Rubradirin, an Inhibitor of Ribosomal Polypeptide Biosynthesis†

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ABSTRACT: The antibiotic rubradirin acts as a potent inhibitor of polypeptide biosynthesis in cell-free systems directed with synthetic messenger ribonucleic acid. Natural messenger RNA mediated protein synthesis is susceptible to rubradirin inhibition if the assay mixtures contain a formylmethionyl-tRNA generating system. An analysis of individual sequential steps occurring within the integrated ribosomal peptide synthesis process suggests that the antibiotic interferes specifically with the function of the peptidyl site of the ribosome.

The antibiotic rubradirin was isolated from the culture broth of *Streptomyces achromogenes* var. *rubradiris*. Preparation, isolation, characterization, and biological properties have been described (Bhuyan *et al.*, 1965; Meyer, 1965). The compound was purified as an amorphous powder which is acidic and has the empirical formula $C_{51}H_{50}N_4O_{21}$, corresponding to a molecular weight of 1055. The free acid is insoluble in water but soluble in most organic solvents; salts are slightly soluble in water. Rubradirin possesses pH indicator properties. The compound is red in acidic and neutral solutions and green in basic solutions or in salt form. Rubradirin is a very potent inhibitor of Gram-positive bacteria *in vitro* and is effective in the systemic treatment of experimental bacterial infections in mice.

Studies pertaining to the mode of action of rubradirin have shown that the antibiotic specifically interferes with protein synthesis in bacterial cell-free systems as described in this paper.

Materials and Methods

The cell-free polyribonucleotide and phage RNA-directed amino acid incorporation systems were essentially as described previously (Reusser, 1969). The S-30 fraction was used as an enzyme source. The exact composition of the reaction mixtures is shown in Tables I and II.

Ribosomes were recovered from the S-30 fraction by centrifugation and washed three times in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.01 M magnesium acetate, 0.5 M NH_4Cl, and 1 mM mercaptoethanol to remove the initiation and elongation factors.

Washed 70S ribosomes were dissociated into subunits and dialyzed as described by Staehelin *et al.* (1969). Isolation and purification of 50S and 30S subunits was achieved by three successive sucrose gradient centrifugation steps in 10–30% sucrose gradients of the same ionic composition as the dialysis buffer mentioned in the reference above. Subunits were concentrated from the appropriate sucrose gradient fractions by precipitation with ethanol (Staehelin *et al.*, 1969). Both the 30S and 50S preparations were activated by incubating them at 30° for 30 min prior to use.

Poly(C) attachment to ribosomes was assessed by the filtration technique of Moore (1966). Poly(U) attachment to ribosomes could not be measured by this technique due to a high degree of self-adsorption of poly(U) to the filters. Poly(U) binding to ribosomes was thus measured by separating the ribosome–poly(U) complex by sucrose density gradient centrifugation (Reusser, 1969).

Escherichia coli soluble RNA (tRNA), stripped, was purchased from General Biochemicals. The methods used to assess the extent of aminoacylation of tRNA and the preparation of [^{14}C]aminoacyl-tRNA were as described previously (Reusser, 1969).

Formation of the ternary [^{14}C]aminoacyl-tRNA–polyribonucleotide–ribosome complexes was determined by the technique of Nirenberg and Leder (1964).

The ribosomal wash fluid used as a source of initiation factors was prepared as described by Lucas-Lenard and Lipman (1967). L-[^{14}C]Phenylalanyl-tRNA was acetylated to *N*-acetylphenylalanyl-tRNA by the procedure of Haenni and Chapeville (1966). The purity of the product was tested by alkaline hydrolysis, followed by thin layer chromatography as described by Grummt and Bielka (1971).

N-Acetylphenylalanyl-tRNA binding to 70S ribosomes was tested in the presence of either 6 or 14 mM Mg^{2+}. In the samples containing 6 mM Mg^{2+}, the reaction mixtures contained in a total volume of 0.25 ml: Tris-HCl, pH 7.4, 12.5 μmol; NH_4Cl, 40 μmol; magnesium acetate, 1.5 μmol; dithiothreitol, 2.5 μmol; poly(U), 20 μg; initiation factors, 120 μg of protein; guanosine 5′-triphosphate (GTP), 0.06 μmol; *N*-acetyl[^{14}C]-phenylalanyl-tRNA, 100 μg containing 8000 cpm; and washed ribosomes as specified in the text. The reaction was stopped by chilling the tubes in ice and dilution with 2 ml of cold 0.01 M Tris-HCl wash buffer, pH 7.4, containing the same molarity of magnesium acetate and NH_4Cl as the assay mixtures. The samples were immediately filtered through 0.45-μ Millipore filters and washed three times with 3-ml portions of wash buffer. Radioactivity retained on the filters was measured by liquid scintillation spectrometry. The samples containing 14 mM Mg^{2+} were of the same composition as the ones described above except for: magnesium acetate, 3.5 μmol; no GTP and no initiation factors.

N-Acetylphenylalanyl-tRNA binding to 30S ribosomal subunits was assessed by the same method given above for 70S ribosomes.

