Amino Acylaminonucleoside Inhibitors of Protein Synthesis. The Effect of Amino Acyl Ribonucleic Acid on the Inhibition*

C. Coutsogeorgopoulos

ABSTRACT: The effect of increasing concentrations of L-phenylalanyl-RNA on the inhibition of the polyuridylic acid (poly U) directed polyphenylalanine synthesis by seven selective inhibitors of protein synthesis has been examined. The inhibitors studied were puromycin, chloramphenicol, gougerotin, blasticidin S, amicetin, streptomycin, and tetracycline. The inhibition caused by these inhibitors was not reversed by increased amounts of L-phenylalanyl-RNA. A graphical analysis showed that, with respect to L-phenylalanyl-RNA, the inhibition caused by puromycin is not competitive. It is suggested that puromycin does not enter the amino acyl-RNA–ribosome–mRNA complex by way of the "amino acid" site (acceptor site) but by way of another site (site S) at which the amino acyl-adenylyl terminus of the bound amino acyl-RNA and the peptidyl-adenylyl terminus of peptidyl-RNA interact to form a peptide bond. Site S might be solely a ribosomal site, a site on the enzyme system that forms the peptide bonds, or a combination of a ribosome–enzyme site. At a constant level of L-phenylalanyl-RNA the inhibition by puromycin, gougerotin, or blasticidin S was greater at 25° than at 37°, whereas the streptomycin inhibition was greater at 37° than at 25°. The inhibition by amicetin, blasticidin S, or gougerotin was much higher at the initial stages of the reaction, than when longer incubation periods were used.

Puromycin, gougerotin, blasticidin S, and amicetin (Figure 1) belong to the class of amino acylaminonucleoside antibiotics (for details on their structure, see review by Fox *et al.*, 1966). They have been found to interfere specifically with protein synthesis in cellular and in cell-free systems (Nathans, 1964; Clark and Chang, 1965; Casjens and Morris, 1965; Yamaguchi *et al.*, 1965; Bloch and Coutsogeorgopoulos, 1966).

The structural resemblance of puromycin to the amino acyl-adenylyl terminus of amino acyl-RNA (Figure 1) has led to the postulate that puromycin inhibits protein synthesis by causing the premature release of polypeptide chains from the ribosome (for a review, see Newton, 1965; Schweet and Heintz, 1966). Gougerotin and blasticidin S, which have a pyrimidine as the aglycone, also interfere with the transfer reaction (Casjens and Morris, 1965; Clark and Chang, 1965; Yamaguchi *et al.*, 1965) and this observation has suggested that, although tRNAs have adenine as the nucleoside base of their amino acyl-adenylyl terminus, amino acylaminonucleosides which have a pyrimidine as the "base" constituent can interfere with the function of amino acyl-RNA or peptidyl-RNA. An analogous postulate based upon the conformational similarity of chloramphenicol (Figure 1) to the pyrimidine amino acylaminonucleoside antibiotics and the peptidyl-adenylyl terminus of peptidyl-RNA has been advanced as an explanation for the inhibition of protein synthesis by chloramphenicol (Coutsogeorgopoulos, 1966).

Based on the structural similarity between the above-mentioned amino acylaminonucleoside antibiotics and amino acyl-RNA, it has been considered possible that these inhibitors bind to the same sites as amino acyl-RNA or peptidyl-RNA and thus interfere with their function. For example, it has been assumed that puromycin is a competitive substrate for the enzyme that catalyzes peptide-bond formation from amino acyl-RNA precursors (Williamson and Schweet, 1965). Furthermore, it has been suggested (Wettstein and Noll, 1965) that puromycin "competes with the incoming amino acyl-RNA" not by blocking attachment of the tRNA portion to its complementary ribosomal site, but by occupying the enzyme site involved in peptide-bond formation. On the other hand, it has been also postulated that puromycin enters the ribosomal structure by way of the ribosomal site that holds the incoming amino acyl-RNA during protein synthesis (Bretscher and Marcker, 1966). Thus, it is not clear at the moment whether the primary site that accepts puromycin is an enzyme site, a ribosomal site, or a combination of both. The hypothesis has also been advanced that gougerotin and puromycin "are bound in the same manner" on the enzyme which catalyzes peptide-bond formation (Casjens and Morris, 1965).

