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Erythromycin, a Peptidyltransferase Effector†

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ABSTRACT: The effect of erythromycin on peptide-bond formation was studied in a modified fragment reaction. Evidence showed that this reaction was a specific assay for peptidyltransferase. The transfer of monoaminoacyl moieties from tRNA to puromycin was consistently stimulated by erythromycin. On the other hand, the transfer of dipeptidyl moieties such as diphenylalanine, *N*-acetyldiphenylalanine, -prolylglycine, -phenylalanylglycine, and -phenylalanylleucine from tRNA to puromycin was inhibited by the antibiotic. Nevertheless, the transfer of two dipeptidyl moieties, *N*-acetyldiglycine and -glycylproline was stimulated by the antibiotic. The degree of stimulation or inhibition was different depending upon the substrates, and complete inhibition was not observed. The maximal effect of erythromycin was observed at an erythromycin concentration of less than 10^{-6} M. Kinetic studies showed that erythromycin altered the rate of peptide-bond formation. The dose–response curve of peptidylpuromycin formation relating to erythromycin concentration coincided with the erythromycin–ribosome binding curve suggesting that the action of erythromycin on peptidyltransferase is a consequence of its binding to ribosomes; and it has been previously established that the binding of erythromycin to ribosomes inhibits bacterial growth. The above results suggest that erythromycin is an allosteric effector. It binds to the 50S ribosomal subunit in the vicinity of the peptidyl-tRNA binding site, induces a conformational change, and thus affects the reaction rate of peptidyltransferase. Considering the results of various donor substrates, several structural factors such as the number of amino acid residues on the donor tRNA, the hydrophobicity of the aminoacyl moiety of the donor tRNA, and the size of the amino acid side chain, seemed to be related to the expression of erythromycin action on peptidyltransferase.

Erythromycin, a macrolide antibiotic, is known to be an inhibitor of protein biosynthesis in bacterial systems (Taubman *et al.*, 1964; Vazquez, 1966; Mao and Wiegand, 1968), but the exact mechanism of action has not yet been elucidated. Nevertheless, the specific step affected by erythromycin has been narrowed down to either peptide-bond formation (Rychlik *et al.*, 1967; Jayaraman and Goldberg, 1968; Černá *et al.*, 1969) or to translocation (Cundliffe and McQuillen, 1967; Igarashi *et al.*, 1969; Oleinick and Corcoran, 1970). Other macrolides (niddamycin, carbomycin, spiramycin, and tylosin) have been convincingly shown to be inhibitors of peptidyltransferase (Monro and Vazquez, 1967; Černá *et al.*, 1969; Mao and Robishaw, 1971a). Since erythromycin competes with other macrolides for the same binding site on the 50S ribosomal subunits (Wilhelm *et al.*, 1967; Mao, 1971) and since its structure is similar to other macrolides, it has been suggested that the action of erythromycin should also be on peptidyltransferase (Mao and Robishaw, 1971a). The evidence supporting this claim is the inhibition by erythromycin of the transfer of the polylysyl moiety from tRNA to puromycin (Černá *et al.*, 1969, 1971), to CpA-Gly[1] (Rychlik *et al.*, 1967) or to Lys-tRNA (Jayaraman and Goldberg, 1968). Recently Tanaka *et al.* (1971) also showed that erythromycin inhibited the transfer of Phe-Phe from tRNA to puromycin. Puromycin has long been considered an analog of aminoacyl-tRNA and has been widely used to study peptide-bond synthesis. Therefore, inhibition of the transfer of a peptidyl moiety to puromycin is a good indication of inhibition of peptide-bond formation.

There are some inconsistencies in the effects of erythromycin on peptidyltransferase. For instance, the transfer of acPhe (Černá *et al.*, 1971), fMet (Monro and Vazquez, 1967; Mao and Robishaw, 1971a), Gly-Phe, Leu-Phe, and Val-Gly-Phe (Tanaka *et al.*, 1971) from tRNA to puromycin was either stimulated or unaffected by erythromycin. Also, erythromycin characteristically caused the accumulation of short peptides in poly(U)- or poly(A)-directed synthesis of Phe_n or Lys_n (Teraoka *et al.*, 1969; Mao and Robishaw, 1971a). In addition, erythromycin did not inhibit synthesis of $acPhe_2$ but did inhibit $acPhe_3$ synthesis (Oleinick and Corcoran, 1970). At first glance these results suggest an effect on something other than peptidyltransferase. The results can also be explained by assuming that the effect of erythromycin on peptidyltransferase is related to the length and nature of the peptidyl moiety (Mao and Robishaw, 1971a; Černá *et al.*, 1971). However, the number of peptidyl donors tested previously are too few to substantiate this explanation.

The availability of the 50S reaction, a modification of the fragment reaction (Monro, 1967), enabled us to test specifically the effect of erythromycin on peptidyltransferase. Although this reaction containing methanol is carried out under unnatural conditions, there is evidence to support the fact that the reaction mechanism is identical with peptide-bond formation in protein biosynthesis (Monro and Vazquez,

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[1] Abbreviations used are: CpA-Gly, cytidylyl-(3′→5′)-2′(3′)-*O*-glycyladenosine; acGly-tRNA, and acPro-Phe-tRNA, etc., *N*-acetylglycyl-tRNA, and *N*-acetylprolylphenylalanyl-tRNA, etc.; fMet-tRNA, *N*-formylmethionyl-tRNA; erythromycin, erythromycin A; acLeu-ACCAC, cytidylyl-(3′→5′)-adenylyl-(3′→5′)-cytidyl-(3′→5′)-cytidylyl-(3′→5′)-2′(3′)-*O*-L-acetylleucyladenosine.

1967; Monro *et al.*, 1968; Monro, 1969; Lucas-Lenard and Lipmann, 1971). Prior to the formation of a peptide bond, binding of peptidyl-tRNA to the 50S subunits is necessary. Since erythromycin does not inhibit binding of peptidyl-tRNA or aminoacyl-tRNA to the donor site or to the acceptor site (Tanaka *et al.*, 1966; Černá *et al.*, 1969; Mao and Robishaw, 1971a), and since other components for protein synthesis are not present, the 50S reaction has become a test solely for peptidyltransferase in this study. By systematically testing different N-blocked or N-unblocked aminoacyl- and dipeptidyl-tRNAs, the mechanism of erythromycin action has emerged.

