STUDIES ON THE FORMATION OF TRANSFER RIBONUCLEIC ACID-RIBOSOME COMPLEXES, XI. ANTIBIOTIC EFFECTS ON PHENYLALANYL-OLIGONUCLEOTIDE BINDING TO RIBOSOMES

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Communicated by Robert W. Berliner, August 15, 1969

Abstract.—The effect of antibiotics on the binding of phenylalanyl-oligonucleotide to ribosomes has been examined. The results show that many classes of antibiotics can interfere with binding of the aminoacyl-oligonucleotide terminus of tRNA to ribosomes: chloramphenicol, sparsomycin, D-WIN-5094, vernamycin A, PA114A, streptogramin, amicetin, gougerotin, tylosin, and spiramycin III. The results are consistent with the hypothesis that these antibiotics inhibit protein synthesis by interfering with the binding of the aminoacyl-end of aminoacyl-tRNA to ribosomes.

The binding of phenylalanyl-oligonucleotides (Phe-oligonucleotide) to ribosomes has recently been reported from this laboratory[1, 2] (Fig. 1). The binding of this terminal T_1 ribonuclease fragment of aminoacyl-tRNA to ribosomes enables one to distinguish between two events involving peptide bond formation (Fig. 2): the binding of the peptidyl- or aminoacyl-tRNA terminus (pCpCpA end) to ribosomes (Steps 1 and 2, respectively) and the subsequent transfer of the nascent peptide to the adjacent aminoacyl-tRNA to form a peptide bond (peptidyl transfer reaction, Step 3).

Studies of the peptidyl transfer reaction[3-6] have generally involved assay of the end product of the reaction rather than the individual steps. The reaction of peptidyl-tRNA,[3-5] fMet-tRNA,[7, 8] or *N*-acetyl-Phe-tRNA[9, 10] with puromycin has been used as an analog of peptide bond formation and is the result of both events. Thus, requirements of formation of the puromycin product characterize the overall reaction rather than the peptidyl transfer reaction itself (Step 3). When the binding of Phe-oligonucleotide to ribosomes was examined, its characteristics and requirements resembled those usually ascribed to the peptidyl-transferase step.[1, 2]

Many antibiotics have been shown to influence the overall reaction.[3-5, 10-17] In fact, we recently showed that chloramphenicol was a competitive inhibitor of puromycin in the overall reaction.[18] In order to localize further their site of action it was, therefore, necessary to determine if chloramphenicol and other antibiotics could influence the binding of Phe-oligonucleotide to ribosomes. In this report, we have, therefore, examined the effect of antibiotics on the binding of Phe-oligonucleotide to ribosomes.

Experimental Procedure.—The binding of Phe-oligonucleotide to ribosomes was determined in 0.050-ml reaction mixtures which contained the following components: 0.05 M Tris-acetate, pH 7.2; 0.05 M potassium acetate; 0.1 M NH_4Cl; 0.04 M magnesium acetate; 3.8 A_{260} units of ribosomes washed four times in 1 M NH_4Cl;[6] 1.3 pmoles of [^{3}H] Phe-oligonucleotide (0.04 A_{260} unit); antibiotic concentration is indicated in each Legend.

Reactions were incubated at 24° for 10 min. At 10 min, reactions were diluted with 3 ml of cold buffer containing the identical concentration of Tris-acetate, potassium acetate, ammonium chloride, and magnesium acetate as the reaction mixture and immediately filtered through a Millipore filter to adsorb the ribosomes;[19] the tube and filter were then washed an additional three times with 3-ml portions of the same buffer. After the filters had been dried, they were counted directly in a scintillation fluor.[20] Specific activity of [^{3}H]phenylalanine was 6300 (New England Nuclear). The [^{3}H]Phe-oligonucleotide was prepared from a T_1 ribonuclease digest of unfractionated [^{3}H]Phe-tRNA as previously reported by Herbert and Smith.[21] The [^{3}H]Phe-tRNA was prepared as previously described from this laboratory.[22] Antibiotics were obtained from the sources previously reported.[18]