If, indeed, the amino acylaminonucleoside inhibitors interfere with amino acyl-RNA at either an enzyme or a ribosomal site, it would be expected that different amounts of amino acyl-RNA affect such inhibition to a different extent. To examine this question, a kinetic analysis was undertaken and the present report describes the effect which increasing concentrations of L-phenylalanyl-RNA (Phe-RNA) have on the inhibition of the poly U directed polyphenylalanine synthesis by

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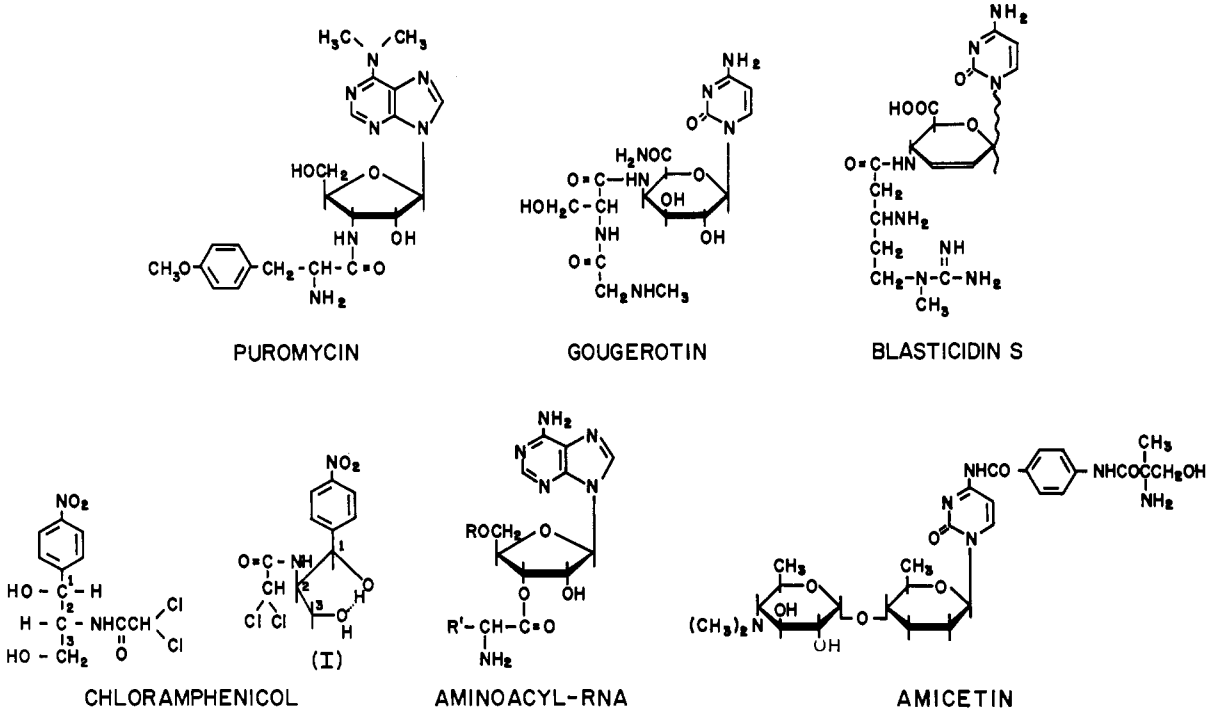

FIGURE 1: Structures of amino acyl-RNA (partial structure) of some amino acylaminonucleoside antibiotics and of chloramphenicol. In the structure of the amino acyl-adenylyl terminus of amino acyl-RNA, R represents the remainder of RNA and R′ the side chain of an amino acid. Structure I of chloramphenicol gives its assumed conformation which has a resemblance to the peptidyl-adenylyl terminus of peptidyl-RNA (Coutsogeorgopoulos, 1966).

puromycin, chloramphenicol, gougerotin, blasticidin S, and amicetin. It has been found that the inhibition exerted by these inhibitors was not reversed by amino acyl-RNA. Streptomycin and tetracycline were examined for comparative purposes.

Materials and Methods

Materials. Deacylated tRNA obtained from *Escherichia coli* B was purchased from General Biochemicals, Chagrin Falls, Ohio, and was used to prepare L[^{14}C]phenylalanyl-RNA[1] according to the procedure of von Ehrenstein and Lipmann (1961). Polyuridylic acid was purchased from Miles Chemical Co. and L-[^{14}C]phenylalanine (uniformly labeled) from the New England Nuclear Corp. The sample of amicetin used was prepared by the Upjohn Co., batch no. 9924. Puromycin was purchased from Nutritional Biochemicals, tetracycline from Chas. H. Pfizer and Co., and streptomycin from E. R. Squibb and Sons. Chloramphenicol was a gift from Parke Davis and Co. The sample of gougerotin was a gift from Dr. J. J. Fox of Sloan-Kettering Institute, New York, N. Y., and blasticidin S was generously provided by Professor Hiroshi Yonehara of the University of Tokyo, Japan.

Ribosomal Preparations. E. coli B cells were harvested in the middle of the logarithmic phase of growth and were frozen until used. Ribosomes (once washed), 100,000*g* supernatant (S-100), and preincubated 30,000*g* supernatant (S-30) were prepared from frozen cells according to the procedure of Nirenberg and Matthaei (1961) and were stored in small aliquots at −70°. The protein content of these fractions was determined according to the method of Lowry *et al.* (1951).