Materials and Methods

Preparation of 50S Ribosomal Subunits. Ribosomes were isolated from *Escherichia coli* B or Q13 according to the procedure described previously (Mao, 1967a) and suspended in a buffer containing 1 M NH_4Cl, 10^{-2} M Tris-HCl (pH 7.5), 1.6×10^{-2} M $MgCl_2$, and 6×10^{-3} M mercaptoethanol at a concentration of 12 mg/ml. After having been stirred overnight at 4°, ribosomes were sedimented and then were dissociated into 50S and 30S subunits by suspension in either (1) 10^{-4} M $MgCl_2$, 5×10^{-2} M NH_4Cl, 10^{-2} M Tris-HCl (pH 7.5), and 6×10^{-3} M mercaptoethanol, or in (2) 0.5 M NH_4Cl, 5×10^{-4} M MgAc–EDTA, and 2×10^{-2} M Tris-HCl (pH 7.5) (Staehelin and Maglott, 1971), and dialyzed against the same buffer for 2 hr. The second procedure dissociated the 70S ribosomes rapidly and completely and, therefore, is recommended. The 50S and 30S subunits were separated by sucrose density gradient centrifugation (Mao, 1967b). The 50S subunits were precipitated by 0.7 volume of cold ethanol after raising the Mg^{2+} concentration to 8×10^{-3} M (Falvey and Staehelin, 1970). The concentrated 50S subunits were purified again by a second sucrose density gradient centrifugation as above. On some occasions the 50S subunits were separated by the sucrose density gradient centrifugation method of Staehelin and Maglott (1971). The 50S subunits purified by the two different procedures had identical peptidyltransferase activities. The purified 50S subunits were suspended in and dialyzed against a solution containing 6×10^{-2} M Tris-HCl (pH 7.5), 0.5 M NH_4Cl, 0.4 M KCl, 2×10^{-2} M magnesium acetate, and 6×10^{-3} M mercaptoethanol and stored in liquid nitrogen in 0.3-ml portions containing about 3 mg of 50S subunits (1 OD_{260} = 60 μg). The peptidyltransferase is a rather stable enzyme since freezing and thawing three times did not decrease its activity. The purified 50S subunits usually contained less than 5% of 30S subunits, and it is known that the peptidyltransferase activity is unaffected by slight contamination with 30S subunits (Monro, 1967).

Preparation of Radioactive Aminoacyl-tRNA and Peptidyl-tRNA. All amino acids used in this work were L-amino acids. For the reason of brevity and similarity of their function in the 50S reaction, *N*-acetylaminoacyl-tRNA, *N*-acetyldipeptidyl-tRNA, and dipeptidyl-tRNA are referred to as peptidyl-tRNA.

Radioactive aminoacyl-tRNAs were prepared by charging *E. coli* tRNA (General Biochemicals) with ^{14}C- or ^{3}H-labeled phenylalanine, glycine, proline, or leucine (International Chemical and Nuclear Corp.) according to Leder and Bursztyn (1966). Specific activities of each amino acid are shown in the legends of pertinent graphs and tables.

N-Acetylation of aminoacyl-tRNA was done by reacting *N*-hydroxysuccinimide ester of acetic acid with radioactive aminoacyl-tRNA (DeGroot *et al.*, 1966). Phe-[^{14}C]Phe-tRNA was prepared by condensation of *N*-hydroxysuccinimide ester of *N*-*o*-nitrophenylsulfenylphenylalanine with [^{14}C]Phe-tRNA followed by removal of the *o*-nitrophenylsulfenyl group according to Hamburger *et al.* (1970). *N*-Hydroxysuccinimide esters of *N*-acetylglycine, *N*-acetylproline, and *N*-acetylphenylalanine were synthesized by coupling *N*-acetylamino acids (Cyclo Chemicals) with *N*-hydroxysuccinimide in the presence of dicyclohexycarbodiimide (DeGroot *et al.*, 1966). The esters were condensed with various radioactive aminoacyl-tRNAs to form *N*-acetyldipeptidyl-tRNA. This reaction was carried out in a minimum amount of freshly distilled dimethyl sulfoxide containing 0.05 M triethanolamine (pH 8.1) at room temperature. In order to obtain complete conversion of aminoacyl-tRNA to *N*-acetyldipeptidyl-tRNA, excess esters, 10^{5-6}-fold the amount of aminoacyl-tRNA, had to be used. The reaction mixture was stirred for 3 hr. Then equal volumes of dimethylformamide and 20% dichloroacetic acid were added. The precipitate containing *N*-acetyldipeptidyl-tRNA and unreacted ester was recovered by centrifugation (15 min at 20,000*g*), resuspended in a minimum amount of potassium acetate buffer (5×10^{-4} M, pH 5.0) and dialyzed against three 1-l. portions of the same buffer over a period of 48 hr.

Identification of Peptidyl-tRNA. A small sample of peptidyl-tRNA was hydrolyzed by 0.3 N KOH at 35° for 30 min. The cleaved peptide was identified by paper chromatography and high-voltage electrophoresis. The unhydrolyzed samples always remained at the origin. Paper chromatography was carried out, together with standard compounds (Cyclo Chemical) on Whatman No. 1 paper strips (2.2 × 57 cm) by the descending technique. The solvent system was 1-butanol–acetic acid–water (78:5:17). Paper electrophoresis was carried out on Whatman No. 3MM paper strips (2.2 × 57 cm) in 5% acetic acid–0.5% pyridine (pH 3.5) at 17.5 V/cm for 4–6 hr. Compounds containing free α-amino groups were detected by ninhydrin spray; while those blocked in the α-amino

TABLE I: Paper Chromatography and Paper Electrophoresis of Amino Acids, *N*-Acetylamino Acids and Peptides.[a]

Compounds	R_F	Electrophoretic Mobility (cm/hr)[b]
Gly	0.05	−1.53
Pro	0.17	−0.80
Phe	0.48	−0.49
Leu	0.42	−0.78
AcGly	0.59	+4.40
AcPro	0.73	
AcPhe	0.89	+3.58
AcLeu	0.92	+2.02
AcPro-Gly	0.57	+3.32
AcPhe-Gly	0.79	+2.13
AcGly-Gly	0.40	+4.30
AcGly-Pro	0.58	+2.84
AcPhe-Pro	0.86	
AcPhe-Phe	0.93	+2.83
AcPhe-Leu	0.91	+1.20
Phe-Phe	0.81	−1.87

[a] The procedure for the above determinations is described under Methods. [b] The negative sign indicates this compound moved toward the cathode. The positive sign indicates this compound moved toward the anode.

