I.

These results demonstrate that rubradirin selectively inhibits the formation of the initiation factor dependent process of initiation without affecting the Mg^{2+}-induced nonenzymatic process.

Effect on Stability of 70S Initiation Complex. The observed overall inhibition of the factor-dependent 70S initiation complex by rubradirin can be due to interference with either the formation of the complex *per se* or the rapid breakdown of the complex as it is formed. To resolve this question the 70S initiation complex was preformed in the absence of rubradirin. Upon addition of rubradirin the stability of the complex was then assessed.

In a first experiment, the initiation complex was preformed in the presence of 15 mM Mg^{2+} and no initiation factors. Addition of rubradirin to this complex did not appreciably affect its stability as shown in Figure 1. If the initiation complex was preformed under enzymatic initiating conditions, addition of rubradirin resulted in a rapid dissociation of this complex (Figure 1). Adjustment of the Mg^{2+} concentration to 15 mM to the rubradirin-dissociated initiation complex

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[1] Abbreviations used are: fMet-tRNA$_f$, formylmethionyl-tRNA, the tRNA being the species which can be formylated after acylation with methionine; A-U-G, adenylyl-uridylyl-guanosine.

TABLE I: Effect of Rubradirin on the Initiation Factor Induced and Mg^{2+}-Induced Formation of the 70S Initiation Complex.[a]

Sample	fMet-tRNA$_f$ Bound (cpm/Sample)	% Inhibition
4 mM Mg^{2+}		
Control	770	0
Rubradirin, 0.05 mM	100	87
15 mM Mg^{2+}		
Control	995	0
Rubradirin, 0.05 mM	990	0

[a] The reaction mixtures contained in 0.15 ml: 50 mM Tris-HCl (pH 7.8), 100 mM NH_4Cl, magnesium acetate as indicated, 4 mM mercaptoethanol, 2 mM GTP, 0.04 OD_{260} unit of A-U-G, 4.5 units of 70S ribosomes, 0.44 unit of [^{3}H]fMet-tRNA$_f$ containing ~140,000 cpm, and 25 μg of crude initiation factors (only present in the samples containing 4 mM Mg^{2+}). Binding was measured after incubation of the reaction mixtures for 10 min at 37°.

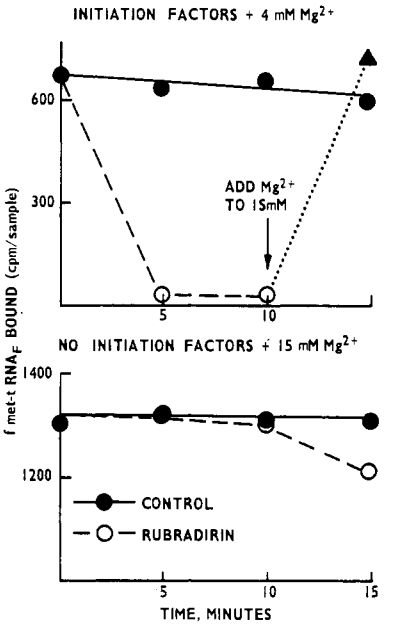

FIGURE 1: Effect of rubradirin on the stability of the 70S initiation complex. Basic reaction mixtures were as described under Table I. Top = 4 mM Mg^{2+} + initiation factors; bottom = 15 mM Mg^{2+}, no initiation factors. The mixtures containing 4 mM Mg^{2+} were preincubated for 15 min, those containing 15 mM Mg^{2+} were preincubated for 20 min; at this point rubradirin (0.05 μmol/ml) was added.

caused an immediate reassociation of the initiation complex (Figure 1). The dissociative action excerted by rubradirin can therefore by reversed by Mg^{2+}.

These studies show that rubradirin is capable to either prevent the formation of the 70S initiation complex or dissociate the 70S complex if these complexes are formed or have been preformed respectively with the active participation of initiation factors. As rubradirin is only capable to interfere with the formation or stability of the enzymatically formed complex but not with the nonenzymatic Mg^{2+}-induced one, it follows that these two types of initiation complexes (enzymatic, nonenzymatic) differ somehow but significantly from each other in their associative structure. An increase of the Mg^{2+} concentration resulted in a rapid reassociation of the rubradirin-dissociated enzymatic initiation complex into a rubradirin stable form. This suggests that fMet-tRNA$_f$ is displaced unmodified from the 70S ribosomes without concomitant deacylation.