Polyphenylalanine Synthesis. The "standard incubation mixture" contained in 0.25 ml: 25 μmoles of Tris-HCl buffer (pH 7.8), 12.5 μmoles of ammonium chloride (pH 7.6), 2.5 μmoles of magnesium acetate, 1.5 μmoles of 2-mercaptoethanol, 0.25 μmole of GTP,[2] 0.5 μmole of trisodium phosphoenolpyruvate, 10 μg of pyruvate kinase from a 10-mg/ml suspension in 2.4 M ammonium sulfate, 2.5 μg of polyuridylic acid, and 1.5 mg (protein) of preincubated S-30 [or where specified, 0.17 mg (protein) of washed ribosomes and 0.17 mg (protein) of S-100]. The L-[^{14}C]phenylalanyl-RNA used had a specific activity of 375 μc/μmole of charged L-[^{14}C]phenylalanine or 7000 cpm/ODU at 260 mμ of L-[^{14}C]phenyl-

[1] In this paper the name L-phenylalanyl-RNA (Phe-RNA) is used to denote the mixture of L-[^{14}C]phenylalanyl-RNA and of the other tRNAs which were present in the original deacylated tRNA used.

[2] Abbreviations used: PCA, perchloric acid; ATP, adenosine triphosphate; GTP, guanosine triphosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2(5-phenyloxazolyl)benzene.

alanyl-RNA and was added as specified, measured in optical density units at 260 mμ (*i.e.*, milliliters of stock solution multiplied by its optical density at 260 mμ; 1-cm light path). The reagents were added in the order cited with L-[^{14}C]phenylalanyl-RNA last. The inhibitors were added after the addition of the polyuridylic acid and before the addition of the ribosomes. The temperature and period of incubation are given in the legends of tables and figures.

Assay for Polyphenylalanine Synthesis (Hot PCA Assay). At the end of the incubation period, two 0.1-ml aliquots were applied on 3 MM Whatman paper disks (2.3 cm in diameter) and assayed for "hot perchloric acid precipitable material" by the following modification of a paper disk method (Bollum, 1959; Mans and Novelli, 1961). After pipetting the incubation mixture onto the disks they were immediately (each duplicate pair at a time) placed in ice-cold 7% PCA (about 10 ml/disk) where they stayed for 15–30 min. If the disks are left to dry in air or in a stream of warm air, further synthesis takes place if the incubation was interrupted at an early stage of the reaction. For kinetic studies it is important to place the disks in the cold perchloric acid as soon as the pipetted liquid has impregnated the paper disk. The disks were subsequently washed by soaking in ice-cold 3.5% PCA for 5 min and then transferred into 3.5% PCA, which was preheated to 80–90° in a boiling water bath, and retained at this temperature for 15 min. Finally the disks were soaked at room temperature, in 3.5% PCA for 5 min, twice in ethanol-ether (1:1) for 1 min, and ether for 1 min. The disks were dried at 75° for 10 min and transferred in scintillation vials containing 10–20 ml of phosphor and counted in a Packard Tri-Carb liquid scintillation spectrometer. The phosphor consisted of 4 g of PPO and 100 mg of POPOP/l. of reagent grade toluene. Two blank disks were included in each assay and the average radioactivity adsorbed by contamination from the other disks was subtracted from all the measured values. Under these conditions 1 μμmole of L-[^{14}C]phenylalanine corresponded to 500 cpm (background 30 cpm).

Assay for Undecomposed L-[^{14}C]phenylalanyl-RNA. The amount of undecomposed L-[^{14}C]phenylalanyl-RNA present at the end of each incubation period was determined by running duplicate incubation mixtures and duplicate disks from each mixture. Hot PCA-precipitable material was determined in one set of disks and cold PCA-precipitable material in the duplicate set of disks. The cold PCA assay was performed exactly like the hot PCA assay mentioned above, the only difference being that the step of heating in 3.5% PCA at 80–90° for 15 min was replaced by a simple soaking in 3.5% PCA for 5 min at 4°, and that all the subsequent washings were done also at 4°. By subtracting the values of the hot PCA assay from the corresponding values of the cold PCA assay the amount of the undegraded Phe-RNA was calculated.

Results

Characteristics of the Uninhibited System. The amount of Phe-RNA substrate necessary to saturate the system depends (a) on the amount of template poly U, and (b) on the ribosomal preparation; for this latter reason all the numerical results given in Tables I and II as well as in Figure 2 were obtained with the same preincubated S-30 preparation. The amount of poly U template used throughout this study was 2.5 μg/0.25 ml of incubation mixture. When levels of poly U higher than 2.5 μg were used, polyphenylalanine synthesis was greatly increased, but the amount of Phe-RNA necessary to saturate the system became very high, rendering the addition of such high levels of Phe-RNA impracticable. On the other hand, when an amount of poly U much lower than 2.5 μg was used, the rate of polyphenylalanine synthesis was very slow. For example, in the presence of unlimiting amounts of Phe-RNA, 0.3 μg of poly U gave 50% of the maximal yield of polyphenylalanine in 12 min at 37°, whereas 2.5 μg of poly U gave 50% of the maximal yield in 3 min.