Results

Effect on Synthetic Messenger RNA Directed Amino Acid

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TABLE I: Effect of Rubradirin on Polyribonucleotide-Directed Amino Acid Incorporation.[a]

Sample	System	Cpm/ Sample	% Inhibition
Control Rubradirin	Poly(U)–phenylalanine incorp.	2600	0
0.05 μM/ml		1060	59
0.02 μM/ml		1270	51
0.01 μM/ml		1580	39
0.005 μM/ml		1670	36
Control Rubradirin	Poly(C)–proline incorp.	3150	0
0.05 μM/ml		1620	48
0.02 μM/ml		2200	30
0.01 μM/ml		2290	27
0.005 μM/ml		2660	15
Control Rubradirin	Poly(A)–lysine incorp.	3030	0
0.05 μM/ml		1440	52
0.02 μg/ml		1630	46
0.01 μM/ml		1870	38
0.005 μM/ml		2020	33

[a] Reaction mixtures contained in a total volume of 0.25 ml: Tris-HCl buffer, pH 7.8, 25 μmol; magnesium acetate, 3.5 μmol; KCl, 15 μmol; ATP, 0.25 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]phenylalanine, [^{14}C]proline, or [^{14}C]lysine, 0.25 μmol containing 1.75 μCi; polyribonucleotide, 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). Incorporation in the absence of messenger amounted to 1–3% of the controls.

TABLE II: Effect of Rubradirin in Phage f_2 RNA-Directed Amino Acid Incorporation Systems.[a]

Sample	Leucovorin	Mg^{2+} Concn (mM)	Cpm/ Sample	% Inhibition
Control	None	14	2950	0
Rubradirin				
0.05 μM/ml	None	14	2930	0
0.025 μM/ml	None	14	2810	4
Control	60 μg/ml	2	1630	0
Rubradirin				
0.05 μM/ml	60 μg/ml	2	920	44
0.025 μM/ml	60 μg/ml	2	1220	25
Control (less leucovorin)		2	810	

[a] Reaction mixtures were those described in Table I, except: f_2 phage RNA, 1.5 OD_{260} units; mixture of 14 [^{14}C]amino acids, 54 Ci/atom C (Amersham/Searle), 0.5 μCi; mixture of six [^{12}C]amino acids, 0.05 μmol per sample. Incorporation in the absence of phage RNA amounted to less than 10% of the controls.

Incorporation Systems. Incorporation of [^{14}C]phenylalanine, [^{14}C]proline, or [^{14}C]lysine directed by synthetic polyribonucleotides was inhibited substantially by rubradirin (Table I). Reasonably strict dose–response relationships were obtained with all these systems. A rubradirin concentration of 0.023 μmol/ml caused 50% inhibition of the poly(U) directed phenylalanine incorporation system. The corresponding values were 0.056 μmol/ml for the poly(C)–proline and 0.035 μmol/ml for the poly(A)–lysine incorporation systems, respectively. Polyphenylalanine synthesis was thus somewhat more susceptible to rubradirin inhibition than polylysine or polyproline synthesis.

Various preincubation times (0–30 min) of the S-30 enzyme with antibiotic prior to assay did not result in an increase of inhibitory activity in the phenylalanine incorporation system. This suggests that the ribosomes have to actively engage in protein synthesis before inhibition can occur. Increasing concentrations of S-30 enzyme (0.18–0.90 mg of protein/sample) in the phenylalanine incorporation system in the presence of a constant amount of drug (0.05 μmol/ml) did not reverse the inhibitory activity of rubradirin. On the contrary, the extent of inhibition was only 44% at the suboptimal level of 0.18 mg of S-30 protein/sample and gradually reached a plateau of approximately 60% inhibition in the samples containing nonlimiting amounts of S-30 enzyme (0.36–0.90 mg of S-30 protein/ml). These results indicate that the assay system is most susceptible to rubradirin inhibition if the assay mixtures contain optimal or excessive amounts of S-30 enzyme. Increasing concentrations of mRNA (7.5–40 μg of poly(U)/sample) in the phenylalanine incorporating system did not cause reversal of the inhibitory activity of rubradirin. The system was inhibited to an extent of 60–70% regardless of the poly(U) concentration. Variation of tRNA in the phenylalanine incorporation system yielded results similar to the ones obtained with various S-30 enzyme concentrations. Rubradirin-induced inhibition of phenylalanine incorporation was lowest at suboptimal tRNA concentrations in the samples and increased gradually to reach a plateau level as tRNA was present in optimal or excessive amounts.

The effect of magnesium concentration in relation to rubradirin inhibition in the phenylalanine incorporation system was studied at relative low Mg^{2+} concentrations (requiring the presence of initiation factors and *N*-acetylphenylalanyl-tRNA) and at high Mg^{2+} concentrations where Mg^{2+} alone serves as an initiator of peptide synthesis. At low Mg^{2+} concentrations (3–7 mM) polyphenylalanine formation was significantly inhibited by rubradirin (Figure 1). The extent of rubradirin-induced inhibition was highest in the presence of an Mg^{2+} concentration of 4 mM (84% inhibition) and only slightly lower at 5 mM (82% inhibition) or 6 mM Mg^{2+} (79% inhibition). At the two extreme Mg^{2+} concentrations tested (3 and 7 mM), inhibition of the reaction by rubradirin was lower and amounted to approximately 66% in each case.

