Inhibition of Protein Synthesis by Polypeptide Antibiotics

III. Ribosomal Site of Inhibition

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(Received June 8, 1966)

SUMMARY

The mechanism by which the antibiotics of the PA 114 complex inhibited protein synthesis in cell-free extracts of bacteria was studied. The antibiotic consists of at least two components belonging to two major groups, A and B. Although each antibiotic of groups A and B was effective individually in inhibiting protein synthesis *in vivo*, in combination they acted synergistically. PA 114 A inhibited protein synthesis best when messenger RNA was not attached to the ribosome and functioning. If the antibiotic was added after the messenger RNA-aminoacyl-tRNA-ribosome complex had formed and was functioning, inhibition of protein synthesis was decreased. This study showed that PA 114 A inhibited polyuridylic acid-directed binding of phenylalanyl-tRNA to 70 S ribosomes. The antibiotic inhibited ribosome function by specifically destroying the functioning of the 50 S ribosome subunit. PA 114 B acted synergistically to inhibit protein synthesis by enhancing the effect of the A component on the 50 S ribosome. These results suggested that the inhibition of protein synthesis by these antibiotics involved an interaction with a site on the 50 S ribosome subunit of the functional 70 S ribosome required for protein synthesis. This site was perhaps the same as or close to the site at which aminoacyl-tRNA bound.

INTRODUCTION

The antibiotics of the PA 114 group exist as a complex of synergistic compounds (1) closely related in structure with vernamycin (2), streptogramin (3), staphylomycin (4), ostreogrycin (5), mikamycin (6), and pristinamycin (7). All the antibiotic complexes consist of at least two components belonging to two major groups, A and B (8). The B component is a peptidic lactone (9–12), and the A component may be a nonpeptide macrolide (9). Each antibiotic of groups A and B is effective itself in inhibiting bacterial growth, but they act synergistically in combination (1, 13, 14). Previous investigations have implicated protein synthesis as the primary site of growth inhibition in intact bacterial cells (15–17) and in cell-free extracts (9, 18–20).[1]

The A group of antibiotics is more active against gram-positive cocci than gram-positive bacilli; and the reverse is true for the antibiotics of group B (13, 17, 21). The antibiotics are essentially inactive against gram-negative organisms (3, 13, 21, 22). However, protein synthesis by cell-free extracts of the gram-negative bacterium *Escherichia coli* is inhibited by the antibiotics, suggesting that the antibiotics are unable to enter gram-negative cells (19).

The A component of the antibiotic complex is a potent inhibitor of polyuridylic acid-directed synthesis of polyphenylala-

[1] Papers I and II of this series were published in *J. Bacteriol.* **90**, 1102 and 1109 (1965).

nine, whereas the B component is inactive in this system (9, 19, 20). However, recent observations indicate that the polyuridylic acid-stimulated system is an exception and that both polycytidylic acid-directed synthesis of polyproline and polyadenylic acid-directed synthesis of polylysine are inhibited by either the A or the B components (20, 23). Investigations in the author's laboratory indicate that the inhibition of polycytidylic acid-directed synthesis of polyproline by PA 114 B is about five times less than that observed with PA 114 A (Ennis, unpublished results).

These antibiotics are potent inhibitors only of the incorporation of amino acids into peptide stimulated by exogenously added messenger RNA. The incorporation due to endogenous messenger RNA is much less susceptible to inhibition than the corresponding stimulated system (9, 19, 24). If the antibiotics are added after the messenger RNA is firmly attached and functioning, inhibition is less than if the antibiotics are added before the messenger RNA-aminoacyl-tRNA-ribosome complex has formed and is functioning (9, 19, 23). This finding suggests that the antibiotics inhibit the formation of an active messenger RNA-aminoacyl-tRNA-ribosome complex, probably by competition for a site or sites on the ribosome required for protein synthesis (9, 19). Consistent with this idea is a recent observation (20, 24) which showed that the A group of antibiotics compete with chloramphenicol for a site on the 50 S ribosome, suggesting that these antibiotics act at that site.