Effect of fMet-tRNA$_f$ Binding to 50S Subunits in the Presence of Methanol (Alcohol Reaction). In the presence of methanol, fMet-tRNA$_f$ binds to the 50S ribosomal subunit without the participation of the 30S subunit and initiation factors. This reaction is commonly referred to as the alcohol reaction. Rubradirin did not interfere with the alcohol reaction (Table II). This suggests that the antibiotic interferes with funtional steps occurring at the level of the 30S rather than the 50S subunit.

Effect on Puromycin Reaction. In studies published previously we demonstrated that rubradirin does not interact with the puromycin reaction (Reusser, 1973). These experiments were carried out in a system containing *N*-acetylphenylalanyl-tRNA prebound to 70S ribosomes which led to the formation of *N*-acetylphenylalanylpuromycin. The conclusion that rubradirin does not inhibit peptide-bond formation as studied with the puromycin reaction was confirmed by the finding that the synthesis of fMet-puromycin was not inhibited either in the presence of rubradirin (Table III).

TABLE II: Effect of Rubradirin on fMet-tRNA$_f$ Binding to 50S Ribosomal Subunits in the Presence of Methanol.[a]

Sample	fMet-tRNA$_f$ Bound (cpm/Sample)	% Control
Control	350	100
Rubradirin, 0.05 mM	420	122

[a] Reaction mixtures contained in a total volume of 0.15 ml: 50 mM Tris-HCl (pH 7.8), 100 mM NH_4Cl, 20 mM magnesium acetate, 4 mM mercaptoethanol, 2 mM GTP, 0.04 unit of A-U-G, 4.5 units of 50S subunits, 0.44 unit of [^{3}H]fMet-tRNA$_f$ containing ~140,000 cpm, and 25% methanol by volume. Reaction mixtures were incubated at 0° for 15 min. The results were corrected for values of control samples run without methanol (approximately 26%).

TABLE III: Effect of Rubradirin on Puromycin Reaction.[a]

Sample	fMet-tRNA$_f$ Bound (cpm/Sample)	% Change from 30-min Control
Control, 20 min	1160	4
Control, 30 min	1210	0
Puromycin only	790	25
Rubradirin only	1160	4
Puromycin + rubradirin	790	25

[a] Reaction mixtures were as described in the legend of Table I (15 mM Mg^{2+}). The 70S initiation complex was formed in the absence of drugs by incubating the reaction mixtures for 20 min. Antibiotics were then added and the mixtures were incubated for an additional 10 min. Puromycin, 1 μmol/ml; rubradirin, 0.05 μmol/ml.

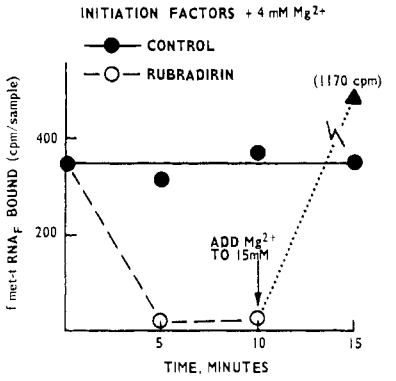

FIGURE 2: Effect of rubradirin on the stability of the enzymatic 30S initiation complex. Basic reaction mixtures are described under Table V. Preincubation before addition of rubradirin (0.05 μmol/ml) was 15 min.

Effect on the Formation of the 30S Initiation Complex. The foregoing results demonstrate that rubradirin inhibits the formation of the initiation factor-dependent 70S initiation complex and causes rapid dissociation of this complex preformed in the absence of the antibiotic. This prompted us to investigate the effect of rubradirin on the formation of the initiation factor-dependent 30S initiation complex. At a concentration of 0.05 μmol/ml, rubradirin completely abolished the enzymatic binding of fMet-tRNA$_f$ to 30S ribosomal subunits (Table IV). As the formation of the 30S initiation complex precedes the formation of the 70S complex it is evident that rubradirin interacts with the formation or stability of the 70S complex by actual interaction with the 30S subunit and processes occurring at that unit. Streptomycin has recently been implicated to interact with the initiation factor related processes (LeLong *et al.*, 1972). We investigated the effect of streptomycin on enzymatic binding of fMet-tRNA$_f$ to 30S subunits and found that streptomycin inhibits this reaction, although it appears somewhat less potent than rubradirin when present at equimolar concentrations (Table IV). This is in contrast to the results of other authors (Okuyama and Tanaka, 1972; Modolell and Davis, 1970) who claim that streptomycin causes breakdown of the 70S complex but has no effect on the formation or stability of the 30S complex. However, their test systems contained to excessive amounts of Mg^{2+} (6–10 mM) to insure exclusive enzymatic binding conditions.