In the assay system containing relative high concentrations of Mg^{2+} when initiation is induced by Mg^{2+} only, polyphenylalanine formation was optimal at Mg^{2+} concentrations of 12–16 mM in the samples containing no drug (Figure 2). In the presence of rubradirin, the reaction was inhibited at all Mg^{2+} concentrations tested. However, the extent of inhibition was highest at optimal Mg^{2+} levels and amounted to 64–67% in the presence of 0.05 μmol of drug/ml.

Effect on Phage f_2 RNA Directed Amino Acid Incorporation Systems. In the presence of a relatively high Mg^{2+} concentra-

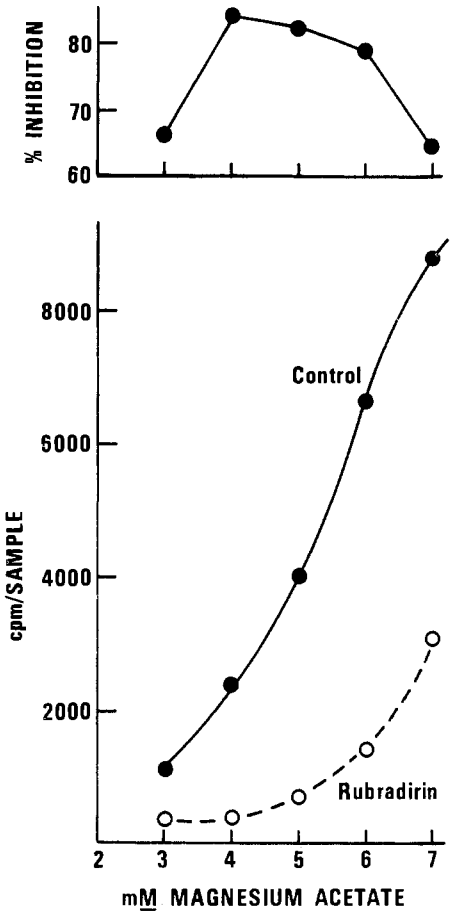

FIGURE 1: Effect of rubradirin on *N*-acetyl[^{14}C]phenylalanyl-tRNA incorporation in the presence of low Mg^{2+} concentrations. Samples contained in a total volume of 0.2 ml: Tris-HCl buffer, pH 7.4, 12.5 μmol; NH_4Cl, 25 μmol; mercaptoethanol, 1.5 μmol; phosphoenolpyruvate, K salt, 2.25 μmol; pyruvate kinase, 10 μg; GTP, 0.1 μmol; poly(U), 15 μg; phenylalanyl-tRNA, 50 μg; *N*-acetyl[^{14}C]phenylalanyl-tRNA, 50 μg containing ~10,000 cpm; S-30 fraction, 430 μg of protein. The samples were incubated at 37° for 30 min. Rubradirin concentration was 0.05 μmol/ml.

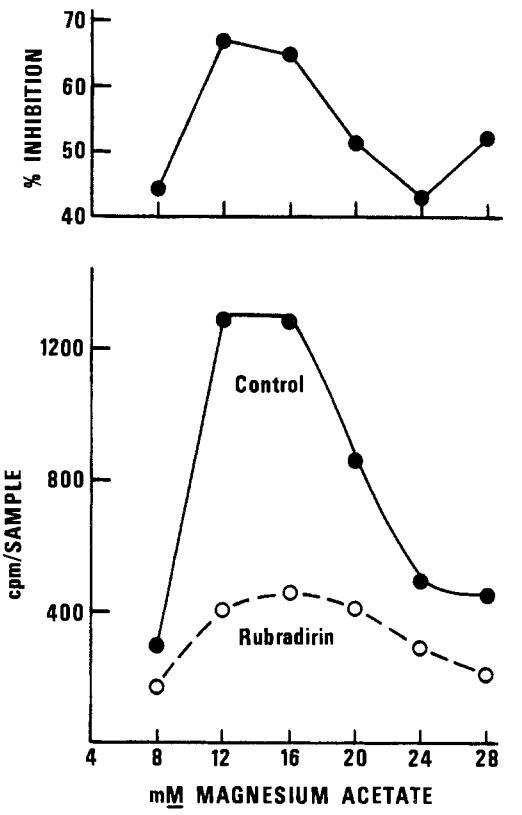

FIGURE 2: Effect of rubradirin on phenylalanine incorporation in the presence of high Mg^{2+} concentrations. Reaction mixtures were those described in Table I. Rubradirin concentration was 0.05 μmol/ml.

tion (14 mM) in the assay system where Mg^{2+} alone serves as an initiator of phage f_2 RNA directed amino acid incorporation, rubradirin showed no inhibition of peptide biosynthesis (Table II).