The present investigation is concerned with elucidating the mechanism by which the antibiotic PA 114 inhibits protein synthesis in cell-free extracts of bacterial cells.

MATERIALS AND METHODS

Biochemical and radioactive materials, methods for preparation of cell extracts and ribosomes have been described (19). *Escherichia coli* B was used as the source of the protein synthesizing system.

The bacterial cell extracts (S-30) were prepared as described previously (19). The S-30 fraction was preincubated (25) and is referred to as the i-S-30 fraction. The supernatant fraction (S-100) containing supernatant enzymes was prepared from the S-30 fraction by four successive centrifugations at 100,000 *g* for 2 hr in the Spinco Model L-2 centrifuge.

The reaction mixture for the synthesis of polyphenylalanine using the i-S-30 fraction was, per milliliter: Tris-HCl buffer (pH 7.8), 100 μmoles; 2-mercaptoethanol, 6 μmoles; magnesium acetate, 12 μmoles; disodium creatine phosphate, 6 μmoles; creatine kinase, 20 μg; ATP, 1 μmole; GTP, 0.12 μmole; KCl, 100 μmoles; polyuridylic acid, 50 μg; ^{14}C-L-phenylalanine, 0.5 μC, 23 mμmoles; i-S-30 fraction. This was used in the experiment described in Table 1. The method for determining the amount of polyphenylalanine synthesized has been described (19).

The reaction mixture for the synthesis of polyphenylalanine by mixtures of the 50 S and 30 S ribosomal subunits was as indicated for the reaction mixture using an i-S-30 except that, per milliliter, 400 μg *E. coli* B stripped tRNA was added and only 1.33 mμmoles phenylalanine was present. An S-100 was also added to provide the supernatant enzymes. This was used in the experiments described in Tables 5–9.

The antibiotics used were PA 114 (the crude mixture of A and B), and the components PA 114 A and PA 114 B. Vernamycin A and vernamycin B α, and streptogramin were also studied. For brevity no data are presented concerning these antibiotics, although their mode of action is not significantly different from that of PA 114. The antibiotics are sparingly soluble in water and were therefore used as nonsterile homogenized suspensions.

RESULTS

Effect of Delayed Addition of PA 114 A on Extent of Inhibition of in Vitro Protein Synthesis

PA 114 was shown to be a potent inhibitor of *in vitro* protein synthesis stimulated by synthetic polynucleotides (9, 19, 20, 24). However, inhibition was decreased if the antibiotic was added after the messenger

TABLE 1

Effect of delayed addition of PA 114 A on extent of inhibition of polyphenylalanine synthesis

The control reaction without antibiotic was started by the addition of an i-S-30 to a final concentration per milliliter of 1.75 mg protein and 1.08 mg RNA. At intervals through 45 minutes 0.2-ml samples were taken and the ^{14}C-phenylalanine incorporated into the hot trichloroacetic acid insoluble peptide was determined. The reaction went to completion by 45 min. At the times indicated in the table, 0.2-ml samples of the same control reaction were added to tubes containing PA 114 A (5 μg/ml final concentration). These reactions were also continued to 45 min and the radioactivity in the peptide was determined. The inhibition of peptide synthesis was determined as follows. The radioactivity incorporated by the control reaction at each time of addition of the antibiotic was subtracted from that amount incorporated at 45 min. The radioactivity incorporated in the antibiotic inhibited reaction was determined in the same way. The inhibition of protein synthesis was determined by dividing the increment in radioactivity incorporated by the antibiotic inhibited reaction after addition of the antibiotic by the increment incorporated in the control reaction during the same period of time. The reaction mixture for the *in vitro* synthesis of polyphenylalanine is described in the Materials and Methods section. Experiment 1 was carried out at 30° and experiment 2 at 37°.

Time antibiotic added (min)	Inhibition of polyphenylalanine synthesis (%)	
	Experiment 1	Experiment 2
0	91	93
1	87	80
2	79	65
5.5	52	29
10.5	37	0
15.5	33	—

RNA-ribosome-aminoacyl-tRNA complex formed and started functioning (9, 19, 23). This initial observation was extended by the experiments summarized in Table 1. The data show that the later the antibiotic was added during the course of normal protein synthesis, the less was the inhibition observed.

