Inhibition by Pactamycin of the Initiation of Protein Synthesis. Binding of *N*-Acetylphenylalanyl Transfer Ribonucleic Acid and Polyuridylic Acid to Ribosomes*

Linda B. Cohen†, Albert E. Herner, and Irving H. Goldberg

ABSTRACT: The inhibition by pactamycin of polyphenylalanine synthesis by extracts from *Escherichia coli* is greatest at low Mg^{2+} concentrations where chain initiation by either *N*-acetylphenylalanyl transfer ribonucleic acid or phenylalanyl transfer ribonucleic acid is rate limiting. This inhibition is decreased by prior incubation of the S-30 extract at 4.5 mM Mg^{2+} with *N*-acetylphenylalanyl transfer ribonucleic acid but not with phenylalanyl transfer ribonucleic acid. Preincubation of the S-30 with pactamycin results in increased inhibition of polypeptide formation, while the delayed addition of pactamycin until after initiation is finished has no effect on polypeptide elongation. At low Mg^{2+} concentrations the binding of *N*-acetylphenylalanyl transfer ribonucleic acid to ribosomes, which depends upon initiation factors and guanosine triphosphate, is significantly inhibited by the antibiotic (10^{-6} M). Pactamycin induces the release of prebound *N*-acetylphenylalanyl transfer ribonucleic acid but does not directly inhibit deacylation caused by puromycin. The effect of pactamycin on the binding reaction is inversely related to the Mg^{2+} and directly related to the NH_4^+ concentrations. Streptomycin at low levels and chlortetracycline at high levels also interfere with the binding reaction. Zonal centrifugation analyses of binding incubations reveal that the bound radioactive *N*-acetylphenylalanyl transfer ribonucleic acid sediments more rapidly during the course of the reaction. In the presence of pactamycin, radioactivity sediments more slowly. While such "particles" may be 50S–30S complexes with varying compactness of structure, it is possible that they represent 70S ribosomes which are disassociating during sedimentation. Pactamycin is viewed as interfering with compact 70S ribosome formation, resulting in more ready disassociation into the component subunits. This effect is accentuated by low Mg^{2+} and high NH_4^+ concentrations which also decrease the stability of the binding and weaken the attraction between the ribosomal subunits. At 20 mM Mg^{2+} or 10 mM spermidine the pactamycin effect is lost. Binding of polyuridylic acid to ribosomes in the presence, but not in the absence, of initiation factors depends upon the addition of *N*-acetylphenylalanyl transfer ribonucleic acid and guanosine triphosphate. Only the former type of binding of polyuridylic acid is inhibited by pactamycin. It is concluded that pactamycin alters the structure of the initiation complex so as to lead to its destabilization and dissociation.

We have previously reported that pactamycin, an antibiotic inhibitor of mammalian and bacterial protein synthesis (Colombo *et al.*, 1966; Bhuyan, 1967), interferes with the specific binding of peptidyl-tRNA (polylysyl-tRNA) to ribosomes but not with peptide-bond formation itself (Cohen and Goldberg, 1967). Since the binding of initiator-tRNA to the peptidyl or donor site on the ribosome is involved in the initiation of protein synthesis, it was of interest to study the effect of pactamycin on the reaction in which *N*-acetyl-L-phenylalanyl-tRNA is used as the initiator (Lucas-Lenard and Lipmann, 1967). In this paper we show that pactamycin interferes with the formation and stability of the initiating complex consisting of *N*-acetyl-L-phenylalanyl-tRNA, ribosome, and poly U, and possibly alters its structure.

Materials and Methods

Assay of Polyphenylalanine Synthesis. *Escherichia coli*, strain B, crude extracts (S-30) were prepared as described by Nirenberg (1964). For measurements of polypeptide synthesis, the S-30 was preincubated for 45 min at 35° with no additions. Polyphenylalanine synthesis was measured by the incorporation of L-[^{14}C]-phenylalanine into hot 10% trichloroacetic acid precipitable material isolated and washed on Millipore filters as described by Nirenberg (1964). The constituents and conditions of incubation are listed in the legend to Figure 1. In each case the radioactivity incorporated in the absence of added poly U (with and without pactamycin) was subtracted from that in its presence.

Preparation of Ribosomes, Initiation Factors, and [^{14}C]N-Acetyl-L-phenylalanyl-tRNA. Ribosomes were prepared according to Lucas-Lenard and Lipmann

* From the Department of Medicine, Harvard Medical School, and the Beth Israel Hospital, Boston, Massachusetts 02215. *Received October 2, 1968.* This work was supported by grants from the National Institutes of Health, U. S. Public Health Service (GM 12575 and CA 10736), and the American Cancer Society. A preliminary report of some of the data included in this paper has appeared (Herner *et al.*, 1968).

† Trainee, supported by Grant T1 CA5167 of the National Cancer Institute.

(1967) or Ohta *et al.* (1967). The ribosomal wash preparation used as a source of initiation factors was prepared according to Lucas-Lenard and Lipmann (1967). L-[^{14}C]Phenylalanyl-tRNA was prepared by the method of Nathans and Lipmann (1961). The product was acetylated to produce [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA using acetic anhydride in the procedure of Haenni and Chapeville (1966). Hydrolysis of an aliquot of the preparation (5000 cpm) in 0.01 N KOH for 30 min at 37° followed by descending paper chromatography in butanol–acetic acid–water (78:5:17, v/v) demonstrated complete N acetylation of the phenylalanine. [^{12}C]*N*-acetyl-L-phenylalanyl-tRNA refers to *E. coli* tRNA charged with a mixture of 20 nonlabeled amino acids and acetylated with acetic anhydride.

Assay of [^{14}C]N-Acetyl-L-phenylalanyl-tRNA Binding. The binding assay of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA to ribosomes was modified after Lucas-Lenard and Lipmann (1967). The conditions and constituents are listed in the legend to Figure 2. Variations from these values are noted where appropriate. Reactions were generally initiated by the addition of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA followed immediately by magnesium acetate. Incubations were terminated by addition of 2 ml of cold buffer containing 10 mM Tris-HCl (pH 7.4) and NH_4Cl and magnesium acetate in the same concentrations as present in the incubation mixtures. The samples were immediately passed through Millipore filters as described by Nirenberg and Leder (1964).