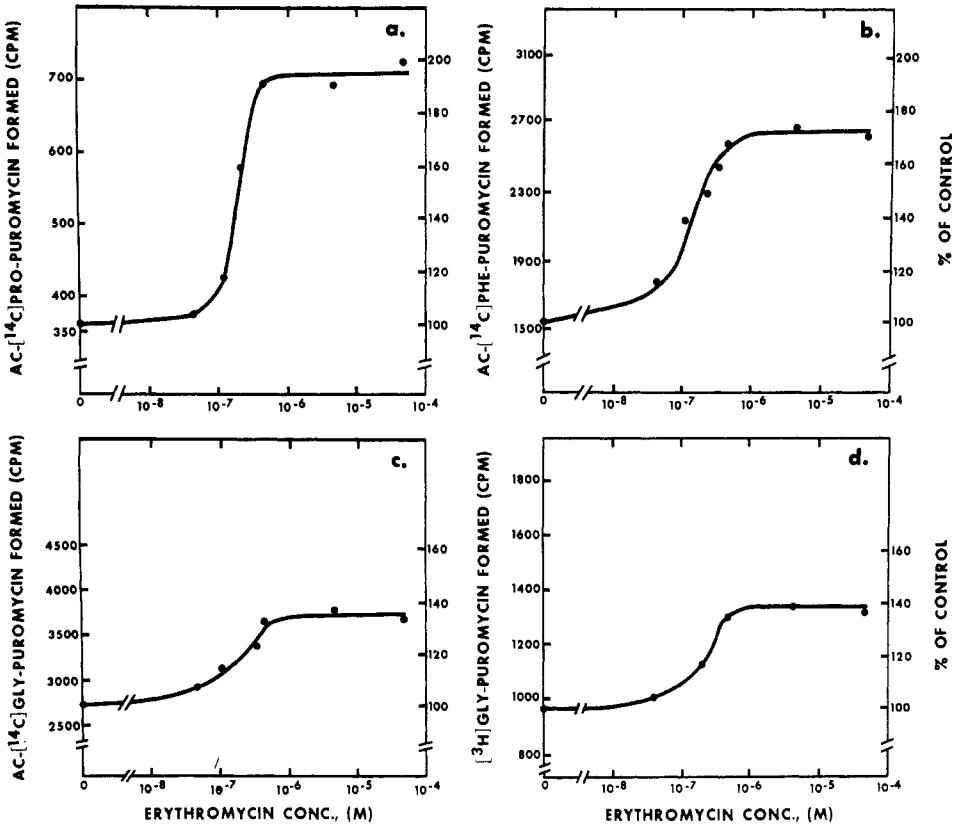

FIGURE 1: Effect of erythromycin on the reaction of puromycin with *N*-acetylaminoacyl-tRNA and aminoacyl-tRNA. The reaction mixtures of 0.15 ml contained 88 μg of 50S ribosomal subunits and (a) 18.5 pmol of ac-[^{14}C]Pro-tRNA (6784 cpm), (b) 17.4 pmol of ac-[^{14}C]Phe-tRNA (11,138 cpm), (c) 94 pmol of ac-[^{14}C]Gly-tRNA (12,950 cpm), and (d) 24.2 pmol of [^{3}H]Gly-tRNA (72,446 cpm). Other components of the reaction mixtures, conditions, and assay procedures are described under Methods.

groups were detected by treatment with chlorine followed by *o*-tolidine–KI (Nitecki and Goodman, 1966). Radioactive spots were detected by cutting 1.5-cm lengths from paper strips and counting in a liquid scintillation spectrometer. R_F values and electrophoretic mobilities of various compounds are given in Table I.

TABLE II: Effect of Fusidic Acid, Chlorotetracycline, Niddamycin, and Chloramphenicol on the 50S Reaction.[a]

Antibiotics (M)	AcPhe-puromycin Formed (cpm)	$AcPhe_2$-puromycin Formed (cpm)
None	2697	2902
Fusidic acid (10^{-4})	2736	2820
Chlorotetracycline (10^{-4})	2657	2857
Niddamycin (10^{-5})	651	682
Chloramphenicol (10^{-5})	2545	2558
(10^{-4})	679	2190
(10^{-3})	206	1475
−50S subunits	75	440

[a] The reaction mixtures of 0.15 ml contained 85 μg of 50S subunits, 9.1 pmol of ac[^{14}C]Phe-tRNA (5260 cpm) or 6.7 pmol of acPhe-[^{14}C]Phe-tRNA (4430 cpm). Other components, conditions and procedure of the assay are described in Methods.

Assay of Peptidyltransferase Activity by the 50S Reaction. This assay is a modification of the fragment reaction (Monro, 1967). Instead of the fragment of fMet-tRNA, whole peptidyl-tRNA was employed as the substrate. Since this reaction is catalyzed by the 50S ribosomal subunits, it will be denoted as the 50S reaction.

Reactions were run in duplicate. Each reaction mixture, including methanol, was 0.15 ml and contained 4×10^{-2} M Tris-HCl (pH 7.5), 0.27 M KCl, 1.3×10^{-2} M magnesium acetate, 3.3×10^{-2} M NH_4Cl, 3.3×10^{-4} M puromycin, 50S ribosomal subunits, and radioactive peptidyl-tRNA. The reaction was started by the addition of 50 μl of methanol. Incubation was at 25° for 50 min. The reaction was stopped by the addition of 10 μl of 10 N KOH and heated 10 min at 40° to hydrolyze methyl esters of peptides (Miskin *et al.*, 1970). After the addition of 1.0 ml of 1 M sodium phosphate (pH 7.0), and 1.5 ml of ethyl acetate, the solution was vigorously stirred for 30 sec. After a low-speed centrifugation to clarify the emulsion, 1.0 ml of the ethyl acetate extract was removed. The aqueous phase was reextracted with an additional 1.0 ml of ethyl acetate, recentrifuged, and 1.0 ml of the extract was removed. By this procedure the reaction products were completely extracted. The radioactivity of the combined ethyl acetate extracts was determined by a liquid scintillation spectrometer. Counting efficiencies for ^{14}C and ^{3}H were 80 and 23%, respectively.