In the presence of low Mg^{2+} concentrations (2 mM) the addition of leucovorin (the calcium salt of 5-formyltetrahydrofolic acid, Lederle) significantly stimulated phage RNA directed amino acid incorporation. This indicates that leucovorin served as a formyl donor for the formation of formylmethionyl-tRNA in the test system. Under these conditions peptide biosynthesis was inhibited substantially in the presence of rubradirin (Table II). A drug concentration of 0.05 μmol/ml inhibited to an extent of 44%; at a concentration of 0.025 μmol/ml the system was inhibited by 25%. Thus, in the presence of a high Mg^{2+} concentration, natural mRNA-mediated amino acid incorporation remained essentially insensitive to rubradirin. On the other hand, in the presence of low Mg^{2+} concentrations and leucovorin, rubradirin now showed substantial inhibition of peptide biosynthesis.

Effect on Polyribonucleotide Attachment to Ribosomes. Attachment of [^{3}H]poly(C) was measured by the Millipore filtration technique. Ribosomal [^{3}H]poly(U) binding was assessed by separation of the ribosome–poly(U) complex on sucrose density gradients due to the high nonspecific adsorption of poly(U) to the filters in the Millipore filter assay. The results of these binding studies indicated that rubradirin did not change the binding capacity of ribosomes toward either poly(C) or poly(U).

Effect on Charging Activity. Both the formation of prolyl-tRNA and phenylalanyl-tRNA remained unaffected in the presence of rubradirin concentrations ranging from 0.01 to 0.1 μmol/ml.

Attachment of Aminoacyl-tRNA to Washed 70S Ribosomes. Attachment of aminoacyl-tRNA was tested in the two systems leading to either the formation of phenylalanyl-tRNA–poly(U)–ribosome complexes or prolyl-tRNA–poly(C)–ribosome complexes. The results obtained indicated that rubradirin inhibits these reactions moderately. Phenylalanyl-tRNA as well as prolyl-tRNA binding both were inhibited by 14% in the presence of 0.05 μmol of rubradirin/ml. Higher antibiotic concentrations did not result in a significant further increase of inhibition in both systems.

Effect of Delayed Addition of Rubradirin on Polyphenylalanine Synthesis. If an antibiotic proves more inhibitory when added at the onset of the reaction rather than any time after initiation of the reaction, this suggests but does not definitely prove that the drug-induced inhibition is limited to chain initiation and not to chain elongation.

The effect of delayed additions of rubradirin to reaction mixtures actively engaged in polypeptide synthesis was tested in systems containing either 6 or 14 mM Mg^{2+}. To the system containing 6 mM Mg^{2+}, antibiotic additions were made from 0 to 7.5 min at 2.5-min intervals after initiation of the reaction

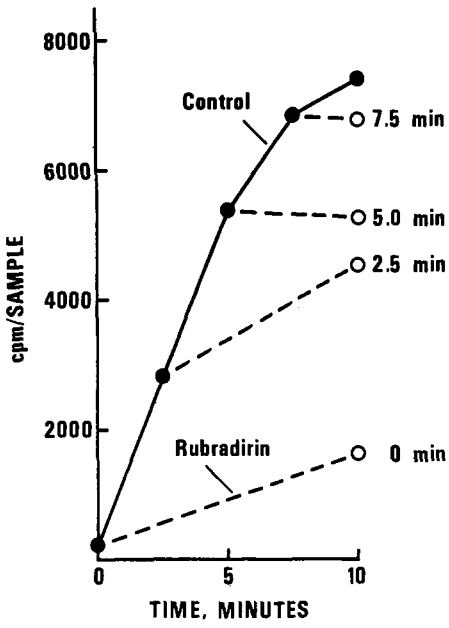

FIGURE 3: Effect of delayed addition of rubradirin on polyphenylalanine synthesis in the presence of 6 mM Mg^{2+}. Reaction mixtures were those described in Figure 1. Rubradirin concentration was 0.05 μmol/ml.

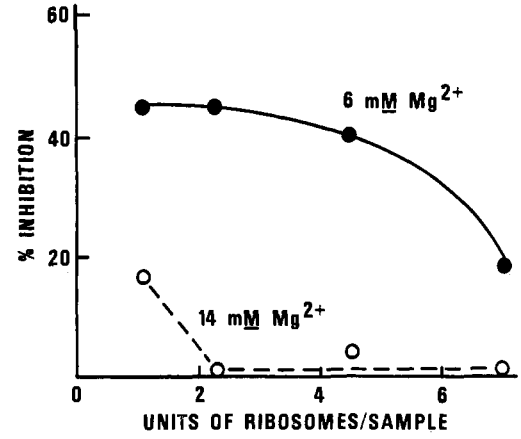

FIGURE 4: Effect of rubradirin on *N*-acetylphenylalanyl-tRNA binding to 70S ribosomes. Reaction mixtures contained in a total volume of 0.25 ml: (a) in the presence of 14 mM Mg^{2+}: Tris-HCl, pH 7.4, 12.5 μmol; NH_4Cl, 25 μmol; dithiothreitol, 2.5 μmol; poly(U), 20 μg; magnesium acetate, 3.5 μmol; *N*-acetyl[^{14}C]phenylalanyl-tRNA, 100 μg containing ~9000 cpm; rubradirin, 0.05 μmol/ml; (b) in the presence of 6 mM Mg^{2+}: same ingredients as above except, magnesium acetate, 1.5 μmol; initiation factors, 120 μg of protein; *N*-acetyl[^{14}C]phenylalanyl-tRNA, 150 μg containing 14,400 cpm; GTP, 0.06 μmol. Reaction mixtures were incubated at room temperature for 15 min for the samples containing 6 mM Mg^{2+} and at 30° for 30 min for the 14 mM Mg^{2+} containing samples. The mixtures were immediately filtered through Millipore filters and rinsed three times with 3-ml portions of Tris–salt buffer.