Radioactivity in all procedures was determined in a Packard scintillation spectrometer at a counting efficiency of 88% for ^{14}C.

Characterization of Bound Radioactivity. The nature of the ribosomal-bound radioactivity was determined by the procedure of Lucas-Lenard and Lipmann (1967). The ribosomes, after incubation with [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA, were collected on Millipore filters and extracted with EDTA. The extracted tRNA was precipitated with alcohol–potassium acetate, treated with 0.2 N KOH, and neutralized with $HClO_4$. The resulting solution was chromatographed with known standards to determine the presence of amino acids and/or oligopeptides. The radioactivity bound to the ribosomes is exclusively in the form of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA.

Sucrose density gradient analysis of *N*-acetyl-L-phenylalanyl-tRNA bound to ribosomes was performed as described below for [^{3}H]poly U.

Determination of Acid-Insoluble Radioactivity. The incubation mixture was precipitated with 5% trichloroacetic acid at 0° for 30 min and the precipitates were collected on Millipore filters. The filters were washed three times with 2-ml portions of cold 5% trichloroacetic acid prior to drying.

Assay of [^{3}H]Poly U Binding to Ribosomes by Sucrose Gradient Centrifugation. The standard reaction mixture (0.2 ml) for measuring binding of [^{3}H]poly U to ribosomes contained the following: 50 mM Tris-HCl (pH 7.4), 5 mM magnesium acetate, 160 mM NH_4Cl, 10 mM dithiothreitol, 3.2 A_{260} units of ribosomes, 8 μg of unlabeled poly U, and 39,600 cpm of [^{3}H]poly U (27 μCi/μmole). Other additions and incubation conditions are indicated in the figure legends. An aliquot (180 μl) was applied to a 12-ml linear sucrose gradient (5–20%) containing 50 mM Tris-HCl (pH 7.4), 5 mM Mg^{2+}, and 160 mM NH_4^+, and centrifuged for the indicated period of time at 41,000 rpm in the SW41 Spinco rotor at 4°. Gradients were analyzed for radioactivity as described elsewhere (Goldberg and Mitsugi, 1967). The counting efficiency for ^{3}H was 23%. Absorbance at 260 mμ was recorded automatically in the Gilford absorbance recorder.

Antibiotic Solutions. Puromycin was dissolved in water (4×10^{-3} or 8×10^{-3} M) and neutralized to pH 7.0 with solid $NaHCO_3$. Pactamycin (10^{-3} M) was dissolved in 10^{-3} M acetic acid. All the other antibiotics were dissolved in water at concentrations of 10^{-2} or 10^{-3} M. These solutions were used as stock solutions in the described experiments. Chlortetracycline solutions were made up fresh on the day of use.

Materials. L-[^{14}C]Phenylalanine (370 μCi/μmole) was obtained from the New England Nuclear Corp. Poly U, [^{3}H]poly U, and 5′-guanylylmethylenediphosphonate were obtained from Miles Chemical Co. *E. coli B* tRNA came from General Biochemicals Co. Puromycin was purchased from Nutritional Biochemicals Co.; streptomycin sulfate from Eli Lilly and Co.; and chlortetracycline from the Lederle Laboratories. Pactamycin was a gift of the Upjohn Co.

Results

Effect of Pactamycin on Initiation of Polypeptide Synthesis. Polyphenylalanine synthesis by S-30 extracts was examined at low Mg^{2+} concentrations where it is dependent upon added *N*-acetyl-L-phenylalanyl-tRNA for initiation and at higher Mg^{2+} where it proceeds without *N*-acetyl-L-phenylalanyl-tRNA (Figure 1). At 5.5 mM Mg^{2+} and below, polyphenylalanine formation, which requires *N*-acetyl-L-phenylalanyl-tRNA, is inhibited by pactamycin, but at 6.5 mM Mg^{2+}, where incorporation has reached a peak, the reaction is completely unaffected by the antibiotic. On the other hand, polyphenylalanine synthesis from phenylalanyl-tRNA not dependent upon *N*-acetyl-L-phenylalanyl-tRNA is sensitive to pactamycin at 10 mM Mg^{2+} and below but becomes resistant by 15 mM Mg^{2+}. In both situations polypeptide synthesis is most sensitive to pactamycin at lower Mg^{2+} concentrations where initiation, whether by *N*-acetyl-L-phenylalanyl-tRNA or phenylalanyl-tRNA, is rate limiting. The extent of polypeptide synthesis in both systems is the same at optimal Mg^{2+} concentrations. While the pactamycin concentration in the experiments shown in Figure 1 is 8×10^{-5} M, identical results were obtained at 8×10^{-6} M pactamycin. Since polyphenylalanine formation dependent upon *N*-acetyl-L-phenylalanyl-tRNA reaches its maximum by 6 mM Mg^{2+}, entrance of phenylalanyl-tRNA into the acceptor site and subsequent amino acid polymerization are presumably efficient at this Mg^{2+} concentration. These steps, therefore, may not be limiting in polypeptide synthesis