Results

Effect of Fusidic Acid, Chlorotetracycline, Chloramphenicol, and Niddamycin on the 50S Reaction. In order to ensure that

TABLE III: Summaries of Reactivities of Various Substrates to Puromycin, Effect of Erythromycin on Substrates, and Erythromycin Concentrations for 50% Maximal Effect.[a]

Substrates	Amount of Substrate (pmol)		% of Substrate Reacted	Max. Effect of Erythromycin (%)	Erythromycin Concn at 50% Max. Effect (M)
	Added	Reacted			
AcPro-tRNA	18.5	0.98	5	+93	3.5×10^{-7}
AcPhe-tRNA	17.4	2.4	14	+72	1×10^{-7}
AcGly-tRNA	94.0	19.8	22	+41	2×10^{-7}
AcLeu-tRNA	2.4	1.7	72	+7	
Gly-tRNA	24.2	0.3	1.3	+37	3.5×10^{-7}
AcPro-Gly-tRNA	9.8	1.0	10	−66	2.4×10^{-7}
AcPhe-Gly-tRNA	105.0	16.1	15	−41	1.5×10^{-7}
AcPhe-Phe-tRNA	9.4	2.3	25	−34	1×10^{-7}
AcPhe-Leu-tRNA	4.5	1.4	31	−25	1×10^{-7}
AcGly-Gly-tRNA	8.0	1.6	19	+33	1×10^{-7}
AcGly-Pro-tRNA	1.9	0.01	0.5	+44	
AcPhe-Pro-tRNA	13.6	0.1	0.9	−64	
Phe-Phe-tRNA	3.9	0.7	18	−43	1×10^{-7}

[a] The reaction conditions were described under Methods. The maximal effects of erythromycin are average values of several experiments which may not be identical to the values shown on the graphs. The positive sign indicates percent of stimulation caused by erythromycin, and the negative sign indicates percent of inhibition caused by erythromycin.

the 50S reaction was a specific assay for peptidyltransferase and did not involve translocation or other steps in protein synthesis, several antibiotics of known mechanism were tested. Fusidic acid, a translocation inhibitor (Tanaka *et al.*, 1968) and chlorotetracycline, an inhibitor of the codon-directed binding of aminoacyl-tRNA to ribosomes (Mao and Robishaw, 1971b), had no significant effect on the formation of acPhe-puromycin and $acPhe_2$-puromycin as expected. On the other hand, chloramphenicol (Julian, 1965) and niddamycin (Mao and Robishaw, 1971a) which are known to be inhibitors of peptide-bond formation, strongly inhibited the formation of acPhe- and $acPhe_2$-puromycin (Table II).

Effect of Erythromycin on the First Peptide-Bond Formation. A previous study (Mao and Robishaw, 1971a), suggested that the action of erythromycin on peptide-bond formation depended on the chain length of the peptidyl moiety of donor substrates. Therefore, two groups of substrates, *N*-acetyl-aminoacyl-tRNA and *N*-acetyldipeptidyl-tRNA, were synthesized. The transfer of acPhe, acPro, acLeu, acGly, and Gly moieties from tRNA to puromycin was used as models of the first peptide-bond formation.

As shown in Figure 1, erythromycin consistently stimulated the first peptide-bond formation with the five above-mentioned substrates. The most sensitive substrate was acPro-tRNA. The formation of acPro-puromycin was stimulated about 93%. Formation of acPhe-puromycin and acGly-puromycin were stimulated 70 and 34%, respectively. AcLeu-tRNA was rather insensitive to erythromycin, the maximal effect observed from several experiments being 10% stimulation (data not shown). An N-unblocked aminoacyl-tRNA, Gly-tRNA, was also tested. The reactivity of Gly-tRNA was much lower than that of acGly-tRNA (Table III), but the effect of erythromycin on these two substrates was similar (Figure 1).

Important observations were made in this set of results. The maximal effect of erythromycin was exerted at a concentration of about 7×10^{-7} M which coincided with the saturation concentration for the binding of erythromycin to ribosomes. Furthermore, the dosage–response curve of peptidyltransferase to erythromycin coincides with the dissociation curve of the erythromycin–ribosome complex (Oleinick and Corcoran, 1969; Weisblum *et al.*, 1971). A time course of acPhe-puromycin synthesis in the absence or presence of macrolide antibiotics is shown in Figure 2. Erythromycin in-

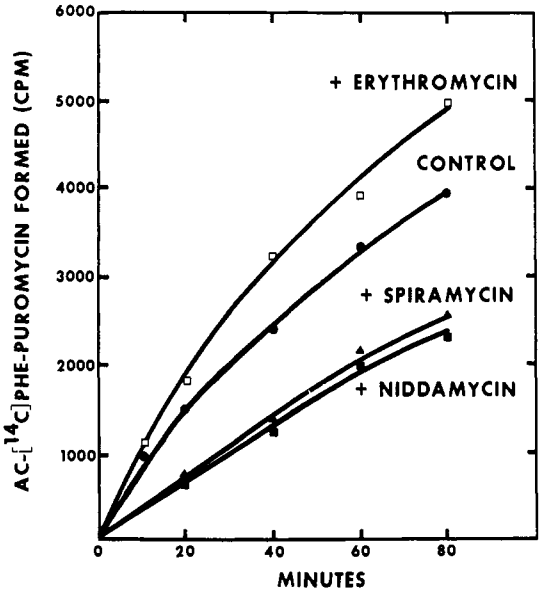

FIGURE 2: Time course of the reaction of puromycin with acPhe-tRNA in the absence or presence of macrolides. Four reaction mixtures of 1.8 ml each contained 2 mg of 50S ribosomal subunits, 220 pmol of ac-[^{14}C]Phe-tRNA (145,030 cpm), and other components as described under Methods. One of the four reaction mixtures contained no antibiotic, others contained 6.6×10^{-5} M erythromycin, 6.6×10^{-5} M spiramycin, or 1.6×10^{-5} M niddamycin Samples of 0.15 ml were taken at the indicated times. Formation of ac-[^{14}C]Phe-puromycin was determined by the ethyl acetate extraction method. Each point is the mean of duplicate estimations after subtraction of the zero-time blank (500 cpm).