The decrease in inhibition by the antibiotics was not due merely to the attachment of aminoacyl-tRNA and messenger RNA to the ribosomes, before the antibiotic could bind, but required a period of peptide synthesis. Table 2 shows that the inhibition of polyphenylalanine synthesis was the same whether the aminoacyl-tRNA was bound to the ribosomes before or after the addition of the antibiotic as long as protein synthesis was not proceeding.

TABLE 2

Inability of aminoacyl-tRNA to protect against against inhibition by PA 114

To each of three tubes was added 1 ml of a complete reaction mixture for *in vitro* synthesis of polyphenylalanine (see Materials and Methods) and containing, in addition to the usual components, 2 μmoles of nonradioactive phenylalanine per milliliter and ribosomes (544 μg ribosomal RNA/ml). To tube 1, PA 114 A (6 μg/ml) was added; to tube 2 ^{14}C-phenylalanyl-tRNA (10,000 cpm, 500 μg/ml) was added; and to tube 3 the ^{14}C-phenylanyl-tRNA was added. All the tubes were incubated for 5 min at 37°. Maximum binding of ^{14}C-phenylalanyl-tRNA and antibiotic occurs by this procedure (19). Then, to tube 1 ^{14}C-phenylalanyl-tRNA and S-100 (88 μg protein/ml) were added simultaneously to start the reaction; to tube 2 PA 114 A (6 μg/ml) and S-100 were added to start the reaction; and to tube 3 S-100 to start the reaction and water to make the final volume equal in all tubes. Each tube was incubated for an additional 20 min at 37°, and the radioactivity incorporated into hot trichloroacetic acid-insoluble peptide was determined (see Materials and Methods).

Reaction	Incorporation of ^{14}C-phenylalanine into peptide	
	Cpm	Inhibition (%)
1	128	88
2	134	88
3	1080	—

Inhibition of Binding of Aminoacyl-tRNA to Ribosomes

These results and those described in previous studies (9, 19, 23) suggested that the antibiotics inhibited functional binding of messenger RNA or aminoacyl-tRNA to ribosomes. Measurements of the binding of ^{14}C-polyuridylic acid to ribosomes failed to substantiate this hypothesis (19). Furthermore, in this same study, no consistent

effect of the antibiotics on polyuridylic acid stimulated binding of ^{14}C-phenylalanyl-tRNA to ribosomes was observed, although occasionally up to 25% inhibition was seen. This observation was reinvestigated and was found to be in error, presumably because saturating amounts of aminoacyl-tRNA were not used in the assay.

In the present investigation the binding of ^{14}C-phenylalanyl-tRNA to ribosomes was performed with washed ribosomes that were stored at −70° until used, and with more than 2 molecules of phenylalanyl-tRNA per 70 S ribosome. If the ratio of aminoacyl-tRNA to ribosomes was less than 2, consistent inhibition by the antibiotics was not always observed.

TABLE 3
Effect of antibiotics on binding of ^{14}C-phenylalanyl-tRNA to ribosomes

The method for the assay of binding of ^{14}C-phenylalanyl-tRNA to ribosomes was as previously described (19, 33). The reaction mixture (0.5 ml) contained 270 μg ribosomal RNA and 960 μg aminoacyl-tRNA (10,600 cpm in ^{14}C-phenylalanine/mg tRNA).

Addition to reaction	Cpm/0.5 ml bound	Inhibition (%)
Control (no antibiotic)	281	—
PA 114 A		
0.5 μg/ml	179	36
1.0 μg/ml	150	47
10.0 μg/ml	118	58
PA 114 (mixture of A and B)		
1.0 μg/ml	195	31
10.0 μg/ml	162	42
No polyuridylic acid	42	—
Control heated for 10 min at 95° in 5% trichloroacetic acid	29	—

The results presented in Table 3 show that the antibiotics inhibited binding of ^{14}C-phenylalanyl-tRNA up to 50%. Moreover, the maximum inhibition of phenylalanyl-tRNA binding was observed at concentrations of the antibiotics at which the maximum inhibition of polyphenylalanine synthesis occurred (19). As has been observed with the tetracyclines (26, 27), 50% inhibition of aminoacyl-tRNA binding is apparently sufficient to give almost complete inhibition of peptide synthesis.