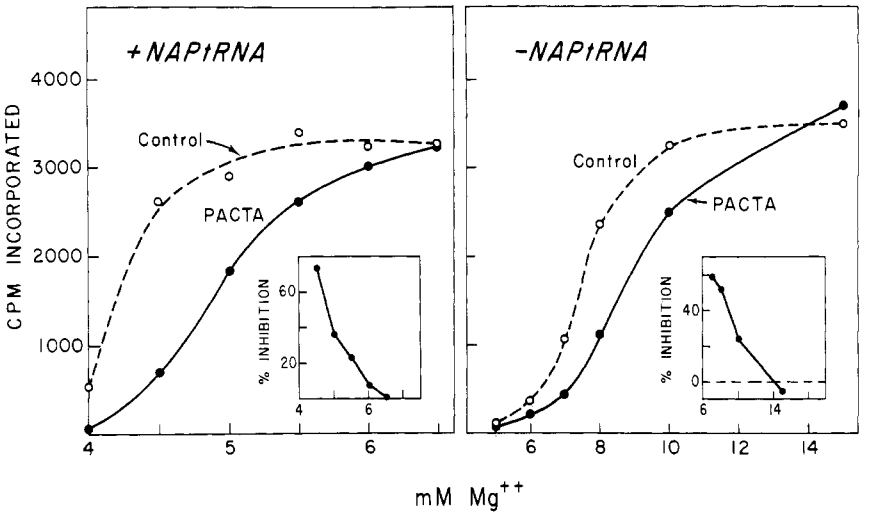

FIGURE 1: Mg^{2+} dependence of pactamycin inhibition of polyphenylalanine synthesis in the presence and absence of *N*-acetyl-L-phenylalanyl-tRNA. The following were incubated at 37° for 10 min in a volume of 0.125 ml: 50 mM Tris-HCl (pH 7.4), 160 mM NH_4Cl, 6 mM β-mercaptoethanol, 9 mM phosphoenolpyruvate, 5 μg of pyruvate kinase, 0.4 mM GTP, 5 μg of poly U, 25 μg of [^{14}C]phenylalanyl-tRNA (8650 cpm, 370 μCi/μmole of [^{14}C]phenylalanine), 15 μl of S-30, and, where indicated, 18.9 μg of [^{12}C]*N*-acetyl-L-phenylalanyl-tRNA and/or 8×10^{-5} M pactamycin, Mg^{2+} concentration was varied as shown in the figure. Polypeptide synthesis was measured as described in Materials and Methods.

independent of *N*-acetyl-L-phenylalanyl-tRNA throughout the Mg^{2+} range where synthesis takes place. This is consistent with the earlier results of Revel and Hiatt (1965).

Preincubation of the S-30 preparation with *N*-acetyl-L-phenylalanyl-tRNA at 4.5 mM Mg^{2+} protects against inhibition by pactamycin in the subsequent incubation with [^{14}C]phenylalanyl-tRNA (Table I). If this preincubated S-30 is treated with pactamycin prior to the final incubation with [^{14}C]phenylalanyl-tRNA, however, the protection afforded by the *N*-acetyl-L-phenylalanyl-tRNA preincubation is lost. Further, if the S-30 is preincubated with [^{14}C]phenylalanyl-tRNA and then incubated with *N*-acetyl-L-phenylalanyl-tRNA with or without pactamycin, there is no protection against pactamycin inhibition.

At 5 mM Mg^{2+}, incubation of the S-30 with pactamycin for 5 min at 37° prior to incubation with *N*-acetyl-L-phenylalanyl-tRNA and [^{14}C]phenylalanyl-tRNA results in greater inhibition of polypeptide synthesis than without preincubation. At 10 mM Mg^{2+}, on the other hand, no inhibitory effect of pactamycin is observed even when the S-30 is preincubated with the antibiotic.

Further evidence for an effect of pactamycin on chain initiation is shown in Figure 2, where the addition

TABLE I: Pactamycin Inhibition of Polyphenylalanine Synthesis. Preincubation of S-30 with *N*-Acetyl-L-phenylalanyl-tRNA or Pactamycin.[a]

	Conditions	Mg^{2+} (mM)	[^{14}C]Phenylalanyl-tRNA Incorp (cpm)		% Inhibn by Pactamycin
			−Pactamycin	+Pactamycin	
Expt I	No preincubation	4.5	3403	1021	70
	Preincubation with *N*-acetyl-L-phenylalanyl-tRNA	4.5	3085	1938	37
Expt II	No preincubation	5	4561	3261	28
		10	4346	4917	−13
	Preincubation with Pactamycin	5	4083	1855	55
		10	4881	5164	−6

[a] Components of the incubation mixture are listed in the legend to Figure 1. In expt I, preincubations (5 min, 37°) were carried out in the presence of *N*-acetyl-L-phenylalanyl-tRNA but without [^{14}C]phenylalanyl-tRNA and pactamycin. The latter components were then added and incubations were continued for 10 min. In expt II, preincubations (5 min, 37°) were carried out in the presence of pactamycin but without [^{14}C]phenylalanyl-tRNA and *N*-acetyl-L-phenylalanyl-tRNA. These two components were then added and incubations were continued for 10 min. Samples were assayed for polypeptide synthesis as described in Materials and Methods.