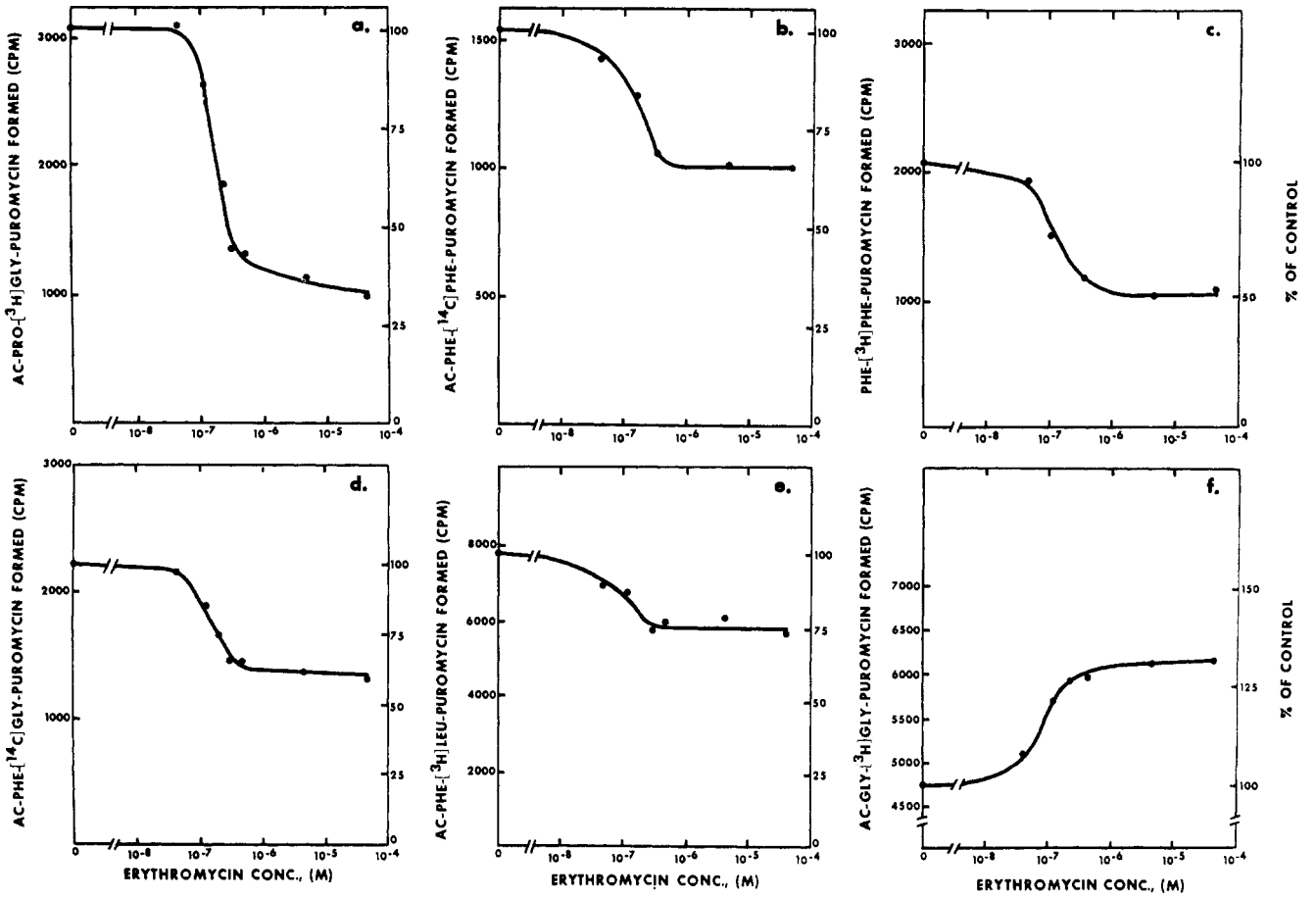

FIGURE 3: Effect of erythromycin on the reaction of puromycin with *N*-acetyldipeptidyl-tRNA and dipeptidyl-tRNA. The reaction mixtures of 0.15 ml contained 87 μg of 50S ribosomal subunits and (a) 9.8 pmol of acPro-[^{3}H]Gly-tRNA (29,335 cpm), (b) 9.4 pmol of acPhe-[^{14}C]Phe-tRNA (6200 cpm), (c) 3.9 pmol of Phe-[^{3}H]Phe-tRNA (11,634 cpm), (d) 105 pmol of acPhe-[^{14}C]Gly-tRNA (14,455 cpm), (e) 2.95 pmol of acPhe-[^{3}H]Leu-tRNA (34,164 cpm), and (f) 8 pmol of acGly-[^{3}H]Gly-tRNA (23,938 cpm). Other components and assay procedures are described under Methods.

creased, but spiramycin and niddamycin decreased, the rate of the first peptide-bond formation.

In all experiments fusidic acid at a concentration of 10^{-4} M had no significant effect on the first peptide-bond formation. Niddamycin at a concentration of 10^{-5} M caused 70, 72, 76, and 64% inhibition of the transfer of acPro, acPhe, acGly, and Gly from tRNA to puromycin, respectively.

Effect of Erythromycin on the Second Peptide-Bond Formation. The transfer of ac-dipeptidyl or dipeptidyl moieties from tRNA to puromycin was used as models of the second peptide-bond formation. Eight substrates were used; six were inhibited and two were stimulated by erythromycin.

Substrates inhibited by erythromycin were acPro-Gly-tRNA, acPhe-Gly-tRNA, acPhe-Phe-tRNA, acPhe-Leu-tRNA, Phe-Phe-tRNA (Figure 3), and acPhe-Pro-tRNA (Table III). Substrates stimulated by erythromycin were acGly-Gly-tRNA (Figure 3) and acGly-Pro-tRNA (Table III). It was noted that erythromycin never completely inhibited the second peptide-bond synthesis among the substrates tested. The most sensitive substrate was acPro-Gly-tRNA, showing a maximal inhibition of 67% at 7×10^{-7} M erythromycin which is the saturation concentration for the binding of erythromycin to the ribosomes. The kinetics of the second peptide-bond formation have been studied with several substrates. A representative result with acPro-Gly-tRNA as the substrate indicates that erythromycin inhibited both the rate and extent of the transfer reaction (Figure 4).

The extent of transfer of acGly-Pro-tRNA and acPhe-Pro-tRNA, was very low, *i.e.*, less than 1% of the substrates reacted with puromycin as compared to more than 10% for other ac-dipeptidyl-tRNA (Table III). This may indicate that peptidyltransferase is adapted to peptidyl donors with an α-amino group at the C terminal, and catalytic activity toward a donor with an α-imino group (proline) at the C terminal is very low.

For all eight substrates tested fusidic acid had no significant effect, and niddamycin at a concentration of 10^{-5} M caused 82, 64, 60, 87, 63, and 79% inhibition of transfer of acPro-Gly, Phe_2, acPhe-Gly, $acPhe_2$, $acGly_2$, and acPhe-Leu to puromycin, respectively.

Products of the 50S Reaction. The product of the transfer reaction was a radioactive, postively charged compound as determined by high-voltage paper electrophoresis. The compounds did not form in the absence of puromycin or 50S subunits, and were strongly inhibited by niddamycin. The N-blocked amino acids or N-blocked dipeptides which were cleaved from tRNA during the KOH treatment, being negatively charged, moved in the opposite direction. Therefore, it is reasonable to assume that the radioactive, positively charged reaction products were peptidyl–puromycin complexes.