Effect on the Stability of the 30S Initiation Complex. The initiation factor dependent 30S initiation complex was preformed in the absence of rubradirin. Subsequent addition of rubradirin induced rapid dissociation of this complex (Figure 2). An increase of the Mg^{2+} concentration to 15 mM resulted in an immediate reassociation of the 30S complex into a form now stable to the dissociative activity of rubradirin. This situation is quite analogous to the one observed with the 70S complex.

Effect on Ribosomal Functions Associated with T- and G-Factors. Rubradirin inhibited the T-factor stimulated binding of phenylalanyl-tRNA to washed 70S ribosomes only negligibly (Table V). G-factor-dependent poly(phenylalanine) formation was inhibited to an extent of 30% in the presence of 0.1 μmol of rubradirin/ml and 23% in the presence of 0.05 μmol/ml (Table VI). Rubradirin inhibits polypeptide formation directed by synthetic messengers such as poly(U) or poly(C). These systems function in the absence of a specific initiator such as fMet-tRNA$_f$. The results presented in the foregoing paragraphs suggest that rubradirin specifically inhibits a step or a sequence of steps occurring during the early initiation of new peptide chains. Since systems directed with synthetic messengers are also inhibited by rubradirin but systems directed with phage RNA in the presence of high Mg^{2+} concentrations are not, one wonders whether chain initiation in the systems programmed by synthetic messengers is effected by a process analogous to the one requiring fMet-tRNA$_f$, initiation factors and A-U-G as the starting codon. In any

TABLE V: Effect on T-Factor-Stimulated Phenylalanyl-tRNA Binding to Ribosomes.[a]

Sample	Phe-tRNA Bound (cpm/Sample)	% Inhibition
Complete	3800	0
Less T-factor	1200	
Rubradirin, 0.1 mM	3380	11
Rubradirin, 0.05 mM	3800	0

[a] Reaction mixtures contained in a total volume of 0.1 ml: 100 mM Tris-HCl (pH 7.2), 20 mM magnesium acetate, 50 mM KCl, 2 units of 70S ribosomes, 1 mM GTP, 10 μg of poly(U), 100 μg of [^{14}C]phenylalanyl-tRNA containing 14,000 cpm, and T-factor, ~1 μg.

TABLE IV: Effect of Rubradirin and Streptomycin on the Formation of the 30S Initiation Complex.[a]

Sample	fMet-tRNA$_f$ Bound (cpm/Sample)	% Inhibition
Control	696	0
Rubradirin, 0.05 mM	48	93
Control	448	0
Rubradirin, 0.05 mM	2	~100
Streptomycin, 0.05 mM	144	63

[a] The basic reaction mixtures are described in the legend of Table I. They contained 4 mM Mg^{2+} and initiation factors. 30S ribosomal subunits = 4.2 units/sample.

TABLE VI: Effect on G-Factor-Dependent Poly(phenylalanine) Formation.

Sample	Incorporation (cpm/Sample)	% Inhibition
Complete	1680	0
Less G-factor	270	
Rubradirin, 0.1 mM	1180	30
Rubradirin, 0.05 mM	1290	23

[a] Reaction mixtures contained in a total volume of 0.2 ml: 50 mM Tris-HCl (pH 7.4), 20 mM magnesium acetate, 50 mM KCl, 10 mM mercaptoethanol, 0.025 mM GTP, 10 μg of poly(U), 100 μg of [^{14}C]phenylalanyl-tRNA containing 14,000 cpm, 1 unit of 70S ribosomes, and ~1 μg of G-factor.

case the observed initiation of G-factor-dependent poly(phenylalanine) formation studied in our system is probably not due to interference of the antibiotic with the G-factor mediated process of translocation as the system directed by natural mRNA should be inhibited as well.

Effect on Codon Misreading. Several properties of rubradirin such as the prevention of the formation of the enzymatic 30S and 70S initiation complexes and the dissociation of the preformed complexes upon addition of rubradirin are shared with streptomycin. Streptomycin also interferes with the ribosomal region involved with codon–anticodon interactions and causes misreading of the codons. We thus investigated the possible misreading effect of rubradirin in an assay system where streptomycin increases the poly(U)-directed incorporation of isoleucine. Rubradirin remained nearly inert in this system and does, therefore, not appreciably affect codon–anticodon interactions in a manner similar to streptomycin (Table VII).