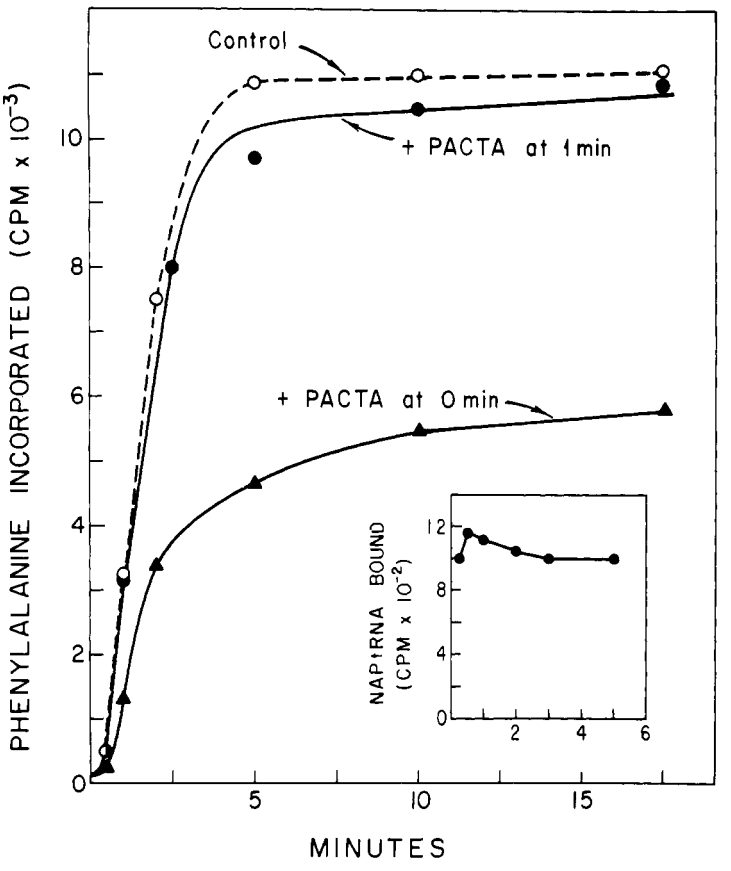

FIGURE 2: Effect of delayed addition of pactamycin on polyphenylalanine synthesis. Conditions for measuring polyphenylalanine synthesis were the same as those described in Figure 1 except that the incubation mixture contained 4.8 mM Mg^{2+}, 50 mM NH_4^+, 152 μg/ml of [^{12}C]*N*-acetyl-L-phenylalanyl-tRNA, and 420 μg/ml (18.3×10^4 cpm/ml) of [^{14}C]Phe-tRNA. Pactamycin, added at zero time or after 1-min incubation at 37°, was present at 10^{-5} M. At the indicated times, 100-μl aliquots were removed and assayed for polypeptide synthesis as described in Materials and Methods.

The same conditions were used for measuring [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA binding to ribosomes (insert) except that the incubation mixture contained 152 μg/ml (7.4×10^4 cpm/ml) of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA and 400 μg/ml of nonradioactive, charged tRNA. At the indicated times, 100-μl aliquots were removed and assayed for [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA binding by the filter technique as described in Materials and Methods. Incorporation of [^{14}C]Phe into polyphenylalanine has been corrected for no poly U controls which were about 15% of the control values and were unaffected by pactamycin.

TABLE II: Effect of Pactamycin on the Puromycin Reaction at Various Mg^{2+} Levels.[a]

	Mg^{2+} (mM)								
	4.8			10.8			18.8		
Additions	Before Puromycin (cpm)	After Puromycin (cpm)	Change (%)	Before Puromycin (cpm)	After Puromycin (cpm)	Change (%)	Before Puromycin (cpm)	After Puromycin (cpm)	Change (%)
None	525	244	−53	631	306	−52	764	642	−16
Pactamycin	374	195	−48	498	257	−49	738	614	−18

[a] Ribosomes were prelabeled with *N*-acetyl-L-phenylalanyl-tRNA (15 min, 25° for 4.8 mM Mg^{2+}; 30 min, 30° for 10.8 and 18.8 mM Mg^{2+}) with or without 2×10^{-5} M pactamycin as described in Materials and Methods. Initiation factors were present only in the incubation at 4.8 mM Mg^{2+}. Puromycin (6×10^{-4} M) was added and incubation was continued for 5 min. Samples (0.1 ml) were then assayed for ribosomal-bound radioactivity as described in Materials and Methods.

of pactamycin before but not after completion of initiation by *N*-acetyl-L-phenylalanyl-tRNA (insert, Figure 2) interferes with [^{14}C]phenylalanine incorporation. In contrast to expt I in Table I where preincubation with *N*-acetyl-L-phenylalanyl-tRNA in the absence of [^{14}C]phenylalanyl-tRNA provided only partial protection against pactamycin, protection is essentially complete when both initiator- and aminoacyl-tRNA are present during initiation. This is probably due to the release of some prebound *N*-acetyl-L-phenylalanyl-tRNA by pactamycin (as shown later in Figure 5) in the former case whereas after formation of the first peptide bond, the complex is relatively immune to the antibiotic.

Pactamycin Inhibition of N-Acetyl-L-phenylalanyl-tRNA Binding to Ribosomes. As has been reported by Lucas-Lenard and Lipmann (1967), the binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes at low

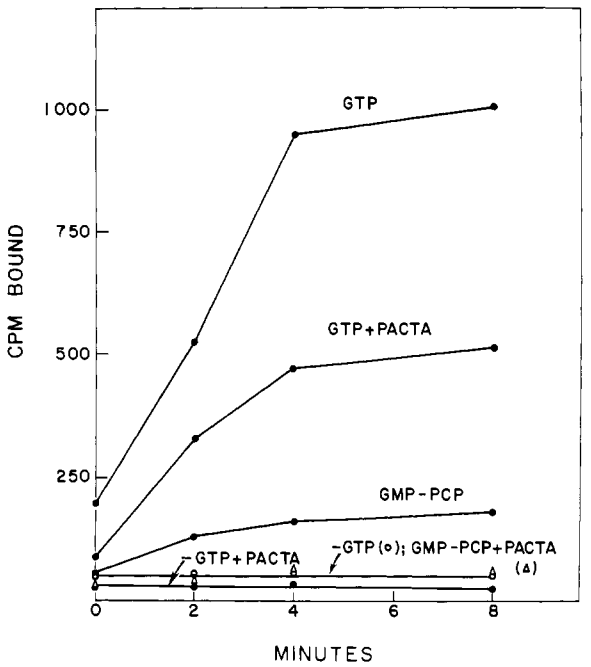

FIGURE 3: Relation of GTP and 5′-guanylylmethylenediphosphonate to the pactamycin inhibition of binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes. The incubation mixture (0.5 ml) contained 50 mM Tris-HCl (pH 7.4), 160 mM NH_4^+, 10 mM dithiothreitol, 40 μg/ml of poly U, 0.24 mM GTP, or 5′-guanylylmethylenediphosphonate where indicated, 4.8 mM magnesium acetate, 13.6 A_{260} units/ml ribosomes, 160 μg/ml of ribosomal wash protein, 3.7×10^4 cpm or 41 $\mu\mu$moles/ml, [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA, and 2×10^{-5} M pactamycin where indicated. Incubation was at 25° for the designated times. Binding of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA by the filter technique was determined as described in Materials and Methods.