Two typical electrophoretic patterns of the ethyl acetate extracts are shown in Figures 5 and 6. Formation of the positively charged compound was either stimulated or inhibited by erythromycin when acGly-tRNA (Figure 5) or acPro-Gly-

TABLE IV: Electrophoretic Analysis of Products of the 50S Reaction.[a]

Substrates	Peptidyl-puromycin Formed (cpm)			Electrophoretic Mobility of Peptidyl-puromycin (cm/hr)
	Control	Erythromycin	Niddamycin	
AcGly-tRNA	2939	4134	1669	2.65
AcPhe-tRNA	938	1546	157	1.18
AcPro-Gly-tRNA	2173	719	630	2.33
AcPhe-Gly-tRNA	1612	924	325	2.05
AcGly-Gly-tRNA	1765	2029	944	2.89
AcPhe-Phe-tRNA	2423	1873	163	1.05
Phe-Phe-tRNA	207	142	96	3.40

[a] The reaction mixtures of 0.15 ml contained 176 μg of 50S ribosomal subunits. The amount of substrates was 94 pmol (12,980 cpm) of ac[^{14}C]Gly-tRNA, 12.2 pmol (8070 cpm) of ac[^{14}C]Phe-tRNA, 9.8 pmol (29,334 cpm) of acPro-[^{3}H]Gly-tRNA, 105 pmol (14,455 cpm) of acPhe-[^{14}C]Gly-tRNA, 8 pmol (23,938 cpm) of acGly-[^{3}H]Gly-tRNA, and 6.7 pmol (4,430 cpm) of acPhe-[^{14}C]Phe-tRNA, or 3.9 pmol (11,684 cpm) of Phe-[^{3}H]Phe-tRNA. The concentration of erythromycin and niddamycin was 10^{-5} M. Other components of the reaction mixture, conditions and procedures for the assay, and electrophoresis are described in Methods and in Figure 5. All peptidyl–puromycin complexes moved toward the cathode.

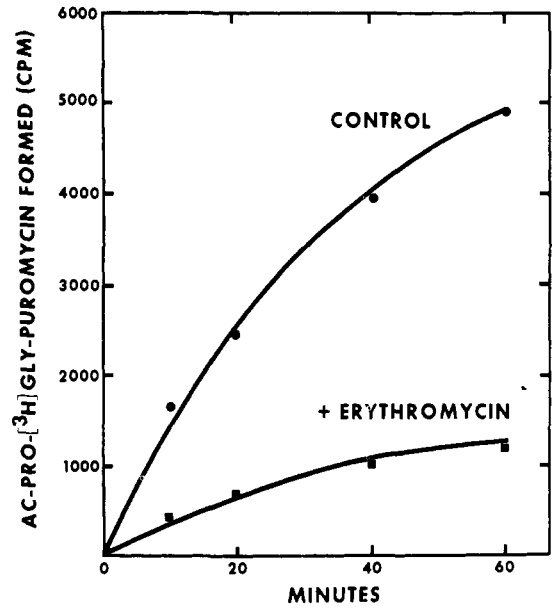

FIGURE 4: Time course of the reaction of puromycin with acPro-Gly-tRNA in the absence or presence of erythromycin. The reaction mixtures of 1.8 ml contained 1 mg of 50S ribosomal subunits, 115.2 pmol of acPro-[^{3}H]Gly-tRNA (345,254 cpm) and other components as described under Methods. Samples of 0.15 ml were taken at the indicated times. Each point is the mean of duplicate estimations after subtraction of a zero-time blank (870 cpm). Erythromycin concentration was 6.6×10^{-5} M.

tRNA (Figure 6) was used as the substrate, respectively. Niddamycin inhibited the formation of both positively charged compounds. Small amounts of acGly and acPro-Gly cleaved from unreacted substrates after KOH treatment were extracted into ethyl acetate and moved toward the anode as shown in the electrophoretograms. The reaction products of several substrates have been analyzed by paper electrophoresis. The radioactivity of the newly formed, positively charged compounds was determined and are listed in Table IV. The effect of erythromycin determined by the electrophoretic method was consistent with that obtained by the ethyl acetate extract method.

Effect of Erythromycin Derivatives on Peptidyltransferase. It has been shown that antibacterial activity of erythromycin is related to its binding to the 50S ribosomal subunits (Wilhelm and Corcoran, 1967; Mao and Putterman, 1969; Černá *et al.*, 1971; Weisblum *et al.*, 1971). Erythromycin derivatives which have different binding affinities for ribosomes and, subsequently, different antibacterial activities were tested in the 50S reaction. The substrate chosen for the assay was acPro-Gly-tRNA because it is the most sensitive to erythromycin inhibition. Table V shows the extent of inhibition of acPro-Gly-puromycin formation by erythromycin and its derivatives, as well as their relative binding affinities for 50S subunits and antibacterial activities. Erythromycin is the most potent inhibitor of bacterial growth, has high binding affinity for ribosomes, and also is the most potent inhibitor of the second peptide-bond formation. Erythromycin C is a less potent inhibitor of bacterial growth, has lower affinity for ribosomes, and also is a less potent inhibitor of peptide-bond formation. Other compounds having low antibacterial activity and low ribosomal binding, if any, showed low inhibitory effect against peptide-bond formation.

Discussion

The action of erythromycin is unique in that it can either stimulate or inhibit peptidyltransferase depending on the nature of the substrate. These dual actions and the incomplete inhibition can not be explained by competition between erythromycin and peptidyl-tRNA. An appropriate explanation consistent with the results is that erythromycin acts as an allosteric effector which binds to the 50S ribosomal subunit in the vicinity of the peptidyl site and induces a conformational change (Figure 7) thus affecting the reaction rate of peptidyltransferase. The possibility that erythromycin changes the binding affinity of donor substrates to the ribosomes does not seem likely since it has been reported that binding of aminoacyl-tRNA, *N*-acetylaminoacyl-tRNA, and Lys_n-tRNA to ribosomes was not affected by erythromycin (Tanaka *et al.*, 1966; Černá *et al.*, 1969; Mao and Robishaw, 1971a).

The testing of many substrates has made it possible to evaluate the structure of substrates in relation to the effect of erythromycin. Erythromycin stimulated the transfer of acPro, acGly, acPhe, acLeu, or Gly moieties from tRNA to puromycin, a reaction which is equivalent to the synthesis of the first peptide bond. Similar results have been reported with acPhe-tRNA (Černá *et al.*, 1969; Tanaka *et al.*, 1971), acLeu-tRNA, acLeu-ACCAC, acPhe-ACCAC (Černá *et al.*, 1971), and fMet-tRNA (Mao and Robishaw, 1971a). The maximal levels of stimulation for each substrate were quite different. For instance, erythromycin stimulated acPro-puromycin synthesis 90–110%, but stimulated acLeu-puromycin synthesis only 5–10%. The different degree of stimulation apparently is not related to the tRNA structure of the various donors since erythromycin caused a similar degree of stimulation with