(Figure 3). The results indicate that the reaction rates were reduced significantly by rubradirin regardless of the time of drug addition. The extents of inhibition amounted to 75 or 60%, respectively, when the antibiotic was added at 0 or 2.5 min after the initiation of the reaction. When the drug was added at 5 or 7.5 min, inhibition was complete.

In the assay system containing 14 mM Mg^{2+}, rubradirin was added to the samples at 5-min intervals from 0 to 20 min after initiation of the reaction. In each case the reaction rate was inhibited to equal extents and amounted to approximately 60% inhibition regardless of the time of antibiotic addition. These results suggested that rubradirin either does not specifically inhibit chain initiation or that the process of initiation in our assay system was continuous and remained constant during the duration of the assay.

Effect on N-Acetylphenylalanyl-tRNA Binding to 70S Ribosomes. Polyphenylalanine synthesis at low Mg^{2+} concentrations depends on chain initiation with *N*-acetylphenylalanyl-tRNA, initiation factors, and GTP (Lucas-Lenard and Lipmann, 1967). GTP and initiation factor promoted *N*-acetylphenylalanyl-tRNA binding to 70S ribosomes can thus be used to assess chain initiation.

In the presence of 6 mM Mg^{2+}, rubradirin interferred significantly with the binding of *N*-acetylphenylalanyl-tRNA to ribosomes (Figure 4). The extent of inhibition was inversely related to the amount of ribosomes present in the reaction mixtures. In the presence of 1.1 OD_{260} units of ribosomes, inhibition was approximately 46%. This value was reduced to 18% in the presence of 7 units of ribosomes. Increasing amounts of ribosomes in the assay system caused, therefore, reversal of the inhibitory effect of rubradirin in this system.

When *N*-acetylphenylalanyl-tRNA binding was studied in the presence of 14 mM Mg^{2+} (Mg^{2+}-induced binding) and constant amounts of rubradirin, *N*-acetylphenylalanyl-tRNA binding to ribosomes was not affected in the presence of various ribosomal concentrations ranging from 2.3 to 7 units/sample (Figure 4). In the presence of 1.1 units of ribosomes some inhibition become apparent amounting to 16%.

N-Acetylphenylalanyl-tRNA prebound to ribosomes was slowly released upon addition of rubradirin (Figure 5). No concurrent decrease of acid-insoluble radioactivity was observed. This indicates that prebound *N*-acetylphenylalanyl-tRNA was released as such by rubradirin and no deacylation into *N*-acetylphenylalanine and tRNA took place.

Effect on N-Acetylphenylalanyl-tRNA Binding to 30S Ribosomal Subunits. Rubradirin did not inhibit the binding of *N*-acetylphenylalanyl-tRNA to 30S ribosomal subunits (Table III) in the presence of poly(U) and 14 mM Mg^{2+} (Mg^{2+}-in-

TABLE III: Effect of Rubradirin on *N*-Acetylphenylalanyl-tRNA Binding to 30S Ribosomal Subunits (14 mM Mg^{2+}).[a]

Sample	30S Particles Units/ Sample)	Cpm/ Sample	% of Control
Control	4	3350	100
Rubradirin	4	3230	96
Control	3	2200	100
Rubradirin	3	2440	111
Control	2	1700	100
Rubradirin	2	1790	105
Control	1	780	100
Rubradirin	1	1050	135

[a] Reactions were carried out as described in the legend to Figure 4 except that 30S ribosomal subunits were present instead of 70S ribosomes. Rubradirin concentration = 0.05 μmol/ml.

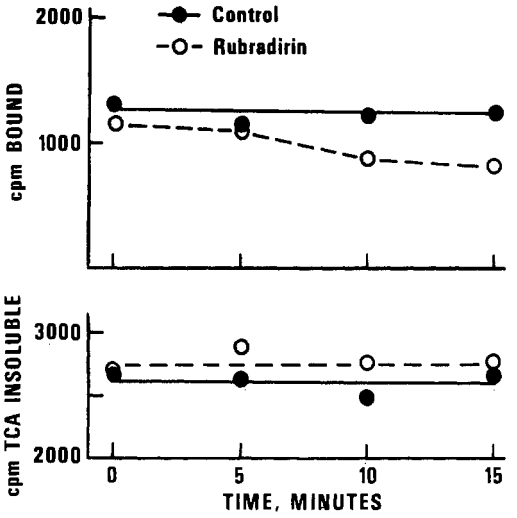

FIGURE 5: Effect of rubradirin on *N*-acetylphenylalanyl-tRNA prebound to 70S ribosomes. Reaction mixtures (6 mM in respect to Mg^{2+}) are described in the legend of Figure 4. The mixtures were preincubated for 15 min at 25°. After preincubation, rubradirin was added (0.05 μmol/ml) and two 0.25-ml sample aliquots were withdrawn at the times specified in the figure and assayed for ribosomal bound and acid insoluble (10% cold Cl_3CCOOH) radioactivity.