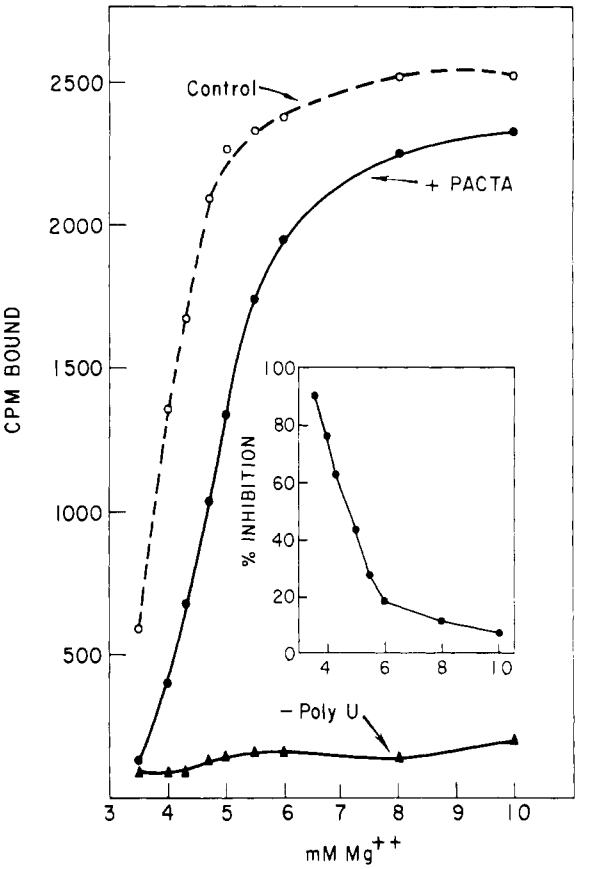

FIGURE 4: Mg^{2+} dependence of pactamycin inhibition of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA binding to ribosomes. Incubation conditions for 100-μl samples were as described in Figure 3 except that the Mg^{2+} concentration was varied as indicated. Samples were incubated at 25° for 15 min. Pactamycin when present was at 10^{-5} M. For calculation of percent inhibition at each Mg^{2+} concentration, values obtained in the absence of poly U were subtracted from those obtained with poly U. In the absence of poly U, binding was not inhibited by pactamycin.

Mg^{2+} concentrations is dependent upon both GTP and initiation factors. At 5 mM Mg^{2+} pactamycin inhibits binding 50–60% (Figure 3). As in the case of *N*-formylmethionyl-tRNA binding to ribosomes (Hershey and Thach, 1967), the methylene analog of GTP, 5′-guanylylmethylenediphosphonate, substitutes partially for GTP in the binding reaction; pactamycin inhibits this reaction by more than 60% (Figure 3).

Table II shows that pactamycin does not interfere with the puromycin reaction as such. Since less *N*-acetyl-L-phenylalanyl-tRNA is bound to ribosomes in the presence of pactamycin, the absolute amount of *N*-acetyl-L-phenylalanyl-tRNA released by puromycin is less but the percentage remains the same. Analysis of the radioactivity remaining trichloroacetic acid precipitable, *i.e.*, where the *N*-acetylphenylalanyl group is still attached to tRNA and has not been transferred to puromycin, also shows that pactamycin affects the puromycin reaction indirectly by the extent to which binding is affected.

Effect of Mg^{2+} and NH_4^+ Concentrations on Pactamycin Inhibition of Binding. The stability of binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes is increased at higher Mg^{2+} and lower NH_4^+ concentrations (Herner *et al.*, 1969). The ability of pactamycin to interfere with *N*-acetyl-L-phenylalanyl-tRNA binding to ribosomes also depends upon the Mg^{2+} concentration (Figure 4). As was found for polypeptide synthesis, the pactamycin inhibition of binding is greatest at low Mg^{2+} levels and disappears at higher Mg^{2+} concentrations.

Release of Bound N-Acetyl-L-phenylalanyl-tRNA by Pactamycin. As shown in Figure 5B, pactamycin causes the release of prebound *N*-acetyl-L-phenylalanyl-tRNA from ribosomes. The pactamycin-induced release is slower than that due to puromycin and is not associated with deacylation (Figure 5A). When both puromycin and pactamycin are present slightly more radioactivity is released from the ribosomes (Figure 5B), while the extent of deacylation (Figure 5A) is less. Again the decrease in the extent of the puromycin-induced deacylation caused by pactamycin can be related to its action on *N*-acetyl-L-phenylalanyl-tRNA binding to ribosomes.

The percentage inhibition of binding by pactamycin is increased at higher levels of NH_4^+ (Table III).

TABLE III: Effect of NH_4^+ on the Pactamycin Inhibition of *N*-Acetyl-L-phenylalanyl-tRNA Binding.[a]

NH_4^+ (mM)	[^{14}C]*N*-Acetyl-L-phenylalanyl-tRNA Bound (cpm)				% Inhibition by Pactamycin
	Control	+Pactamycin	−Factors	−Poly U	
50	1590	920	150	110	42
100	1710	870	160	100	49
160	1360	500	100	85	63

[a] Incubation conditions for 100-μl samples for 15 min were as described in Figure 3 except that the NH_4^+ concentration was varied as indicated and that pactamycin, when present, was at 10^{-4} M.

Pactamycin Concentration Curve. Inhibition of binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes is found as low as 10^{-7} M pactamycin but at 4.8 mM Mg^{2+} the maximal effect reached by 10^{-6} M pactamycin does not exceed 55% (Figure 6). A similar pattern is found when the pactamycin-induced release of prebound *N*-acetyl-L-phenylalanyl-tRNA is studied. Streptomycin at low concentrations also interferes with this reaction but high levels of chlortetracycline are required for inhibition. The latter curve (Figure 6) is complicated at about 10^{-3} M chlortetracycline by precipitation of the antibiotic with magnesium. It may be significant that at 10^{-4} M chlortetracycline, a level sufficient to inhibit polyphenylalanine synthesis maximally (Suarez and Nathans, 1965), *N*-acetyl-L-phenylalanyl-tRNA binding is inhibited only 20%. Since at levels of 10^{-4} M chlortetracycline and greater the chelation of Mg^{2+} by the antibiotic (see Laskin, 1967) may significantly reduce the Mg^{2+} available for binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes, caution must be exercised in interpreting effects at such chlortetracycline concentrations.