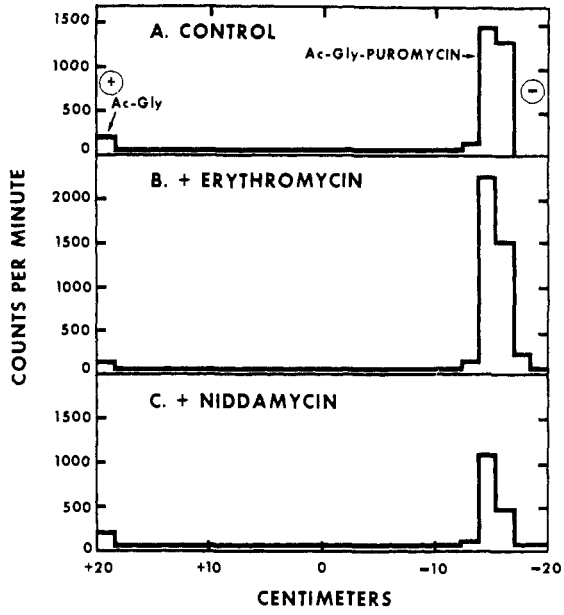

FIGURE 5: Paper electrophoretic analysis of the reaction product formed during the reaction of puromycin with acGly-tRNA. The reaction mixtures of 0.15 ml contained 209 μg of 50 S ribosomal subunits and 94 pmol of ac-[^{14}C]Gly-tRNA (12,980 cpm). Other components of the reaction mixture, conditions, and procedures for the assay are described under Methods. Antibiotic concentration was 6.6 $\times$ 10^{-5} M for erythromycin and 1 $\times$ 10^{-5} M for niddamycin. The product was extracted into ethyl acetate and evaporated to dryness. The residue was redissolved in 50 μl of 5% acetic acid–0.5% pyridine (pH 3.5), applied at the middle of the paper strip and developed with the same solvent, at 17.5 V/cm for 6 hr. Radioactivity on paper was located by immersing 1.5-cm strips in scintillation fluid and measured in a liquid scintillation spectrometer.

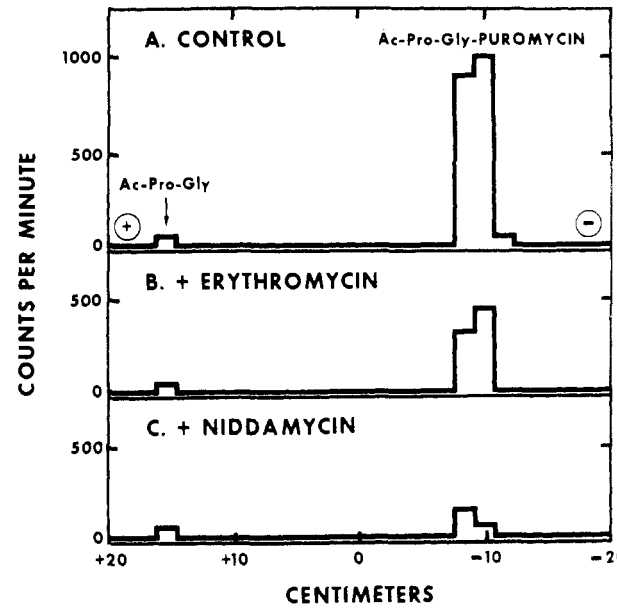

FIGURE 6: Paper electrophoretic analysis of the product formed during the reaction of puromycin with acPro-Gly-tRNA. The reaction mixtures of 0.15 ml contained 209 μg of 50S ribosomal subunits and 19.6 pmol of acPro-[^{3}H]Gly-tRNA (58,560 cpm). Other components of the reaction mixture, conditions, and procedures for the assay are described under Methods. The antibiotic concentration was 6.6 $\times$ 10^{-5} M for erythromycin and 1 $\times$ 10^{-5} M for niddamycin. Procedures for paper electrophoretic analysis were described in the legend of Figure 5.

TABLE V: Effect of Erythromycin and Its Derivatives on the Reaction of Puromycin with acPro-Gly-tRNA, with Data of Its Affinity to Ribosome and Antibacterial Activity.[a]

Antibiotics	% Inhibn of Peptidyl-transferase	Rel Binding Affinity[b]	Rel Antibacterial Act.[c]
Erythromycin	69	100	100
Erythromycin C	51	62	30
2′-Benzoylerythromycin	10	6	2
3′-De(dimethylamino)-erythromycin	8	2	1
N-Oxide erythromycin	7	2	2
Anhydroerythromycin	3	5	2
3-Mycarosylerythronolide B	6	2	1
5-Desosaminylerythronolide B	10	5	1

[a] The reaction mixtures of 0.15 ml each contained 85 μg of 50S subunits, 9.6 pmol of acPro-[^{3}H]Gly-tRNA (28,733 cpm), and erythromycin or its derivatives at a concentration of 10^{-6} M. Other components, conditions, and procedure for the assay are described under Methods. A sample without antibiotic gave 3609 cpm (1.2 pmol) in the ethyl acetate extracts. The blank containing no 50S subunits gave 19 cpm only. [b,c] Data are calculated from a previous report (Mao and Putterman, 1969).

acPhe-tRNA and acPhe-ACCAC (Černá *et al.*, 1971). Thus the amino acid side chains (R_1 and R_2 in Figure 7) are responsible for the sensitivity of substrates to the action of erythromycin. It appears that hydrophilicity and size of the amino acid side chains are predominant factors determining the sensitivity of a substrate to erythromycin. The order of water solubility of amino acids used is acPro > acGly > acPhe > acLeu. The order of stimulation was acPro > acPhe > acGly > acLeu (Table III). The reversed order of Gly and Phe with respect to water solubility and stimulation by erythromycin could be due to the size of the amino acid side chains. It is reasonable to assume that since glycine lacks a side chain, the influence of the conformational change of the peptidyl binding site is less pronounced. Thus, monoaminoacyl-tRNA substrates containing small or hydrophobic side chains were relatively unaffected by erythromycin.

Erythromycin caused inhibition of dipeptidyl-puromycin formation with six substrates (acPro-Gly-, acPhe-Gly-, acPhe-Phe-, acPhe-Leu-, acPhe-Pro-, and Phe-Phe-tRNA) and caused stimulation with two (acGly-Gly- and acGly-Pro-tRNA). In addition, Tanaka *et al.* (1971) have reported that erythromycin had no significant effect on the formation of Gly-Phe-, Leu-Phe- and Val-Gly-Phe-puromycin but inhibited Phe-Phe-puromycin formation. The various responses can be related to hydrophilicity and size of the amino acid side chains. The order of hydrophilicity of the various *N*-acetyl dipeptides is acPro-Gly > acPhe-Gly > acPhe-Phe > acPhe-Leu. The maximal inhibition of these substrates by erythromycin is in the same order (Table III). The reaction of acPhe-Pro-tRNA and acGly-Pro-tRNA with puromycin was too low for meaningful discussion (Table III). Gly-Phe-, Leu-Phe-, and Val-Gly-Phe-tRNA with which erythromycin causes no significant effect (Tanaka *et al.*, 1971), has either glycine or leucine at the second position (R_2 in Figure 7). According to

our hypothesis these two amino acids either because of the small size or high hydrophobicity of the side chains should be resistant to the action of erythromycin.