PHENYLALANYL - OLIGONUCLEOTIDE BINDING TO RIBOSOMES

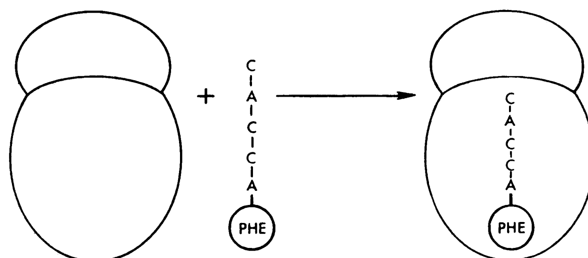

FIG. 1.—Schematic illustration of the binding of phenylalanyl-oligonucleotide (CACCA-Phe) to ribosomes.

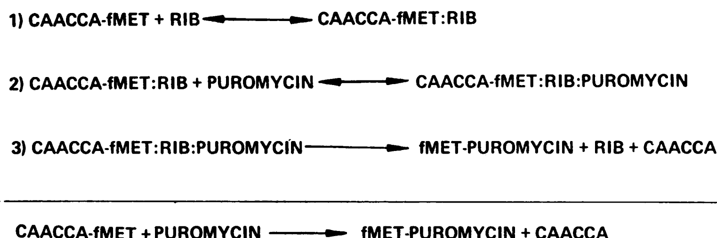

FIG. 2.—The reactions involved in the formation of formylmethionyl-puromycin from the formylmethionyl-hexanucleotide fragment (CAACCA-fMet) produced by ribonucleose T_1 action on formylmethionyl-tRNA.[7] Step 1 represents the binding of the CCA-terminus of peptidyl-tRNA to ribosomes (RIB), namely, the binding of CAACCA-fMet to ribosomes. Step 2 represents the binding of puromycin to the ribosome, which is equivalent to the binding of the CCA-terminus of aminoacyl-tRNA and Phe-oligonucleotide to ribosomes. Step 3 represents the peptidyl-transfer reaction, with the resultant formation of a peptide bond.

Results and Discussion.—The results (Table 1) indicate that several different groups of antibiotics significantly affect the binding of Phe-oligonucleotide to ribosomes. Chloramphenicol, sparsomycin, and D-WIN-5094 inhibit the binding of the Phe-oligonucleotide to ribosomes at 10^{-5} M. The streptogramin A group of antibiotics (streptogramin, vernamycin A, and PA114A) markedly inhibit the binding at 10^{-5} M. PA114B significantly stimulates the binding of Phe-oligo-

TABLE 1. *Effect of antibiotics on Phe-oligonucleotide binding.*

Antibiotic	Per Cent of Value Without Antibiotic		
	10^{-5} M	10^{-4} M	10^{-3} M
No Antibiotic	100	100	100
Chloramphenicol	42	19	...
Sparsomycin	34	15	...
D-WIN-5094	80	52	...
Vernamycin A	10	9	...
PA114 A	9	9	...
Streptogramin	10	10	...
PA114 B	...	133	194
Amicetin	...	39	32
Gougerotin	...	28	21
Pactamycin	109	110	...
Edeine	74	64	...
Streptomycin	70	72	...
Neomycin	86	73	...
Spectinomycin	...	102	96
Tetracycline	96	82	...
Tylosin	51	56	...
Erythromycin	113	120	...
Spiramycin III	...	36	39
Fusidic acid	...	112	95
Bottromycin	...	106	124
Aurintricarboxylic acid	...	107	107

Reactions were performed as indicated under *Experimental Procedure.* Antibiotic concentrations are indicated in the Table. One pmole was equivalent to 4000 cpm. The binding of [^{3}H]Phe-oligonucleotide to ribosomes is expressed as a percentage of the binding to ribosomes in the absence of any antibiotic. In the absence of antibiotics, 0.33 pmole of [^{3}H]Phe-oligonucleotide was bound to ribosomes. Streptogramin was a mixture of types A, B, and G.

nucleotide to ribosomes; this resembles its stimulation of aminoacyl-tRNA binding to ribosomes.[23] The pyrimidine antibiotics, amicetin and gougerotin, produce substantial inhibition. Two macrolide antibiotics, tylosin and spiramycin III, produced inhibition although erythromycin did not. Although edeine produced inhibition at 10^{-5} M, this inhibition of Phe-oligonucleotide binding to ribosomes was much smaller than its inhibition of Phe-tRNA binding to ribosomes at comparable concentrations.[24] Tetracycline had an inhibitory effect at 10^{-4} M. Fusidic acid, bottromycin, aurintricarboxylic acid, pactamycin, and spectinomycin produced no evident inhibition of Phe-oligonucleotide binding to ribosomes.