Binding of N-Acetyl-L-phenylalanyl-tRNA to Ribosomes on Sucrose Gradients; Effect of Pactamycin. When the binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes is followed by sucrose density gradient analysis, it is found that both the optical density pattern and the sedimentation properties of ribosome-bound [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA depend upon the concentrations of Mg^{2+} and NH_4^+. Further, a time-dependent shift of radioactivity from lighter to heavier forms is noted. This shift is accentuated by low Mg^{2+} and high NH_4^+ concentrations. Pactamycin decreases the sedimentation velocity of the labeled "particles" and, at lower Mg^{2+} concentrations, further diminishes the stability of binding of the *N*-acetyl-L-phenylalanyl-tRNA so as to lead to its release.

In Figures 7–9 are shown sucrose gradient analyses of incubations carried out for different times at various Mg^{2+} and NH_4^+ levels. Comparison of the optical density patterns in these figures reveals that at 10 mM Mg^{2+} and 50 mM NH_4^+ the main peak is at 70 S (Figure 7), while at 10 mM Mg^{2+} and 160 mM NH_4^+ (Figure 8) or 5 mM Mg^{2+} and 50 mM NH_4^+ (Figure 9) the main peak is at 60 S. Even at 1-min incubation with [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA most of the radioactivity in Figure 7 is under the 70S peak, although some is found in the 60S region; with time the radioactivity shifts slightly to heavier forms. Although not shown in Figure 7, the radioactivity curve at 10 min falls between those at 1 and 60 min. At 10 mM Mg^{2+} and 160 mM NH_4^+ most of the [^{14}C]*N*-acetyl-L-

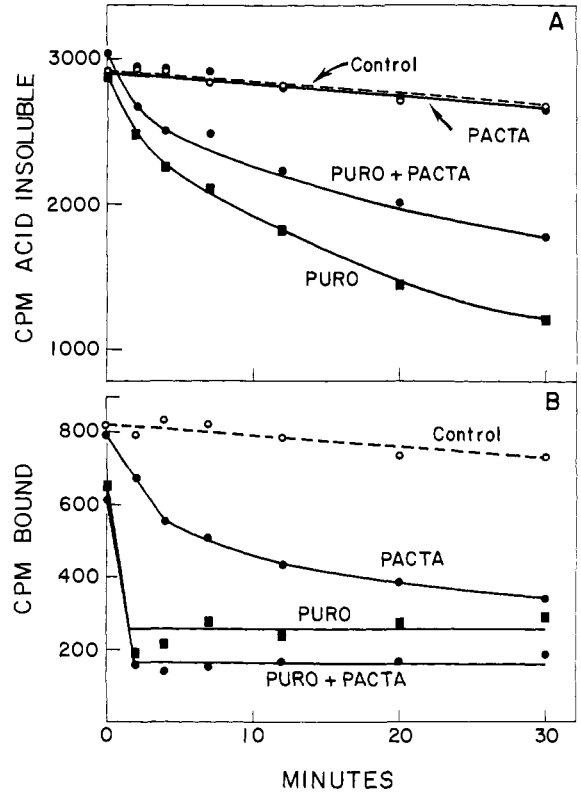

FIGURE 5: Effect of pactamycin and puromycin on *N*-acetyl-L-phenylalanyl-tRNA prebound to ribosomes. Ribosomes were preincubated with [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA for 10 min in 1.5-ml reaction mixtures as described in Figure 3. After the preincubation, aliquots (100 μl) were removed for determination of binding to ribosomes and radioactivity remaining precipitable by cold 5% trichloroacetic acid (zero time); pactamycin (2×10^{-5} M) and/or puromycin (4×10^{-4} M) were added and incubation was continued for the indicated times when 100-μl aliquots were removed for assay as described.

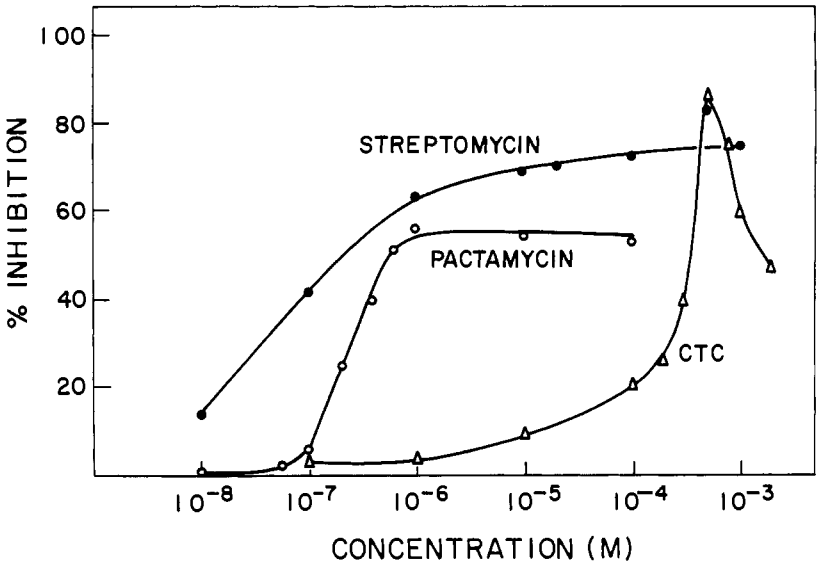

FIGURE 6: Concentration curves for pactamycin, streptomycin, and chlortetracycline. Incubation conditions for 100-μl samples for 15 min were as described in Figure 3. Pactamycin, streptomycin, and chlortetracycline were varied as indicated.