TABLE VII: Effect of Rubradirin on Poly(U)-Directed Incorporation of Isoleucine.[a]

Sample	Ile Incorp (cpm/Sample)	% of Control
Control	92	100
Streptomycin, 0.1 mM	202	219
Streptomycin, 0.05 mM	209	217
Rubradirin, 0.1 mM	112	121
Rubradirin, 0.05 mM	79	86

[a] Reaction mixtures contained in a total volume of 0.25 ml: Tris-HCl buffer (pH 7.8), 25 μmol of magnesium acetate, 3.5 μmol; KCl, 15 μmol; ATP, 02.5 μmol; GTP, 0.0075 μmol; mercaptoethanol, 1.5 μmol; phosphoenolpyruvate, K salt, 18.75 μmol; pyruvate kinase (Calbiochem), 10 μg; [^{12}C]amino acid mixture of 19 amino acids, 0.05 μmol each; [^{14}C]isoleucine, 0.25 μmol containing 1.75 μCi; poly(U), 15 μg; S-30 enzyme, 430 μg of protein. The samples were incubated at 37° for 15 min. Further processing was carried out according to published procedures (Reusser, 1969).

Discussion

Rubradirin prevents the formation of a stable 30S initiation complex formed with *E. coli* 30S ribosomal subunits, A-U-G and fMet-tRNA$_f$ if the reaction is carried out in the presence of low Mg^{2+} concentrations and dependent on initiation factors. If the factor-induced 30S initiation complex is preformed in the absence of rubradirin, subsequent addition of this antibiotic causes rapid dissociation of the complex. The rubradirin-dissociated complex reaggregates upon adjustment of the Mg^{2+} concentration to 15 mM to a form now resistant to rubradirin. In addition to the 30S complex, initiation factor induced 70S initiation complexes are also unstable in the presence of rubradirin. Again an increase of the Mg^{2+} concentration to 15 mM results in the reassociation of the 70S complex into a form now resistant to rubradirin.

These results indicate that rubradirin functions as a selective inhibitor of the initiation factor dependent peptide-chain initiation process (enzymatic initiation). Rubradirin has no effect on the Mg^{2+}-induced nonenzymatic process. The products of these two types of initiation differ, therefore, in some significant way from each other as rubradirin is capable to interact only with the enzymatically formed complex. These results corroborate our previous findings which showed that the enzymatic binding of *N*-acetylphenylalanyl-tRNA to 70S ribosomes was inhibited by rubradirin but not the Mg^{2+}-induced binding (Reusser, 1973).

As mentioned above rubradirin acts as a specific inhibitor of enzymatic initiation. As the antibiotic inhibits the formation of the 30S initiation complex, rubradirin must affect the functions of the 30S ribosomal subunit concerned with the early steps of new chain initiation. This includes the ribosomal regions undergoing interaction with the initiation factors.

The discussed interactions of rubradirin with the ribosomal chain initiation processes closely mimic the ones observed with streptomycin. If streptomycin is bound to 30S ribosomal subunits isolated from a phenotypically masked *E. coli* mutant, the subunits remain nonfunctional after removal of the antibiotic. They can be rendered functional upon exposure to high salt concentrations (Biswas and Gorini, 1972). Similarly, 30S and 70S enzymatic initiation complexes, dissociated by rubradirin can be reassociated by exposure to high Mg^{2+} concentration. It was also recently shown that the binding of radioactively labeled streptomycin to 70S and 30S ribosomes can be partially suppressed by the addition of combinations of purified initiation factors, F_1, F_2, and F_3 (LeLong *et al.*, 1972). In conjunction with our results it appears that the region of streptomycin interaction on the ribosome extends from the initiation factor mediated chain initiation functions to the codon–anticodon recognition functions. Rubradirin, on the other hand, affects the initiation functions but does not affect the codon–anticodon recognition functions. The action or rubradirin is, therefore, limited to the upstream portion of the region affected by streptomycin.

Rubradirin effectively inhibits peptide synthesis directed by synthetic messengers (*e.g.*, poly(U)–phenylalanine, poly(C)–proline incorporation) with S-30 cell extracts and inhibits the G-factor-dependent polymerization of phenylalanyl-tRNA by washed ribosomes. Natural mRNA-directed protein synthesis, on the other hand, is only susceptible to rubradirin inhibition if the system contains an fMet-tRNA$_f$ generating system but not if the system functions in the presence of high Mg^{2+} concentrations. These observations deserve some comment as these systems are presumed to initiate new chains without the participation of initiation factors and fMet-tRNA$_f$ as a specific initiator. It is possible that the process of initiation in the systems directed with synthetic messengers resembles more closely the ones occurring with fMet-tRNA$_f$ and initiation factors or that an additional step is inhibitable by rubradirin which remains insensitive in other systems such as the Mg^{2+}-induced phage RNA directed system.