The effects of chloramphenicol and sparsomycin concentration on Phe-oligonucleotide binding to ribosomes are shown in Figures 3 and 4, respectively. Chloramphenicol is markedly inhibitory to this binding at concentrations which inhibit protein synthesis *in vivo*.[25, 26] Puromycin also inhibits Phe-oligonucleotide binding to ribosomes, but does not react with it to form phenylalanyl-puromycin (Fig. 5).

The present results indicate that many antibiotics can interfere with the binding of the Phe-oligonucleotide to ribosomes and, therefore, probably interfere with functional attachment of the aminoacyl-end of tRNA to ribosomes. This may be the primary mode of action of chloramphenicol, sparsomycin, D-WIN-5094, vernamycin A, PA114A, streptogramin, amicetin, gougerotin, and perhaps of tylosin and spiramycin III. It may be a secondary effect for edeine, strepto-

mycin, neomycin, and tetracycline produced by induction of conformational changes in the ribosome through their interaction with the 30*S* subunit.

Sparsomycin stimulates the binding of CACCA-Leu-Ac but not the unblocked CACCA-Leu to ribosomes.[27] The sparsomycin stimulated complex CACCA-

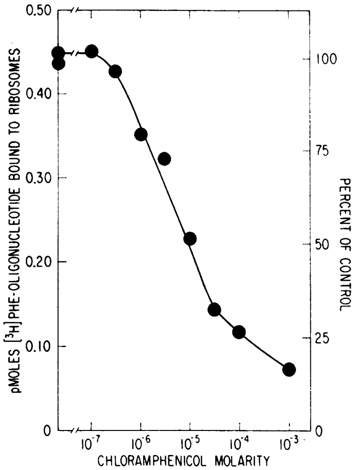

FIG. 3.—The binding of Phe-oligonucleotide to ribosomes as a function of chloramphenicol concentration. Each 0.050-ml reaction contained the components indicated under *Experimental Procedure* with the following changes: 0.16 M NH_4Cl; 0.02 M magnesium acetate; 4.3 pmoles of [^{3}H]Phe-oligonucleotide (0.05 A_{260} unit; specific activity, 1840 mc/mmole). Chloramphenicol concentration is given on the abscissa.

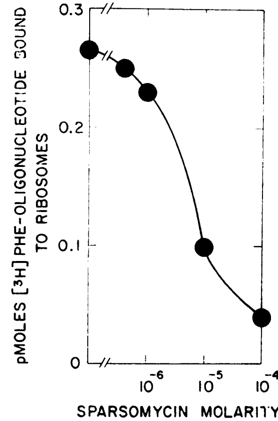

FIG. 4.—Binding of Phe-oligonucleotide to ribosomes as a function of sparsomycin concentration. Each 0.050-ml reaction mixture contained the following components: 0.050 M Tris-acetate, pH 7.2; 0.45 M KCl; 0.1 M NH_4Cl; 0.04 M magnesium acetate; 3.8 A_{260} units of ribosomes; 1.3 pmoles of [^{3}H]-Phe-oligonucleotide (0.036 A_{260} unit; 6300 mc/mmole); sparsomyicn concentration as indicated on the abscissa. Reactions were performed as indicated under *Experimental Procedure.*

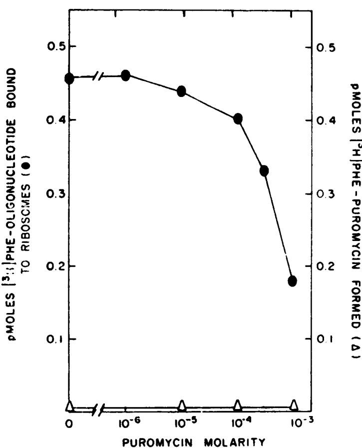

FIG. 5.—Binding of Phe-oligonucleotide to ribosomes and Phe-puromycin formation as a function of puromycin concentration. Reactions contained the components indicated in the legend to Fig. 4; puromycin concentration is indicated on the abscissa.