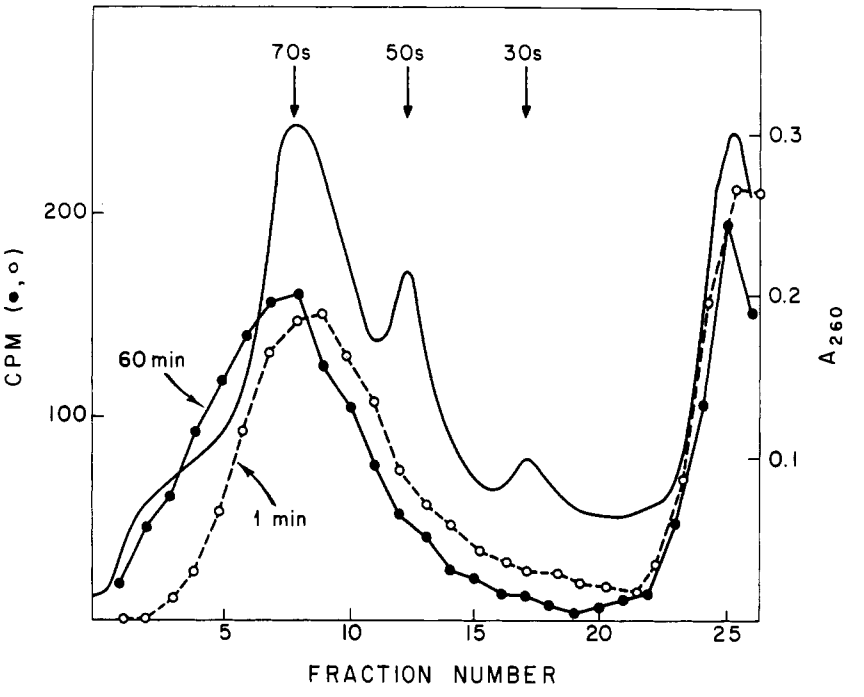

FIGURE 7: Binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes at 10 mM Mg^{2+} and 50 mM NH_4^+. Incubations (0.15 ml) were performed as described in Figure 3 except that each tube contained 3.2 A_{260} units of ribosomes, 10 mM Mg^{2+} and 50 mM NH_4^+. At the indicated times aliquots (0.1 ml) were applied to 5–20% sucrose gradients containing 10 mM Tris-HCl (pH 7.4), 10 mM magnesium acetate, and 50 mM NH_4Cl. Gradients were centrifuged at 41,000 rpm in the Spinco SW41 rotor for 3 hr at 4° and were analyzed for A_{260} and radioactivity as described in Materials and Methods. Since the A_{260} pattern was identical at both incubation times, only that at 60 min is shown.

phenylalanyl-tRNA is in the 50S region by 1 min but with further incubation becomes mainly 60 S with some radioactivity in the 70S region (Figure 8). The sedimentation distribution from 50 to 70 S of these *N*-acetyl-L-phenylalanyl-tRNA-bearing "particles" and the progression with incubation to more rapidly sedimenting forms suggests that they may represent stages in the maturation of the 30S–50S initiation complex prior to the onset of polypeptide synthesis. Since puromycin strips all the label from these "particles," 50S subunits are involved in such structures. Presumably at the earliest times, especially at Mg^{2+} and NH_4^+ concentrations which weaken the attraction between 30S and 50S subunits (Watson, 1964), such "particles"

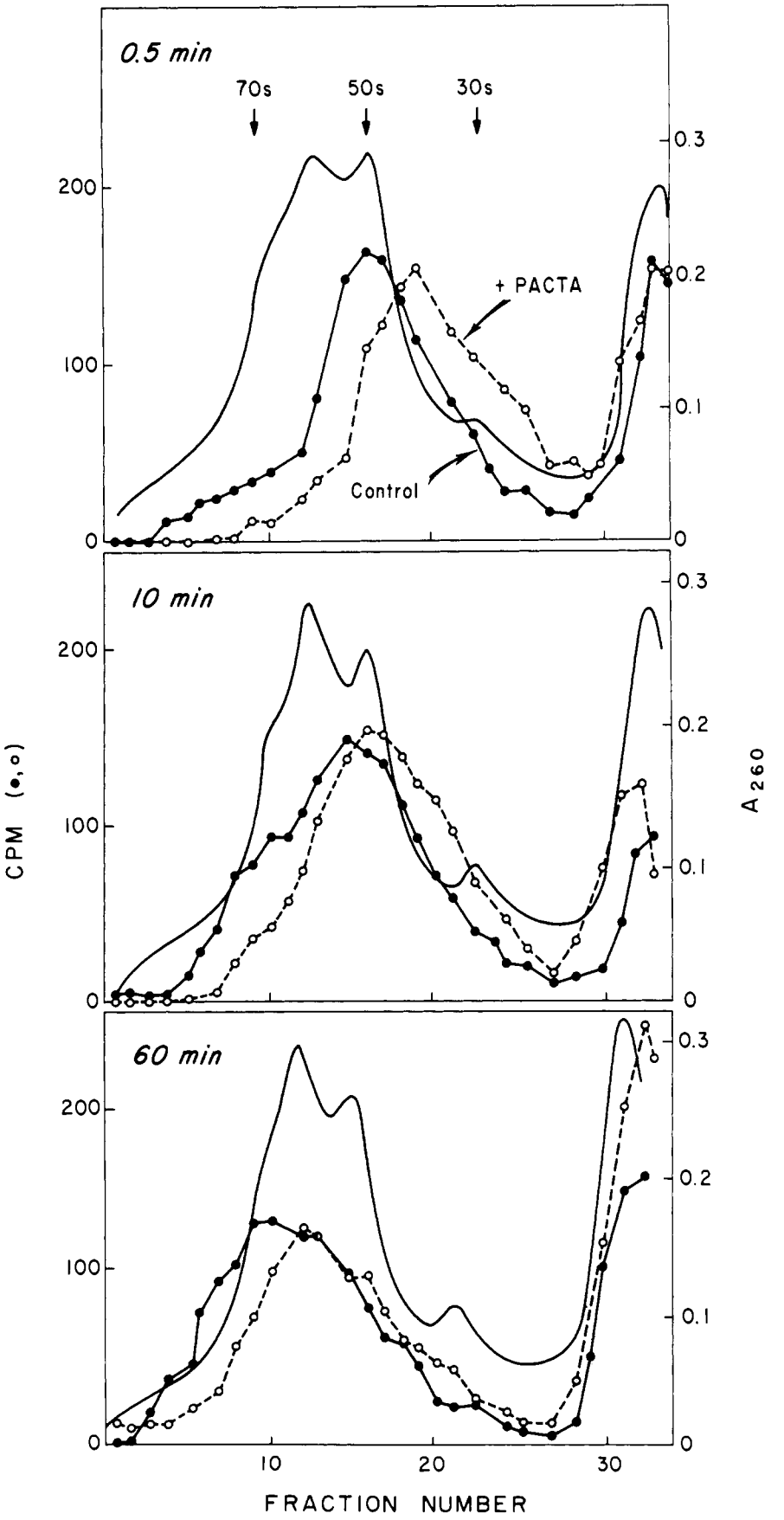

FIGURE 8: Effect of pactamycin on binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes at 10 mM Mg^{2+} and 160 mM NH_4^+. Incubation conditions and analysis were identical with those in Figure 7 except that the NH_4^+ concentration in the incubation mixtures and in the sucrose gradients was 160 mM. Incubations were carried out for 0.5, 10, and 60 min. Where indicated pactamycin was present at 5×10^{-5} M. The A_{260} pattern shown (control) was identical to that with pactamycin present.