The same argument pertains to the observation that G-factor-dependent poly(phenylalanine) synthesis is inhibited by rubradirin. As the Mg^{2+}-initiated phage system is not susceptible to rubradirin inhibition and translocation functions normally in this system, it is unlikely that rubradirin interacts with the specific event of translocation but probably inhibits the G-factor-dependent phenylalanine incorporation system by interfering with the initiation of this system.

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Misreading, a Fundamental Aspect of the Mechanism of Action of Several Aminoglycosides†

D. Lando,* M. A. Cousin, and M. Privat de Garilhe

ABSTRACT: The effect of various aminoglycoside antibiotics on the cell-free synthesis of total protein and of late T4 phage mRNA-induced lysozyme has been studied with ribosomes from streptomycin sensitive and resistant *Escherichia coli*. Lysozyme biosynthesis is more sensitive to aminoglycosides than total protein synthesis in both systems, indicating a high level of misreading, except in the case of kasugamycin and spectinomycin for which both effects are identical. The Mg^{2+} concentration significantly influences the effect of all the misreading inducing aminoglycosides. The effect of these amino glycosides on the initiation step was also investigated. On the basis of the results obtained, the antibiotics may be classified into three groups: (1) those inducing no misreading, *i.e.*, kasugamycin and spectinomycin, (2) an antibiotic acting both on initiation and fidelity of translation, namely, streptomycin, (3) those inducing a high level of misreading, *i.e.*, with a deoxystreptamine moiety.

Aminoglycoside antibiotics inhibit protein synthesis in sensitive bacteria (Erdos and Ullman, 1959), their site of action being located in the ribosomes (Spotts and Stanier, 1959; Jacoby and Gorini, 1967; Weisblum and Davies, 1968; Traub and Nomura, 1968; Ozaki *et al.*, 1969). In the study of their effect on translation mechanism in subcellular ribosomal systems, two fundamental observations have been made. (1) For blockage of the initiation of polypeptide chains, Modolell and Davis (1970) and Lelong *et al.* (1971) have shown that streptomycin inhibits the initial steps of mRNA translation *in vitro*, leading to the release of formylmethionyl-tRNA (fMet-tRNA) from the initiation complex without chain elongation; Okuyama *et al.* (1971) have established that initiation complex formation is inhibited on 30S ribosomes by kasugamycin, on 70S ribosomes by kanamycin A and gentamicin C. (2) Infidelity in the translation of the genetic code ("misreading effect"): this effect was primarily observed in studies on cell-free extracts programmed with synthetic polynucleotides (Davies *et al.*, 1965, 1966; Davies and Davis, 1968; Davies, 1970). Moreover, studies with viral mRNA have shown inhibited or stimulated polypeptide synthesis as measured by radioactive amino acid incorporation (van Knippenberg *et al.*, 1965). In these experiments, however, no specific enzyme activity was measured and thus the importance of misreading under conditions approximating biological conditions could not be clearly demonstrated. To pursue the study of the mechanism of action of aminoglycosides, we therefore selected the mRNA induced by late T4 phage infected *Escherichia coli* which codes for the translation of a specific enzyme, lysozyme, and studied the misreading effect of aminoglycosides by measuring not only the incorporation of a radioactive amino acid into protein, but also lysozyme biosynthesis. Preliminary accounts of these experiments have already been presented (Cousin *et al.*, 1971; Lando, 1972). Results for streptomycin-sensitive ribosomes were compared with those for streptomycin-resistant ribosomes, which according to Jacoby and Gorini (1967), are less sensitive to misreading. The effects on fidelity of translation were compared to those on the initiation step.

Experimental Section

Materials

Streptomycin-sensitive (Sm^S)[1] and streptomycin-resistant (Sm^R) *E. coli* MRE 600 were used to prepare ribosomes and crude translation factors; *E. coli* D_{10} was used to prepare wild T4 phage mRNA.

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[1] Abbreviations used are: A-U-G, adenylyl-3′,5′-uridylyl-3′,5′-guanosine 3′-phosphate; Gm, gentamicin; Kg, kasugamycin; Km, kanamycin; Kn, kanendomycin; Nea, neamine; Nm, neomycin; Pa, paromamine; Pm, paromomycin; Sm, streptomycin; Sp, spectinomycin; ID_{50}, inhibition dose 50%.