—●—, Phe-oligonucleotide binding to ribosomes, assayed as described in *Experimental Procedure.*

—△—, Phenylalanyl - puromycin formation was determined by extracting Phe-puromycin formed into ethyl acetate after making reaction mixtures alkaline with ammonium hydroxide.[10]

Leu-Ac with ribosomes does not readily react with puromycin. It is possible that in the presence of sparsomycin the ribosome is fixed in a conformation which is unable to bind puromycin, Phe-oligonucleotide, or the aminoacyl-end of aminoacyl tRNA; but binds peptidyl-tRNA tightly. Chloramphenicol, spiramycin III, streptogramin A, amicetin, and gougerotin inhibit the binding of CACCA-Leu-Ac stimulated by sparsomycin.[27] They may accomplish this by inhibiting the binding of sparsomycin to the ribosome or by interfering with the binding of CACCA-Leu-Ac itself. By studying the binding of N-acylated and unblocked aminoacyl-oligonucleotides to ribosomes it should be possible to clarify further the action of these antibiotics and the nature of the ribosomal sites. Also, it should be considered that the Phe-oligonucleotide may be binding to ribosomal sites other than those participating in peptide bond formation.

The hypothesis that chloramphenicol inhibits protein synthesis by interfering with the binding of the aminoacyl-end of aminoacyl-tRNA to ribosomes is consistent with these results. It is possible that the binding of peptidyl-tRNA also may be inhibited by chloramphenicol. Coutsogeorgopoulos[11] suggested that chloramphenicol may compete with the aminoacyl-end of tRNA for binding to ribosomes. On the other hand, Das, Goldstein, and Kanner[28] suggested that chloramphenicol may be an analog of the C-terminal amino acid of peptidyl-tRNA.

The present findings are consistent with and help to explain the varied effects of chloramphenicol on bacterial protein synthesis. The paradoxical observation that the inhibition of polypeptide synthesis is dependent on the template[29, 30] is probably a function of the trichloroacetic acid precipitability of the various polypeptides synthesized rather than of any real influence of the template on chloramphenicol action. For although polylysine synthesis appears to be inhibited more than polyphenylalanine synthesis, analysis of the oligolysine products shows an increase of shorter chain-length material so that net peptide bond synthesis is only slightly inhibited.[31] In the case of phenylalanine, where the short peptides are precipitable with trichloroacetic acid,[32] there is thus a small difference in the presence and absence of chloramphenicol. Nevertheless, diphenylalanine synthesis is often stimulated while the production of longer chains is inhibited,[13] which is a finding consistent with the polylysine results.

Also, it should be noted that sparsomycin and chloramphenicol, antibiotics which inhibit termination,[33] also inhibit Phe-oligonucleotide binding to ribosomes. It is, therefore, possible that these antibiotics inhibit termination by interfering with proper binding of the peptidyl- or aminoacyl-tRNA terminus to the ribosome rather than by inhibiting the synthesis of a labile peptide intermediate. Furthermore, it should be emphasized that the binding of aminoacyl-tRNA to ribosomes is probably a multistep process during which several portions of each tRNA molecule associate with specific areas of ribosomes in a defined temporal and spacial sequence with which many antibiotics may interfere.

I thank Miss Elena Smith for her capable assistance in the preparation of this manuscript.

Phe-oligonucleotide, phenylalanyl-oligonucleotide (also CACCA-Phe); CAACCA-fMet, CACCA-Leu, and CACCA-Leu-Ac, the formylmethionyl-, leucyl-, and *N*-acetyl-leucyl-oligo-

nucleotides, respectively, prepared from a T_1 ribonuclease digest of their respective tRNA's; Phe-puromycin, phenylalanylpuromycin.

Note added in proof: Lincomycin was also found to inhibit Phe-oligonucleotide binding to ribosomes.

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