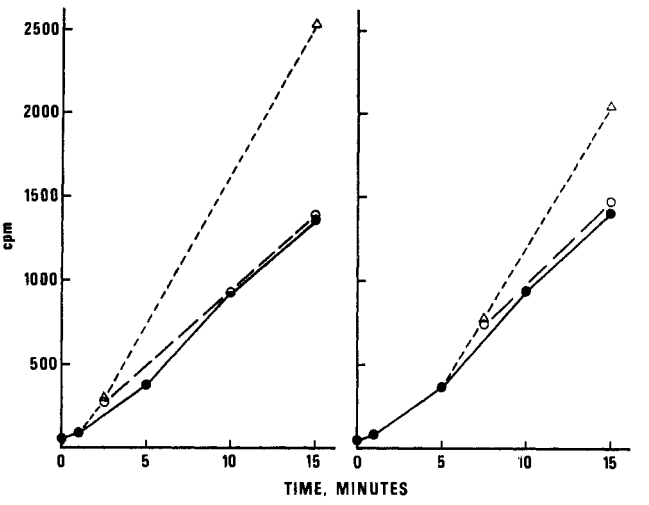

FIGURE 6: Effect of pactamycin and rubradirin on polyphenylalanine formation in the presence of 14 mM Mg^{2+}. Assay mixtures are defined in Table I: pactamycin, 0.02 μmol/ml; rubradirin, 0.05 μmol/ml. Left (●) control; (Δ) pactamycin only at 1 min; (○) pactamycin at 1 min, rubradirin at 2.5 min. Right: (●) control: (Δ) pactamycin only at 5 min; (○) pactamycin at 5 min, rubradirin at 7.5 min.

duced binding). On the contrary, at the low level of 1 unit of subunits/sample, some stimulation of binding by rubradirin become apparent. If the 30S initiation complex was preformed in the presence of rubradirin and low amounts of 30S subunits (1 unit/sample), the subsequent addition of 50S subunits to form the 70S complex resulted in a further stimulation of *N*-acetylphenylalanyl-tRNA binding (Table IV). These findings were somewhat unexpected. The experiments with 70S ribosomes indicated that rubradirin inhibits the Mg^{2+}-induced formation of the 70S complex if low amounts of 70S ribosomes are present during the initiation of the reaction as discussed in the preceding paragraph.

If the 30S initiation complex was formed in the presence of low Mg^{2+} concentrations (6 mM), initiation factors, and GTP, the complex proved unstable in the absence of rubradirin. Subsequent addition of 50S subunits did stabilize the complex somewhat. The lability of the complex is probably attributable to the presence of aminoacyl-tRNA hydrolases contained in the crude initiation factor preparation used. Such hydrolases were recently described by Vogel *et al.* (1971). It was thus not feasible to assess the effect of rubradirin in this very labile system. However, in the presence of rubradirin the extent of *N*-acetylphenylalanyl-tRNA binding was always lower than in the control samples. Addition of 50S subunits did not prevent this effect.

TABLE IV: Addition of 50S Particles to 30S Initiation Complex Preformed in the Presence of Rubradirin (14 mM Mg^{2+}).[a]

Sample	Time after Addn of 30S (min)	Cpm/ Sample	% of 20-min Control
Control			
30S subunits only	20	550	100
30S subunits only	40	570	105
30S + 50S subunits	40	510	94
Rubradirin			
30S subunits only	20	740	135
30S subunits only	40	730	134
30S + 50S subunits	40	930	170

[a] Basic reaction mixtures were as described in the legend to Figure 4 except that 1 unit of 30S ribosomal subunits were added per sample to initiate the reaction. Two units of 50S subunits were added 20 min after initiation of the reactions where applicable.

Effect of Pactamycin in Conjunction with Rubradirin. Pactamycin present in low concentrations was reported to prevent chain initiation during polyphenylalanine synthesis in cell-free systems (Cohen *et al.*, 1969). Thus, upon addition of pactamycin shortly after initiation of the reaction, chain initiation should cease and further net synthesis would reflect chain elongation only. Further inhibition (reflecting the portion not inhibitable by pactamycin) resulting from the addition of a second inhibitor would indicate that the second inhibitor inhibits a reaction step occurring after chain initiation.

This experimental approach was used to test rubradirin in conjunction with pactamycin. However, at 14 mM Mg^{2+} in the test system, pactamycin alone stimulated polyphenylalanine synthesis (Figure 6). Incorporation was almost doubled when pactamycin was added 1 min after initiation of the reaction. The addition of rubradirin as a secondary inhibitor at 2.5 min after initiation of the reaction resulted in total abolishment of the pactamycin-induced stimulation. Similar results were obtained when pactamycin was added 5 min after initiation of the reaction. In this case the extent of the pactamycin-induced stimulation was somewhat less and was approximately 150% of the control value. Rubradirin added at 7.5 min after the reaction was initiated again abolished the pactamycin-induced stimulation.