There are 2 sites on the 70 S ribosome to which aminoacyl-tRNA can bind, one on the 30 S subunit and another on the 50 S subunit (28–30). If the site of inhibition of tetracycline and the PA 114 group were different, one might expect that the inhibition of binding of aminoacyl-tRNA using mixtures of the two antibiotics would be additive. On the other hand, if the site of inhibition of the two antibiotics were the same, no such additive effect should be observed. Table 4 presents the results of one such experiment. Tetracycline itself consistently inhibited binding to a greater extent than PA 114 A. In this particular experiment the inhibition was 61% for tetracycline and 48% for PA 114 A. Mixtures of the two antibiotics also gave a consistently greater degree of inhibition of binding of aminoacyl-tRNA than either antibiotic alone, but this value never approached 100%.

TABLE 4
Effect of antibiotics on binding of ^{14}C-phenylalanyl-tRNA to ribosomes

The method for the assay of binding of ^{14}C-phenylalanyl-tRNA to ribosomes was as previously described (19, 33). The reaction mixture (0.5 ml) contained 270 μg ribosomal RNA and 820 μg aminoacyl-tRNA (10,600 cpm in ^{14}C-phenylalanine/mg tRNA).

Addition to reaction	Cpm/0.5 ml bound	Inhibition (%)
Control (no antibiotic)	515	—
PA 114 A		
1.0 μg/ml	282	45
10.0 μg/ml	305	41
100.0 μg/ml	271	47
PA 114 B		
100.0 μg/ml	378	27
Tetracycline		
100.0 μg/ml	201	61
Tetracycline 100.0 μg/ml plus PA 114 A 10.0 μg/ml	167	68
No polyuridylic acid	35	—

Table 4 also shows that PA 114 B, which alone cannot inhibit polyuridylic acid-

stimulated synthesis of polyphenylalanine (9, 19, 20), slightly inhibited binding, but only at very high concentrations.

Ribosomal Site of Inhibition by PA 114

Since radioactive antibiotic was unavailable, experiments designed to locate the site of binding to the ribosomes were not possible in this way. However, in this regard,

TABLE 5

Effect of antibiotics on functioning of 50 S and 30 S ribosomes

In this experiment the 50 S and 30 S ribosomes were collected directly from sucrose density gradients made up in Tris-HCl, 10^{-2} M, pH 7.8, 5×10^{-4} M Mg^{++} and 5×10^{-2} M KCl as previously described (34). Each of the ribosome subunits was then made 10^{-2} M with respect to Mg^{++}. The antibiotics (each at 10 μg/ml final concentration) or an equal volume of distilled water in the case of the control, were added directly to aliquots of each of the ribosomal subunits and incubated at 37° for 5 min. Maximum inhibition of function of the 50 S ribosome by the antibiotics occurred using this procedure. The suspensions of ribosomes were then dialyzed for 16 hr against 100 times the volume of standard buffer (35) at 4°. Other experiments have shown that all unbound antibiotic was removed from the ribosome suspension by this treatment. The ribosome subunits were then reconstituted in various combinations and tested for their ability to synthesize peptide by adding them to a complete reaction mixture. No antibiotic was present during the incubation of these reactions. The radioactivity incorporated into polyphenylalanine was corrected for small amounts of peptide synthesized in the absence of ribosomes and by each of the 50 S and 30 S components incubated by itself in the complete reaction mixture. The reaction mixture (0.5 ml) contained 33 μg RNA in 50 S ribosomes, 23 μg RNA in 30 S ribosomes, and 80 μg protein in the S-100.