Polyphenylalanine synthesis is usually measured at 37°. Some ribosomal preparations used in this study were so active that an incubation temperature of 25° had to be used in order to measure initial rates. However, as will be shown, the effects of inhibitors were different at the two temperatures.

Effect of Temperature and Period of Incubation on the Inhibition. It has been customary to examine the effects

TABLE I: Effect of Temperature and Length of Incubation on the Inhibition of Polyphenylalanine Synthesis by Various Selective Inhibitors of Protein Synthesis.[a]

Inhibitor (M)	% Inhibition			
	Incubation at 25°		Incubation at 37°	
	8 min	40 min	3 min	20 min
Puromycin (2.4×10^{-5})	64	65	20	41
Chloramphenicol (10^{-3})	61	50	60	46
Gougerotin (4×10^{-5})	51	25	34	11
Blasticidin S (10^{-6})	70	52	54	35
Amicetin (4×10^{-5})	64	34	70	46
Streptomycin (2.0×10^{-5})	51	51	70	65
Tetracycline (1.2×10^{-5})	59	52	67	65

[a] The complete system was that given under Methods. Poly U (2.5 μg), 0.75 mg (protein) of preincubated S-30, 8.0 ODU at 260 mμ of Phe-RNA. Temperature and time of incubation as indicated. Polyphenylalanine synthesis (hot PCA assay) was linear in the presence or absence of inhibitors for the first 10 min at 25° or the first 5 min at 37°. The percentage inhibition was constant during the time interval in which both reactions were linear. The L-[^{14}C]phenylalanine incorporation/0.1 ml of incubation mixture in the absence of inhibitors was (counts per minute): at 25°, for 8 min 5098, for 40 min 8502; at 37°, for 3 min 4894, for 20 min 10,226.

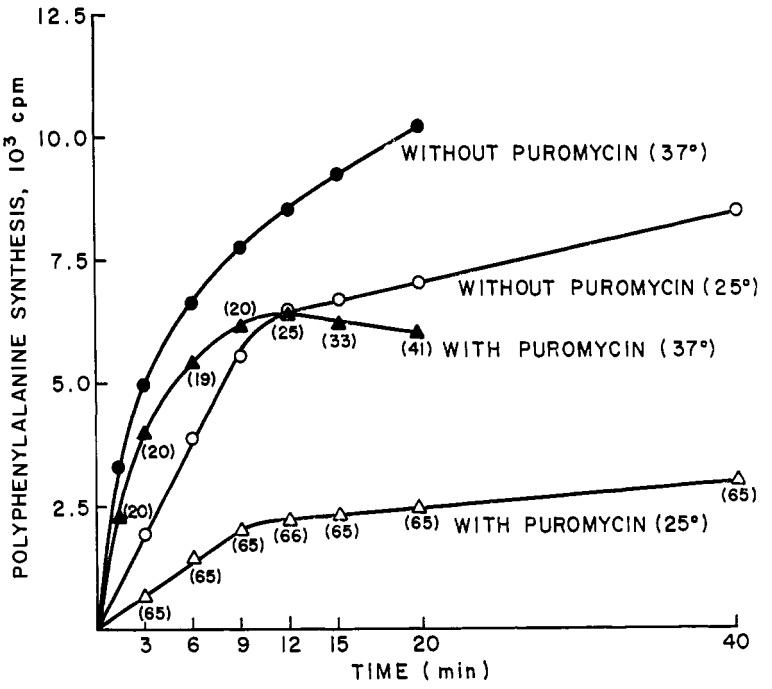

FIGURE 2: Effect of temperature and period of incubation on the inhibition of polyphenylalanine synthesis by puromycin at 2.4×10^{-5} M; (●-●-●) minus puromycin, 37°; (▲-▲-▲) plus puromycin, 37°; (○-○-○) minus puromycin, 25°; (△-△-△) plus puromycin, 25°. The numbers in parentheses give the per cent inhibition.