In the presence of 6 mM Mg^{2+} in the reaction mixtures requiring *N*-acetylphenylalanyl-tRNA and initiation factors for initiation, pactamycin did cause inhibition of peptide synthesis (Figure 7). Addition of rubradirin shortly after the

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

Sample	Mg^{2+} Concn (mM)	Cpm/ Sample	% Inhibition
Control	6	3550	0
Puromycin	6	1430	60
Rubradirin	6	3530	0
Rubradirin + puromycin	6	1330	62
Control	14	4190	0
Puromycin	14	2830	32
Rubradirin	14	4250	0
Rubradirin + puromycin	14	2940	31

[a] The reaction mixtures were those described in Figure 4 and contained 9 units of ribosome per sample. Ribosomes were prelabeled with *N*-acetyl[^{14}C]phenylalanyl-tRNA (10 min, room temperature for 6 mM Mg^{2+}; 30 min, 30° for 14 mM Mg^{2+}) in the presence or absence of 0.05 μmol of rubradirin/ml. Puromycin (1 μmol/ml) was then added and the samples were incubated for an additional 15 min.

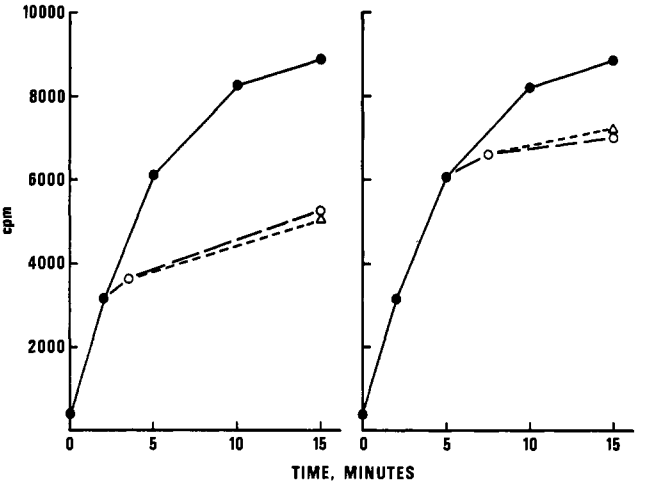

FIGURE 7: Effect of pactamycin and rubradirin on polyphenylalanine formation initiated by *N*-acetylphenylalanyl-tRNA (6 mM Mg^{2+}). Assay mixtures are defined in Figure 1; pactamycin, 0.02 μmol/ml; rubradirin, 0.05 μmol/ml. Left: (●) control; (△) pactamycin only at 2 min; (○) pactamycin at 2 min, rubradirin at 3.5 min. Right: (●) control; (△) pactamycin only at 5 min; (○) pactamycin at 5 min, rubradirin at 7.5 min.

addition of pactamycin did not result in any further inhibition of the reaction.

Effect on Puromycin Reaction. In the presence of relative high amounts of ribosomes, inhibition of *N*-acetylphenylalanyl-tRNA binding to ribosomes by rubradirin is minimal as shown above. Addition of puromycin to ribosomal-bound *N*-acetylphenylalanyl-tRNA resulted in the release of *N*-acetylphenylalanylpuromycin from the ribosomes. Rubradirin did not inhibit this reaction regardless of whether it was carried out in the presence of 6 or 14 mM Mg^{2+} (Table V).

Discussion

Rubradirin inhibits synthetic mRNA-directed amino acid incorporation into peptides in cell-free systems. Phage RNA-mediated peptide biosynthesis remains insensitive to rubradirin inhibition in a system containing high Mg^{2+} concentrations where chain initiation is effected by Mg^{2+} only. In the presence of low Mg^{2+} concentrations and leucovorin the system becomes susceptible to rubradirin inhibition. Under these conditions leucovorin will serve as a donor of formyl groups for the formation of formylmethionyl-$tRNA_f$. This aminoacyl-tRNA species is essential for chain initiation in whole bacterial cells. The complete absence of rubradirin inhibition in the amino acid incorporation system directed by natural mRNA, where chain initiation is induced by Mg^{2+} only, as opposed to the substantial inhibition observed in the system where chain initiation is induced by formylmethionyl-$tRNA_f$, strongly suggests that rubradirin interferes with the process of peptide chain initiation.

Polyphenylalanine formation is effectively inhibited in systems containing high Mg^{2+} concentrations (Mg^{2+}-induced initiation) and in systems low in Mg^{2+}, where initiation is induced by *N*-acetylphenylalanyl-tRNA, initiation factors, and GTP. Inhibition is optimal at optimal concentrations of Mg^{2+} in both systems. This indicates that there is no direct relationship between Mg^{2+} concentration and the extent of rubradirin inhibition. The systems studied proved most sensitive to rubradirin inhibition while synthesizing at optimal rates.