Constitution of ribosome hybrids		Incorporation of ^{14}C-phenylalanine into peptide	
50 S	30 S	Cpm	Inhibition (%)
Control (no antibiotic)	Control	1972	—
Control	PA 114	1482	25
Control	PA 114 A	1407	29
PA 114	Control	87	96
PA 114	PA 114	38	98
PA 114 A	Control	630	68
PA 114 A	PA 114 A	482	76

TABLE 6

Effect of PA 114 A on functioning of 50 S and 30 S ribosomes subunits isolated from antibiotic-treated 70 S ribosomes

Two reactions were set up containing all the components necessary for a complete amino acid synthesizing system (see Materials and Methods) in a final volume of 6 ml. To one reaction tube PA 114 A (2 μg/ml) was added, and to the other an equal volume of water. An i-S-30 fraction containing 2.2 mg RNA and 3.5 mg protein per milliliter reaction was added to each reaction and the tubes were incubated at 37° for 30 min. The reaction mixtures were then chilled rapidly and centrifuged at 100,000 *g* for 2 hr to sediment the 70 S ribosomes. Each ribosome pellet was rinsed and suspended in buffer containing Tris-HCl 10^{-2} M, pH 7.8, 5×10^{-4} M Mg^{++} and 5×10^{-2} M KCl and dialyzed overnight against 100 times the volume of the same buffer at 4°. This treatment dissociates the 70 S ribosomes into the 50 S and 30 S components. An aliquot of each ribosome suspension was then run on sucrose density gradients (5–20%) containing Tris-HCl, 10^{-2} M, pH 7.8, 5×10^{-4} M Mg^{++} and 5×10^{-2} M KCl, and the 50 S and 30 S ribosomes were isolated. The ribosomes were then made 10^{-2} M with respect to Mg^{++}. The 50 S and 30 S ribosomes from the antibiotic inhibited and control reactions were reconstituted in all possible combinations, and the ability of these hybrids to synthesize polyphenylalanine in a complete protein synthesizing system stimulated by polyuridylic acid (see Materials and Methods) was determined. The reaction (0.5 ml) contained 69 μg RNA in 50 S, 26 μg RNA in 30 S, and 23 μg protein in the S-100. No antibiotic was present during the incubation of these reactions. The radioactivity incorporated was corrected for small amounts of trichloroacetic acid-insoluble peptide formed in the absence of ribosomes and formed by each of the 50 S and 30 S components incubated by itself in the complete reaction mixture.

Constitution of ribosome hybrids		Incorporation of ^{14}C-phenylalanine into peptide	
50 S	30 S	Cpm	Inhibition (%)
Control (no antibiotic)	Control	610	—
Control	PA 114 A	601	2
PA 114 A	Control	261	57
PA 114 A	PA 114 A	229	62

an earlier investigation had shown that when the antibiotic was added to functioning ribosomes, these ribosomes, when reisolated and dialyzed free of unbound antibiotic, were incapable of supporting further protein synthesis (19). The results indicated that the antibiotic either interacted with a specific site on the ribosome which was also important in protein synthesis, or that the antibiotic destroyed the functional integrity of the entire ribosome. Therefore, the inability of the antibiotic-treated ribosomes (or ribosome subunits) to support protein synthesis in a complete *in vitro* protein synthesizing system could be used to locate the site of action of the antibiotics.

In one such experiment the 70 S ribosome was dissociated into its 50 S and 30 S subunits, and the subunits were isolated and treated with the antibiotics (see legend to Table 5 for the experimental details). The effect of the antibiotics on the functioning of the ribosome subunits was then studied by reconstituting antibiotic-treated and untreated ribosome subunits in all combinations. Table 5 shows that the antibiotics markedly inactivated the 50 S subunit and were less effective in inhibiting the activity of the 30 S subunit. Furthermore, crude PA 114, which is a mixture of the A and B components, was more effective in binding to the 50 S ribosome and rendering it incapable of carrying out protein synthesis than the A component by itself.

The inhibition of the activity of the 50 S ribosome was also obtained if the 70 S ribosome was first treated with PA 114 A system, and then dissociated into its 50 S in a complete *in vitro* protein synthesizing and 30 S components (Table 6).