TABLE II: Effect of Increasing Concentrations of Phenylalanyl-RNA on the Inhibition of Polyphenylalanine Synthesis.[a]

Inhibitor (M)	ODU at 260 mμ of Phe-RNA (% inhibn)								
	4.0	8.0	12.0	16.0	20.0	24.0	32.0	36.0	40.0
Puromycin (2.4×10^{-5})	67	65	66	63	62	62	61	60	56
Chloramphenicol (10^{-3})	56	53	53	53	50	50	47	47	46
Gougerotin (4×10^{-5})	52	52	56	57	58	60	61		
Blasticidin S (10^{-6})	58	61	65	66	64	67	67		
Amicetin (4×10^{-5})	65	68	70	70	72	72	72		
Streptomycin (2×10^{-5})	32	47	48	52	53	54	55		
Tetracycline (1.2×10^{-5})	57	61	62	63	64	65	63		

[a] The complete system was that described under Methods; 2.5 μg of poly U, 0.75 mg (protein) of preincubated S-30, incubation at 25° for 8 min. Polyphenylalanine synthesis was linear for the first 10 min (25°) in the presence or absence of inhibitors. The optical density units at 260 mμ of Phe-RNA are given for the whole (0.25 ml) incubation mixture, whereas polyphenylalanine synthesis (hot PCA assay) is given for the assayed 0.1 ml of the incubation mixture. At each level of Phe-RNA a blank value for hot PCA-precipitable material in the absence of poly U was subtracted. By increasing the concentration of the L-[^{14}C]phenylalanyl-RNA added, the increase in the incorporation of L-[^{14}C]phenylalanine (hot PCA assay)/0.1 ml of incubation mixture in the absence of inhibitors was as follows in counts per minute (the numbers in parentheses represent the optical density units at 260 mμ of Phe-RNA added in 0.25 ml of incubation mixture): 2960 (4.0), 5052 (8.0), 6725 (12.0), 7812 (16.0), 8601 (20.0), 9410 (24.0), 10,143 (32.0), 10,685 (36.0), and 11,291 (40.0) Each optical density unit at 260 mμ of Phe-RNA added, corresponded to 2600 cpm input in the 0.1 ml of the incubation mixture assayed.

of inhibitors of protein synthesis in cell-free systems under conditions which give maximal yields of polypeptides. However, as Table I shows, the length and the temperature of incubation affect the inhibition. Chloramphenicol, gougerotin, blasticidin S, and amicetin were much stronger inhibitors during the initial stages of the reaction. Furthermore, a lower incubation temperature gave higher inhibition for puromycin, gouge-

rotin, and blasticidin S. Conversely, streptomycin inhibited more strongly at 37 than at 25°, whereas the inhibition exerted by amicetin and tetracycline was almost independent of temperature. The puromycin inhibition showed peculiarities not encountered with the rest of the inhibitors examined. First, Figure 2 shows that, at 25° the inhibition by puromycin did not change with time, whereas at 37°, the inhibition was low and constant until polyphenylalanine synthesis stopped prematurely. Second, the concentration of 2.4×10^{-5} M puromycin was inhibitory with certain ribosomal preparations. However, with some preparations this concentration of puromycin was completely ineffective, whereas all the other inhibitors examined were equally effective in both types of preparations at the concentrations given in Table I. Those preparations which were not inhibited by 2.4×10^{-5} M puromycin gave much higher rates as well as maximal synthesis of polyphenylalanine, at an equal level of Phe-RNA. For example, using the standard incubation mixture, we have encountered ribosomal preparations which compared with those that were inhibited by 2.4×10^{-5} M puromycin synthesized approximately double "maximal" amounts of polyphenylalanine, at double the rate, and needed as high as 10^{-3} M puromycin for 50% inhibition (initial rates measured at 25°).

Effect of Increasing Concentrations of L-*Phenylalanyl-RNA on the Inhibition.* Table II gives the inhibition of polyphenylalanine synthesis by the seven inhibitors studied, when increasing concentrations of Phe-RNA precursor were used. That "initial rates" were measured was deduced from the fact that both the inhibited and the uninhibited reactions were linear throughout the entire incubation period. However, as will be further discussed, during this period a substantial amount of substrate Phe-RNA is consumed in secondary reactions.[3] When the concentration of Phe-RNA was gradually increased up to tenfold there was only a slight decrease of the inhibition caused by chloramphenicol and puromycin. In contrast, the inhibition exerted by streptomycin under the same conditions was clearly increased. A slight increase of inhibition was observed with amicetin, gougerotin, blasticidin S, and tetracycline. When data from Table II were plotted according to Lineweaver and Burk (1934) the graphs of Figure 3 were obtained. When examined at a second concentration of inhibitor different from that given in Table II, the nature of the inhibition was found to be the same. In these graphs, the values given for the substrate Phe-RNA are the concentrations added at zero time. However, a certain amount of the input Phe-RNA is consumed in secondary reactions.[3] Thus, the true concentrations of this substrate were less than those plotted. A correction for this error has not been attempted but, since initial rates were measured and since at the end of the incubation period a substantial amount of Phe-RNA was still present,[4] the observations regarding the nonreversal of the inhibition by increasing amounts of Phe-RNA are still valid. That at least part of the undecomposed Phe-RNA found at the end of the 8-min (25°) incubation period was still functional is evidenced by the fact that the reaction could proceed at an undiminished rate for an additional 2 min (see legend of Table II).