As the data show, erythromycin stimulated the transfer of acPhe and acGly but inhibited the transfer of acPhe-Phe and acGly-Gly. This suggests that the peptide chain length is another factor along with size and hydrophilicity related to the expression of erythromycin action. Therefore, a general assumption that inhibition of tripeptide but not dipeptide synthesis indicates translocation as the step of inhibition may not be correct.

To account for all ranges of stimulation and inhibition of dipeptidyl transfer, one can assume that the conformational change induced by erythromycin at the C-terminal aminoacyl site (R_1 in Figure 7) increases peptidyltransferase activity; the conformational change at the second aminoacyl site (R_2 in Figure 7) decreases peptidyltransferase activity. The overall effect of erythromycin on dipeptidyl-tRNA is the sum of the effects on R_1 and R_2. For example, erythromycin stimulated the transfer of acGly-Gly but inhibited the transfer of acPro-Gly. This can be explained as follows. The effect of erythromycin on C-terminal Gly which is a common component of these two substrates is stimulatory, as evident by the stimulation of acGly transfer by erythromycin. When the second amino acid is glycine which has no side chain, the influence from erythromycin is small as compared to the influence on the C-terminal glycine; therefore, the overall result of acGly-Gly transfer is stimulation. On the other hand, when the second amino acid is proline which is highly hydrophilic and has a sizeable side chain, it should be extremely sensitive to the conformational change induced by erythromycin. Therefore, the overall results of acPro-Gly transfer is inhibition. These rules can generally be applied to predict the action of erythromycin on various dipeptidyl-tRNA.

An inversed relationship was noted between the extent of reaction of various substrates with puromycin and the maximal levels of effect of erythromycin among the N-blocked substrates (Table III). Those which reacted extensively with puromycin and were affected little by erythromycin are more hydrophobic. Those which reacted less with puromycin and were more affected by erythromycin are less hydrophobic. These results suggest that the binding site for the peptidyl moiety on the 50S subunit is a hydrophobic area. It can be expected that more hydrophobic peptidyl moieties stabilize ribosomal conformation at the peptidyl site more effectively than less hydrophobic peptidyl moieties and consequently, are affected less by erythromycin.

There is a question whether the mechanism of action of erythromycin observed in the highly simplified 50S reaction also operates in the bacterial cell. There are several areas of similarities in *in vivo* and *in vitro* results. The saturation concentration of erythromycin affecting peptidyltransferase determined with eleven substrates in the 50S reaction was about 7×10^{-7} M (0.5 μg/ml) which is in the range of minimum inhibitory concentrations of erythromycin for sensitive bacteria. Six erythromycin derivatives which have no antibacterial activity also had no effect on peptidyltransferase. Erythromycin C which has antibacterial activity less than that of erythromycin A also had less effect on the activity of peptidyltransferase (Table V). It is well known that erythromycin binds to the 50S ribosomal subunits and forms a 1:1 ratio complex (Mao, 1967b), and there is overwhelming evidence relating the antibacterial activity of erythromycin to its binding to ribosomes (Wilhelm and Corcoran, 1967; Mao *et al.*, 1970; Tanaka *et al.*, 1971; Saito *et al.*, 1971; Černá *et al.*, 1971; Weisblum *et al.*, 1971). If the inhibition of peptidyltransferase is indeed the mechanism of action of erythromycin, the erythromycin concentration causing 50% of the maximal effect, whether stimulation or inhibition, should be identical with the dissociation constant of the erythromycin–ribosome complex which has been reported to be $1\text{–}2 \times 10^{-7}$ M (Oleinick and Corcoran, 1969; Weisblum *et al.*, 1971). In addition it should also be identical to the erythromycin–bacterium dissociation constant which has been determined to be 1.1×10^{-7} M for *Staphylococcus aureus* and 3.4×10^{-7} M for *Bacillus subtilis* (Mao and Putterman, 1968). From the data presented in this paper, the erythromycin concentration causing 50% of the maximal effect on peptidyltransferase was $1\text{–}3.5 \times 10^{-7}$ M with an average of 1.8×10^{-7} M (Table III), which agrees with the dissociation constants of erythromycin–ribosome and with erythromycin–bacterium complexes. These correlations between (1) the binding of erythromycin to ribosomes, (2) the effect of erythromycin on peptidyltransferase, and (3) the inhibition of bacterial growth by erythromycin, strongly support the theory that the antibiotic action is due to an effect on peptidyltransferase.

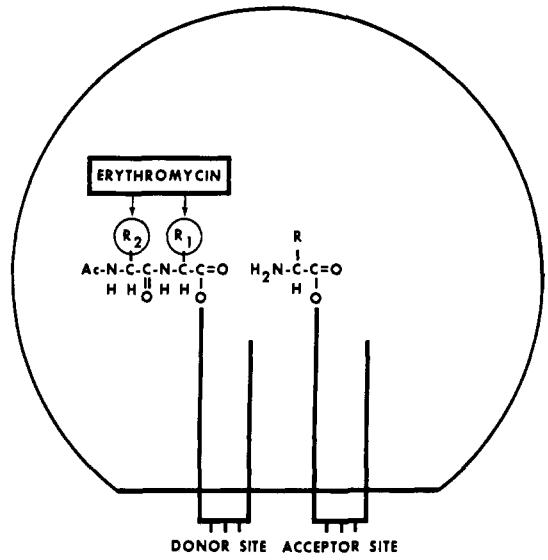

FIGURE 7: Proposed mechanism of action of erythromycin. An erythromycin molecule binds to the 50S ribosomal subunits in the vicinity of the peptidyl binding site, and induces a conformational change which affects at least two aminoacyl residues (R_1 and R_2) of the donor tRNA. The effect of erythromycin on R_1 usually increases the reaction rate of peptidyltransferase; and, therefore, erythromycin stimulates the transfer of monoaminoacyl moieties from the donor tRNA to an acceptor. The effect of erythromycin on R_2 usually decreases the reaction rate of peptidyltransferase. The overall result of the transfer of a dipeptidyl moiety from the donor tRNA to an acceptor is the sum of the effect of erythromycin on R_1 and R_2. Erythromycin may be inhibitory or stimulatory to the transfer of dipeptides depending on the nature of the amino acids (see Discussion). In this study, aminoacyl-tRNA on the acceptor site was replaced by puromycin.

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