PA 114 B was ineffectual alone in inhibiting the activity of the ribosomal subunits. This is in agreement with the finding that the B component did not inhibit polyphenylalanine synthesis in a complete *in vitro* protein synthesizing system (9, 19, 20).

Synergistic Action of Mixtures of the A and B Antibiotic Components

The results presented in Table 5 showed that the crude mixtures of the A and B components of the antibiotic complexes were far superior in rendering the 50 S ribosome inactive, than the A component alone. This occurred even though the B component was itself ineffectual in stopping protein synthesis. Previous *in vivo* and *in vitro* experiments showed that the B component of the antibiotic complex potentiated the inhibitory effect of the A component on protein synthesis (17, 19). The results given in Table 7 show that, indeed, mixtures of the A and B components of

TABLE 7

Synergistic action of mixtures of PA 114 A and PA 114 B on the functioning of 50 S and 30 S ribosomes

The procedure was the same as that outlined in the legend to Table 5. The concentrations of antibiotics used was as follows: 5 μg/ml PA 114 A plus 5 μg/ml PA 114 B; 10 μg/ml PA 114 A (when used alone). The reaction mixture (0.5 ml) contained 22 μg RNA in 50 S ribosomes, 11 μg RNA in 30 S ribosomes, and 23 μg protein in the S-100.

Constitution of ribosome hybrids		Incorporation of ^{14}C-phenylalanine into peptide	
50 S	30 S	Cpm	Inhibition (%)
Control (no antibiotic)	Control	936	—
Control	PA 114 A	775	17
Control	PA 114 A + PA 114 B	830	11
PA 114 A	Control	367	61
PA 114 A	PA 114 A	290	69
PA 114 A + PA 114 B	Control	16	98
PA 114 A + PA 114 B	PA 114 A + PA 114 B	3	100

TABLE 8

Effect of mixtures of PA 114 A and PA 114 B on functioning of 50 S ribosomes: Addition of PA 114 A before PA 114 B

Purified 50 S ribosomes suspended in standard buffer (35) were divided into two portions. To one (portion 2) 5 μg/ml PA 114 A was added and to the other (portion 1) an equal volume of distilled water. The ribosomes were incubated at 37° for 5 min, and then dialyzed for 7 hr at 4° against 200 times the volume of standard buffer. The ribosome suspension 2 was divided into two equal portions. To one portion designated (D) 5 μg/ml PA 114 B was added, and to the other portion (C) an equal volume of water. The ribosome suspension 1 was treated similarly, to (B) 5 μg/ml PA 114 A was added and to (A) an equal volume of water. The ribosome suspensions were incubated again for 5 minutes at 37° and then dialyzed as above for 18 hr. The 50 S ribosomes were then added to purified untreated 30 S ribosomes and the reconstituted ribosomes were tested for their ability to synthesize peptide as described in Materials and Methods. The reaction mixture (0.5 ml) contained 37 μg RNA in 50 S ribosomes, 23 μg RNA in 30 S ribosomes, and 44 μg protein in the S-100.

	Conditions of incubation of 50 S ribosomes		Incorporation of ^{14}C-phenylalanine into peptide	
Sample	First incubation	Second incubation	Cpm	Inhibition (%)
A	No antibiotic	No antibiotic	1235	—
B	No antibiotic	PA 114 A	518	58
C	PA 114 A	No antibiotic	558	55
D	PA 114 A	PA 114 B	92	93

the PA 114 complex were more effective in destroying the activity of the 50 S ribosome than the A component by itself.