The same pattern of results given in Table II was also observed when washed ribosomes and "supernatant" were used in place of preincubated S-30, the difference being that a higher level of template poly U was used in order to achieve greater yields of polyphenylalanine synthesis while still retaining the ability to half-saturate the system with *ca.* 20 ODU at 260 mμ of Phe-RNA.

The deacylated tRNA used for charging with L-[^{14}C]-phenylalanine was unfractionated and, as a result, the preparation of [^{14}C]Phe-RNA contained all the other contaminating amino acid specific species of tRNA, but presumably not esterified with amino acids. Thus, when the concentration of L-[^{14}C]phenylalanyl-RNA was increased, the concentration of deacylated tRNA was increased at the same time. To determine whether the deacylated tRNA influences the inhibition observed, we have examined (at constant levels of L-[^{14}C]phenylalanyl-RNA) the effect of increasing concentrations of the same deacylated tRNA, treated under the conditions of amino acid charging but in the absence of L-[^{14}C]-phenylalanine. It has been found that the inhibition of polyphenylalanine synthesis by the seven inhibitors examined was not changed. This indicates that the effects on the inhibition discussed in this work are due to the increased concentrations of L-[^{14}C]phenylalanyl-RNA *per se* and not to the contaminating tRNAs.

Discussion

Puromycin. It has been proposed (Warner and Rich, 1964; Wettstein and Noll, 1965; Bretscher and Marcker,

[3] When polyphenylalanine synthesis (Table II) and undecomposed Phe-RNA[4] are expressed as per cent of input Phe-RNA, the difference of the sum of these percentages from 100 gives the percentage of Phe-RNA input consumed in secondary reactions. The amount of Phe-RNA lost in these reactions decreased with increasing amounts of input Phe-RNA. For a change in Phe-RNA input from 4 to 32 ODU at 260 mμ, the Phe-RNA consumed in secondary reactions decreased (as per cent of input): for the uninhibited reaction from 20 to 14%; in the presence of chloramphenicol from 25 to 12%; in the presence of puromycin from 35 to 22%; in the presence of gougerotin from 25 to 12%; in the presence of amicetin from 24 to 14%; in the presence of blasticidin S from 22 to 14%; in the presence of tetracycline from 24 to 12%; and in the presence of streptomycin from 25 to 16%. The concentrations of inhibitors used were those given in Table I or II.

[4] When initial rates were followed (conditions of Table II) the fraction of the L-[^{14}C]phenylalanyl-RNA which remained undecomposed at the end of the incubation period (measured by the difference between "cold PCA assay" and "hot PCA assay") was higher, the higher the input of Phe-RNA used. For an increase in Phe-RNA input from 4 to 32 ODU at 260 mμ, the undecomposed Phe-RNA increased (as per cent of input): for the uninhibited reaction from 52 to 74%; in the presence of chloramphenicol from 62 to 72%; in the presence of puromycin from 55 to 73%; in the presence of gougerotin from 60 to 83%; in the presence of amicetin from 66 to 83%; in the presence of blasticidin S from 66 to 82%; in the presence of tetracycline from 64 to 84%; and in the presence of streptomycin from 56 to 78%. The concentrations of inhibitors used were those given in Table I or II.