Initiation factor dependent binding of *N*-acetylphenylalanyl-tRNA to 70S ribosomes at low Mg^{2+} levels is greatly reduced by rubradirin and can be reversed partially by increasing amounts of ribosomes in the test system. On the other hand, *N*-acetylphenylalanyl-tRNA binding in the presence of high Mg^{2+} levels remains less sensitive to rubradirin inhibition. Mg^{2+}-induced binding of *N*-acetylphenylalanyl-tRNA to 30S ribosomal subunits is not affected by the antibiotic. In the presence of low Mg^{2+} concentrations and initiation factors the 30S complex formed proved very labile and the effect of rubradirin on the formation of this complex could not be assessed satisfactory.

It is assumed that *N*-acetylphenylalanyl-tRNA binds to the peptide site of the ribosome and that initiation factor dependent binding of *N*-acetylphenylalanyl-tRNA closely resembles the chain initiation process induced by formylmethionyl-$tRNA_F$ in bacteria (Lucas-Lenard and Lipmann, 1967). The observation that the factor-mediated binding process of *N*-acetylphenylalanyl tRNA to 70S ribosomes and probably also to 30S subunits is more susceptible to rubradirin inhibition than the one mediated by Mg^{2+} only suggests that rubradirin inhibits peptide synthesis by interaction with the assembly of the initiation complex.

The preformed 70S initiation complex is only slowly degraded upon addition of rubradirin and remains relatively stable in the presence of the antibiotic. Under these conditions, *N*-acetylphenylalanyl-tRNA is released as such and no evidence of concomitant deacylation is found.

The conclusion that rubradirin interferes somehow with the function of the peptidyl site of the ribosome is also sustained by the results obtained with mixtures of rubradirin and pactamycin. Pactamycin was reported to inhibit chain initiation (Cohen *et al.*, 1969) which might be true in systems low in Mg^{2+}. However, at Mg^{2+} concentrations higher than the ones studied by Cohen *et al.* pactamycin markedly stimulates peptide synthesis.

Under conditions where pactamycin will effect inhibition of peptide synthesis (low Mg^{2+} levels), rubradirin added in addition to pactamycin does not cause a further increase of the

pactamycin-induced inhibition. At high Mg^{2+} levels where pactamycin will stimulate peptide synthesis, addition of rubradirin abolishes the pactamycin-induced stimulation. As far as pactamycin is concerned, these results indicate that pactamycin definitely interferes somehow with the chain initiation process which, depending on the Mg^{2+} concentration present in the system, results in inhibition of this process at low Mg^{2+} levels, no interference at intermediate Mg^{2+} levels (see Cohen *et al.*, 1969), and enhancement at high Mg^{2+} concentration. Inhibition of polypeptide biosynthesis by rubradirin is not directly related to the Mg^{2+} concentration present. In addition, rubradirin does not potentiate pactamycin-induced inhibition at low Mg^{2+} levels and abolishes pactamycin-induced stimulation at high Mg^{2+} levels. This suggests that both antibiotics interact at the same functional level of the ribosome, namely the peptidyl site. Rubradirin does not interfere with the peptide bond formation step studied with the puromycin reaction. It is also unlikely that rubradirin affects the chain termination process. Synthetic messenger-directed amino acid incorporation leads to the accumulation of peptide chain products which remain bound to the ribosomes, and practically no release of peptides occurs under these conditions. Inhibition of chain termination would thus remain obscured in these test systems.

The results presented therefore indicate that rubradirin interacts somehow with the function of the peptidyl site of the ribosome resulting in inhibition of polypeptide biosynthesis.

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Isoleucyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli* B. Effects of Magnesium and Spermine on the Amino Acid Activation Reaction†

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ABSTRACT: We have investigated the interaction of magnesium and spermine with Ile-tRNA synthetase under conditions in which catalysis of L-isoleucine activation is observed. We used a fluorimetric method and titration and stopped-flow techniques. The results support the previous findings by Cole and Schimmel ((1970), *Biochemistry 9*, 3143) that ATP reacts as the magnesium salt. At concentrations higher than 1 mM, magnesium becomes inhibitory with an inhibition constant of 3–5 mM. Spermine is found to inhibit accumulation of enzyme-bound L-isoleucyl adenylate. Inhibition proceeds *via* two routes; one is binding of spermine to an effector site and the other is the formation of catalytically inert spermine–ATP. Presumably, binding to the effector site is followed by a conformation change of the enzyme, leaving the Michaelis–Menten complex less reactive. Binding of the effector is associated with an enhancement of the fluorescence intensity of the reporter group, 2-*p*-toluidinylnaphthalene-6-sulfonate, which is complexed with the enzyme. This response is in contrast to the fluorescence quenching observed for binding of substrates and products. Spermine binds to ATP. The strength of the interaction is comparable with that for magnesium. Spermine–ATP appears to bind to the enzyme, however, ability to participate in the catalysis of the amino acid activation reaction is lost.

The catalysis of an amino acid specific ATP-[^{32}P]PP_i exchange reaction together with the isolation of an enzyme-bound aminoacyl adenylate were taken as strong evidence for the formation of an intermediate as part of the specific charging reaction of a cognate tRNA catalyzed by an aminoacyl-tRNA synthetase (Berg, 1958; Bergmann *et al.*, 1961; Berg *et al.*, 1961; Norris and Berg, 1964). The rate of exchange as well as the preparative accumulation of the intermediate have been found to possess a substantial magnesium dependence. A

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