Experiments were designed to provide more information concerning the mechanism by which synergism between the antibiotic components is achieved. The first experiment, given in Table 8, shows that the B antibiotic component was capable of potentiating the action of the A component already bound to the 50 S ribosome. In this study, 50 S ribosomes were incubated first with the A component, dialyzed free of antibiotic unbound to the ribosomes,

TABLE 9

Effect of mixtures of PA 114 A and PA 114 B on functioning of 50 S ribosomes: Addition of PA 114 B before PA 114 A

Purified 50 S ribosomes suspended in standard buffer (35) were divided into two portions. To one (portion 2) 5 μg/ml PA 114 B was added and to the other (portion 1) an equal volume of distilled water. The ribosomes were incubated at 37° for 5 min and then dialyzed for 7 hr at 4° against 200 times the volume of standard buffer. The ribosome suspension 2 was divided into 2 equal portions. To one portion designated (D) 5 μg/ml PA 114 A was added and to the other portion (C) an equal volume of water. The ribosome suspension 1 was treated similarly; to (B) 5 μg/ml PA 114 A was added and to (A) an equal volume of water. The ribosome suspensions were incubated again for 5 min at 37° and then dialyzed as above for 18 hr. The 50 S ribosomes were then added to purified untreated 30 S ribosomes, and the reconstituted ribosomes were tested for their ability to synthesize peptide as described in Materials and Methods. The reaction mixture (0.5 ml) contained 37 μg RNA in 50 S ribosomes, 23 μg RNA in 30 S ribosomes, and 44 μg protein in the S-100.

	Conditions of incubation of 50 S ribosomes		Incorporation of ^{14}C-phenylalanine into peptide	
Sample	First incubation	Second incubation	Cpm	Inhibition (%)
A	No antibiotic	No antibiotic	1146	—
B	No antibiotic	PA 114 A	393	66
C	PA 114 B	No antibiotic	946	17
D	PA 114 B	PA 114 A	330	71

and then tested for their ability to carry out peptide synthesis. A portion of this same batch of treated and dialyzed ribosomes was then incubated with the B component, dialyzed, and again tested for its ability to carry out peptide synthesis. Table 8 shows that the ribosomes treated only with PA 114 A were inhibited 55%, while those treated sequentially with PA 114 A and then with PA 114 B were inhibited by 93%.

However, 50 S ribosomes treated as above but first with PA 114 B and then with PA 114 A gave only that degree of inhibition expected for PA 114 A-treated ribosomes alone (Table 9).

DISCUSSION

Previous investigations have shown that the antibiotics of the PA 114 (and also vernamycin and streptogramin) complexes specifically inhibit protein synthesis in intact bacterial cells and in cell-free extracts prepared from these cells (9, 16, 17, 19, 20, 23, 24, 31). The antibiotics inhibit protein synthesis optimally only when messenger RNA is not attached to the ribosome and functioning. The present investigation has further shown that the longer the cell-free protein synthesizing system is allowed to proceed before PA 114 A is added, the less is the final inhibition of peptide synthesis. This suggests that the length of the nascent peptide chain may play a role in preventing inhibition by sterically interferring with the attachment of the antibiotic to the inhibiting site on the ribosome.

The present investigation is concerned with elucidating the specific mechanism by which the antibiotics inhibit the formation of an active messenger RNA-aminoacyl-tRNA complex. Direct measurements of ^{14}C-polyuridylic acid binding to ribosomes showed that the physical attachment of this polynucleotide is unaffected by concentrations of antibiotic 10-fold greater than that required to maximally inhibit *in vitro* protein synthesis (19). This same investigation also showed that the polyuridylic acid-directed binding of phenylalanyl-tRNA to ribosomes was not consistently inhibited by this same concentration of antibiotic. However, the present study has shown that this finding was in error, presumably because a saturating amount of aminoacyl-tRNA was not used in the assay. Studies using at least 2 molecules of phenylananyl-tRNA per 70 S ribosome showed that the antibiotics do, in fact, inhibit polyuridylic acid directed binding of phenylalanyl-tRNA to ribosomes. Moreover, the inhibition of binding closely correlated with the inhibition of *in vitro* polyphenylalanine synthesis. For example, maximum inhibition of aminoacyl-tRNA binding (about 50%) was achieved at approximately 1 μg PA 114 A per milliliter, which is about the same concentration required for maximal (about 95%) inhibition of the *in vitro* synthesis of polyphenylalanine (19). The work on the mode of action of the tetracyclines has also indicated that 50% inhibition of aminoacyl-tRNA binding is sufficient to maximally inhibit *in vitro* protein synthesis (26, 27).