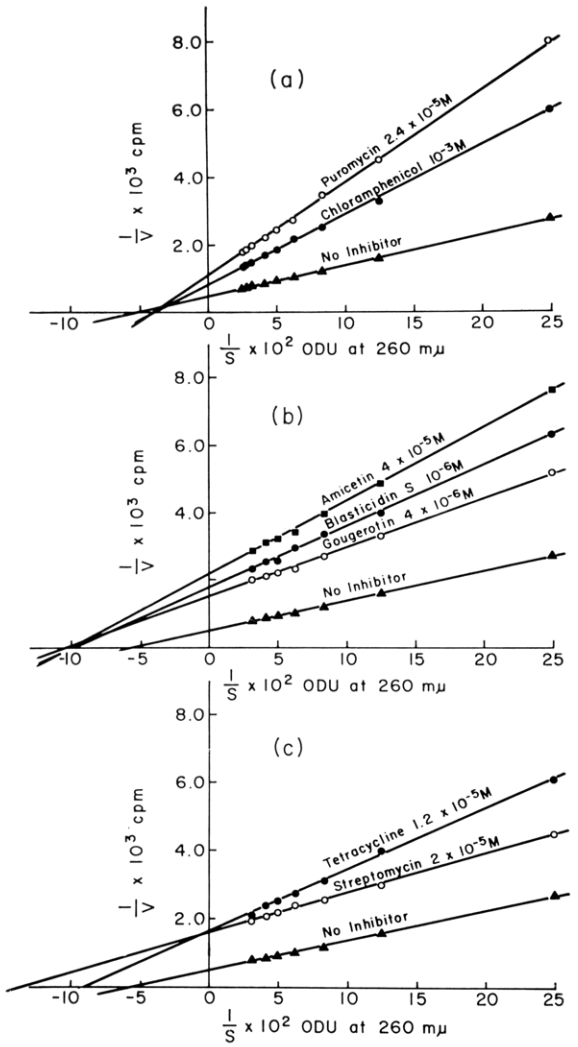

FIGURE 3: Nature of the inhibition of polyphenylalanine synthesis, (▲—▲—▲) by: (a) puromycin (○—○—○) and chloramphenicol (●—●—●); (b) gougerotin (○—○—○), blasticidin S (●—●—●), and amicetin (■—■—■—■); and (c) streptomycin (○—○—○) and tetracycline (●—●—●). The graphs were obtained by plotting the data from Table II. V = polyphenylalanine synthesis (counts per minute/0.1 ml of incubation mixture) per min at 25°. S = optical density units at 260 mμ of Phe-RNA in the whole incubation mixture (0.25 ml).

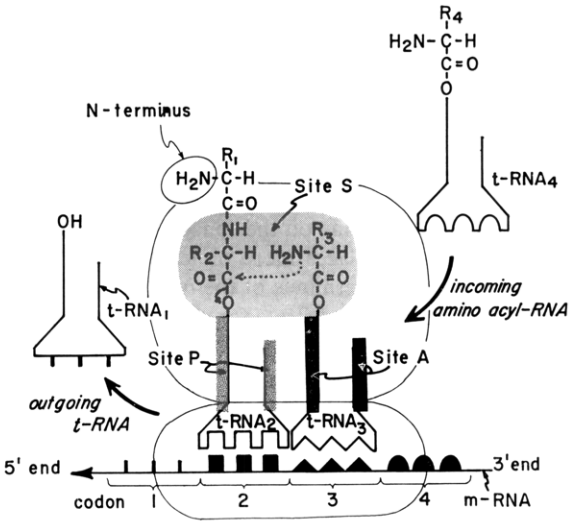

FIGURE 4: Schematic diagram of peptide-bond formation on the ribosome. Adapted from Schweet *et al.* (1964). Sites A, P, S, and tRNAs are not drawn to scale; site S is exaggerated for purposes of discussion.

1966; Heintz *et al.*, 1967) that during the stepwise elongation of polypeptide chains, the amino acyl-RNA and the peptidyl-RNA remain bound to the mRNA–ribosome complex at two specific ribosomal sites, the "amino acid site" ("acceptor site," site A in Figure 4) and the "peptide site" ("donor site," site P in Figure 4), respectively. It has been tacitly assumed that during the formation of each peptide bond the amino acyl-adenylyl terminus of amino acyl-RNA and the peptidyl-adenylyl terminus of peptidyl-RNA interact on a region which we shall call site S (Figure 4). Site S could be (a) solely a ribosomal site, (b) an enzyme site, perhaps a region of the "peptide synthetase" (Arlinghaus *et al.*, 1964), or (c) a combination of a ribosome–enzyme site. Although site S could be viewed as a continuation of sites A and P, the latter sites, as defined, are primarily associated with the tRNA parts of amino acyl-RNA and peptidyl-RNA whereas site S is to be considered a separate site, as will become apparent from the following discussion. Peptide-chain release by puromycin involves the formation of a peptide bond between the free amino group of puromycin and the tRNA-bound carboxy terminus of the growing polypeptide chain (Smith *et al.* (1965) and references therein). This reaction has been used as a model for the study of peptide-bond formation. It has been assumed that due to its structural similarity to amino acyl-RNA, puromycin enters the ribosomal structure by way of the amino acid site A and reacts further with the peptidyl-RNA bound to site P (Bretscher and Marcker, 1966). If puromycin occupies at any time part of the ribosomal site A, which is the site that accepts the incoming amino acyl-RNA, it might be expected that amino acyl-RNA could compete with puromycin for site A and, when added in increasing amounts, could reverse the inhibition caused by puromycin. However, the results of this study show that the puromycin inhibition is essentially not reversed by increasing amounts of amino acyl-RNA. An analogous observation has been made by Allen and Schweet (1962).