There are two sites on the 70 S ribosome to which aminoacyl-tRNA can bind, one on the 50 S subunit, and another on the 30 S subunit (28–30). It is reasonable to assume that if the antibiotics maximally inhibit binding of aminoacyl-tRNA by only 50%, then only one of these two is affected. Our experiments have shown that, indeed, PA 114 A and mixtures of A and B, but not PA 114 B alone, interact with only the 50 S ribosome subunit. The treated 50 S ribosome subunits are inactive in supporting further protein synthesis in a complete *in vitro* protein synthesizing system. Some recent experiments are pertinent in this connection. Chloramphenicol binds to the 50 S ribosome subunit (20, 24). PA 114 A interferes with the binding of chloramphenicol, while PA 114 B does not. It was shown that PA 114 A interacts with the 50 S ribosome and makes it incapable of binding chloramphenicol. PA 114 A acts on the ribosome at a site closely related to, but not the same as, that of the attachment of chloramphenicol. This is quite reasonable because chloramphenicol does not interfere with aminoacyl-tRNA binding (32) while PA 114 A does. These data, together with the experiments presented in the

present study, give compelling evidence that the antibiotics affect a site involved in protein synthesis located on the 50 S ribosome subunit.

These data by themselves do not prove that the site on the 50 S ribosome that is destroyed by the antibiotics is the same as that involved in the functional binding of aminoacyl-tRNA. However, the circumstantial evidence argues in favor of this idea.

PA 114 B is alone unable to inhibit the functioning of the 50 S or 30 S ribosome subunits. This is in agreement with the finding that the B component does not inhibit polyphenylalanine synthesis in an *in vitro* system (9, 19, 20). However, mixtures of the A and B components are far superior in destroying the function of the 50 S ribosome than the A component alone (Table 7). This ability of the B component to enhance the activity of the A component is consistent with previous work in intact bacteria and cell-free extracts (17, 19) which showed that certain combinations of PA 114 A and B are much more effective in inhibiting protein synthesis than each component by itself.

The conclusions which can be derived from the present investigation are circumstantial, but reasonable. The antibiotics inhibit the binding of aminoacyl-tRNA to the ribosomes, and destroy or modify the activity of a site necessary for protein synthesis on the 50 S ribosome. Although direct evidence is lacking we can reasonably suggest that the antibiotic competes with the site involved in aminoacyl-tRNA function on the 50 S ribosome. If one accepts this conclusion then the antibiotic interferes with protein synthesis by displacing one molecule of aminoacyl-tRNA from the 50 S ribosome.

Although this proposed mode of action may be correct, other interpretations are not excluded by the data. It could be argued that the site on the 50 S ribosome which the antibiotic inactivates is, in fact, not the site at which aminoacyl-tRNA binds. The antibiotic, however, by an allosteric effect could change the structure of the ribosome, making the ribosome unable to bind aminoacyl-tRNA. The end result is the same in both instances. Protein synthesis is inhibited by changes in the ribosome which alter the affinity for the binding of aminoacyl-tRNA, and the effect is directly on aminoacyl-tRNA binding.

Another possibility is that the inhibition of binding of aminoacyl-tRNA may be only an incidental reflection of other changes in the ribosome structure and the mode of action of the antibiotics may have nothing at all to do with aminoacyl-tRNA binding.

ACKNOWLEDGMENTS

I wish to thank W. D. Celmer, Chas. Pfizer and Co., Inc., for the PA 114, PA 114 A, and PA 114 B; R. Donovick, Squibb Institute for Medical Research, for the vernamycin A and B α; F. J. Wolf, Merck, Sharp and Dohme, for the streptogramin; and Mrs. R. Tirey for her assistance in some of the experiments.

This investigation was supported by Public Health Service Grant GM-12359-02 from the Division of General Medical Sciences, by Grant GB-3521 from the National Science Foundation, and by the American Lebanese Syrian Associated Charities (ALSAC).

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