The explanation we favor for evaluating the present results is the following. By virtue of the rest of the tRNA molecule which is not present in puromycin, the incoming amino acyl-RNA can, during peptide-bond formation, bind to the ribosome–mRNA complex at site A without interfering with the binding of puromycin. If we accept *the structural similarity of puromycin to the amino acyl-adenylyl terminus of amino acyl-RNA as the*

reason for the puromycin action, we should further accept that the site of puromycin attachment is part of what we defined as site S (Figure 4). During peptide-chain elongation the incoming amino acyl-RNA binds strongly at site A whereas the amino acyl-adenylyl terminus of the bound amino acyl-RNA occupies part of site S. In other words, the substrate amino acyl-RNA binds at both sites A and S. Depending on the concentration of puromycin the latter displaces more or less effectively the amino acyl-adenylyl terminus of the *bound* amino acyl-RNA and, acting as a substrate for site S, reacts further with the peptidyl-RNA at site P. This explanation is in accord with a suggestion made by Wettstein and Noll (1965) who, based on a completely different analysis, postulated that puromycin "competes with the incoming amino acyl-RNA" not by blocking the attachment of the tRNA portion to its complementary ribosomal site, but by occupying the enzymic site involved in peptide-bond formation. However, as the present study suggests (Figure 3a) there is no strict competition in a kinetic sense.

Chloramphenicol. On the propositions that chloramphenicol: (a) interferes with a function of mRNA (Wolfe and Hahn: 1965), (b) acts by altering the ribosomal structure (Vazquez, 1966), or (c) acts by competing with the carboxy-terminal amino acid of the growing polypeptide chain for a ribosomal binding site (Das *et al.*, 1966), one would not expect any effect by amino acyl-RNA on the chloramphenicol inhibition. However, the fact that chloramphenicol interferes with the puromycin release of polypeptide chains (Traut and Monro, 1964) suggests that chloramphenicol may bind at the same site at which puromycin attaches. It was considered, therefore, that the chloramphenicol inhibition may also be affected by amino acyl-RNA. Wolfe and Hahn (1965) have reported that increasing amounts of tRNA did not influence the inhibition exerted by chloramphenicol on the poly U directed polyphenylalanine synthesis from *free* L-phenylalanine, although a graphical analysis of their results showed competitive inhibition.

The present results show that the chloramphenicol inhibition of the poly U directed polyphenylalanine synthesis is essentially not reversed by Phe-RNA (Table II) and that there is no strict competition between chloramphenicol and this substrate (Figure 3a). No definite conclusions can be drawn as to the site where chloramphenicol acts. However, it is suggested that this site is not the ribosomal site A (the amino acid site).

Gougerotin has been shown to inhibit the puromycin release of polypeptides from the ribosome (Clark and Chang, 1965; Casjens and Morris, 1965) and since this inhibition was found to be competitive, it was proposed that gougerotin and puromycin are bound "in the same manner" on the peptide-bond-forming enzyme (Casjens and Morris, 1965). Since puromycin was expected to compete with amino acyl-RNA, it was reasonable to expect that the gougerotin inhibition could also be reversed by amino acyl-RNA. However, in analogy with our findings on puromycin, the present study shows that Phe-RNA did not reverse the inhibition of polyphenylalanine synthesis by gougerotin. On the contrary a slight increase of the inhibition was consistently observed (Table II). A graphical analysis of these results (Figure 3b) shows that the inhibition by gougerotin is not strictly noncompetitive but it contains an uncompetitive element as well (Dixon and Webb, 1964).

Blasticidin S and Amicetin. In the present study blasticidin S and amicetin behaved very much like gougerotin in their response to increased concentrations of Phe-RNA (Table II, Figure 3b). Although it is tempting to speculate that they interact at the same sites as gougerotin, definitive conclusions on the mechanism of action of these antibiotics should await further investigation.

Streptomycin and Tetracycline. In order to see whether the system under examination would respond differently to selective inhibitors of protein synthesis structurally related to neither amino acyl-RNA nor peptidyl-RNA, streptomycin and tetracycline were included in this study. It is seen that in the case of streptomycin the inhibition of polyphenylalanine synthesis is clearly increased by increasing amounts of Phe-RNA (Table II, Figure 3c). Analogous results have been reported by Davies *et al.* (1965) who showed that the streptomycin inhibition of polyphenylalanine synthesis from *free* L-phenylalanine, in the presence of the 19 other nonradioactive-free amino acids, was increased by increasing amounts of tRNA.

The inhibition of polyphenylalanine synthesis by tetracycline is essentially not reversed by increasing concentrations of Phe-RNA (Table II, Figure 3c). Analogous results have been reported by Suarez and Nathans (1965).

Other Conclusions. These results show that compounds screened for inhibitory activity of cell-free polypeptide synthesis should be tested not only at time intervals in which polypeptide synthesis is maximal, but also at times corresponding to the linear portion of the time curve of the reaction. It is further shown that the temperature and the length of incubation are critical for the inhibition measured.

The present study indicates that although the ribosomal polypeptide-synthesizing system is complex, the effects of inhibitors on a rate-limiting step can be examined if the conditions are carefully controlled. However, definitive conclusions, from such a kinetic study, on the nature of the inhibition and its mechanistic implications should await the use of more resolved and purified systems.

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