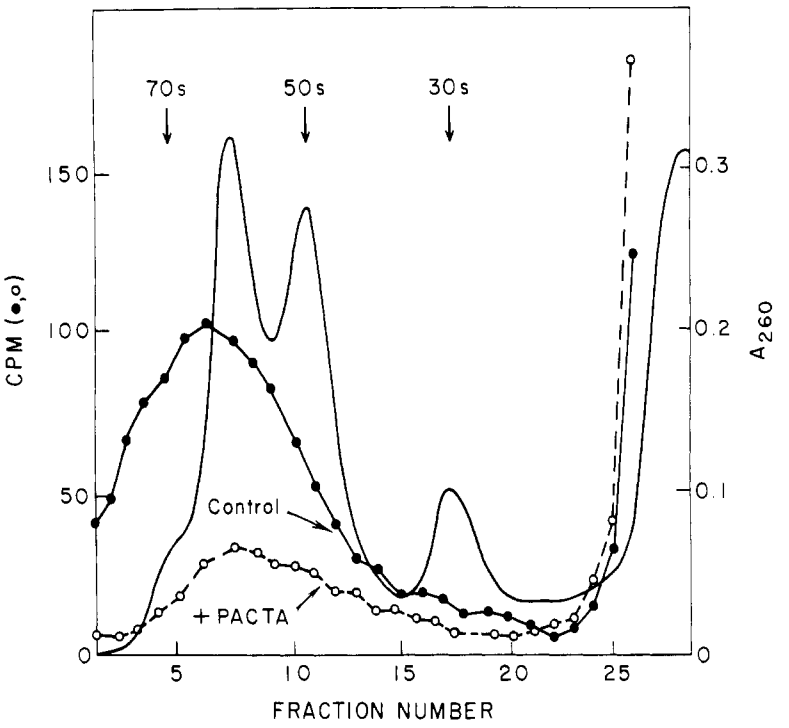

FIGURE 9: Effect of pactamycin on binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes at 5 mM Mg^{2+} and 50 mM NH_4^+. Incubation conditions and analysis were the same as in Figure 7 except that (1) each tube contained 2.4 A_{260} units of ribosomes, (2) the Mg^{2+} concentration in the incubation mixtures and in the sucrose gradients was 5 mM, (3) the reaction was initiated by addition of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA instead of Mg^{2+}, (4) incubation was for 40 min, and (5) an 0.13-ml aliquot was applied to each gradient. The A_{260} pattern shown (control) was identical with that with pactamycin (10^{-5} M) present.

(30S–50S complex) would have an unfolded, less compact structure in which the subunits have yet to assume their final relationship to each other and may reversibly disassociate during sedimentation. Since this sequence depends upon having *N*-acetyl-L-phenylalanyl-tRNA and poly U bound to the ribosomes (the A_{260} pattern does not change), these components are intimately involved in such changes in structure.

When pactamycin is included in the incubation, [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA is bound to "particles" sedimenting more slowly than the controls at each time point (Figures 8–10). At 10 mM Mg^{2+} the decrease in the amount of ribosome-bound *N*-acetyl-L-phenylalanyl-tRNA by pactamycin is relatively small (Figure 8). At 5 mM Mg^{2+} and 50 mM NH_4^+ pactamycin significantly decreases binding in the 60S to 80S region of the gradient (Figure 9). At 5 mM Mg^{2+} and 160 mM NH_4^+, however, much of the radioactivity is in the region of the 30S subunit; pactamycin decreases binding significantly and causes the radioactive peak to sediment at less than 30 S, suggesting that unbinding occurs during sedimentation (Figure 10). The lack of coincidence between the radioactive peaks and the A_{260} peaks indicates that only a fraction of the ribosomes bind [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA. It should be noted that the antibiotic affects only those ribosomes involved in the binding of *N*-acetyl-L-phenylalanyl-tRNA since at the concentrations of pactamycin used there is no alteration in the optical density pattern.

In other experiments the addition of pactamycin for 1 min after binding of the *N*-acetyl-L-phenylalanyl-tRNA is completed also causes similar shifts to more slowly sedimenting forms.

As was shown in the filter experiments, at high Mg^{2+} levels (20 mM) pactamycin has no effect on *N*-acetyl-L-phenylalanyl-tRNA binding (or on polyphenylalanine synthesis); the effect of the antibiotic on the sedimentation property of the *N*-acetyl-L-phenylalanyl-tRNA bearing particle is also eliminated (Figure 11). At this Mg^{2+} concentration the main A_{260} and radioactivity peaks are at 70 S.

Effect of Pactamycin on Binding of Poly U to Ribosomes. We have previously noted that pactamycin does not interfere with the nonenzymatic attachment of poly U to ribosomes (Cohen and Goldberg, 1967). This result is shown in Figure 12 where binding of [^{3}H]poly U to ribosomes at 5 mM Mg^{2+} and 160 mM NH_4^+ in the absence of GTP and factors is studied by sucrose density gradient centrifugation. The bound radioactivity appears as a sharp peak between the 30S and 50S areas and is not decreased by pactamycin. In the presence of GTP and factors (Figure 13), however, only very slight binding of poly U to ribosomes is found and this is unaffected by pactamycin. It is likely that the labeled poly U is rapidly degraded by nucleases contaminating the crude factor preparation. The addition of [^{12}C]*N*-acetyl-L-phenylalanyl-tRNA, however, has a marked stimulatory effect on poly U binding to ribosomes and the peak appears in the 30S–40S region

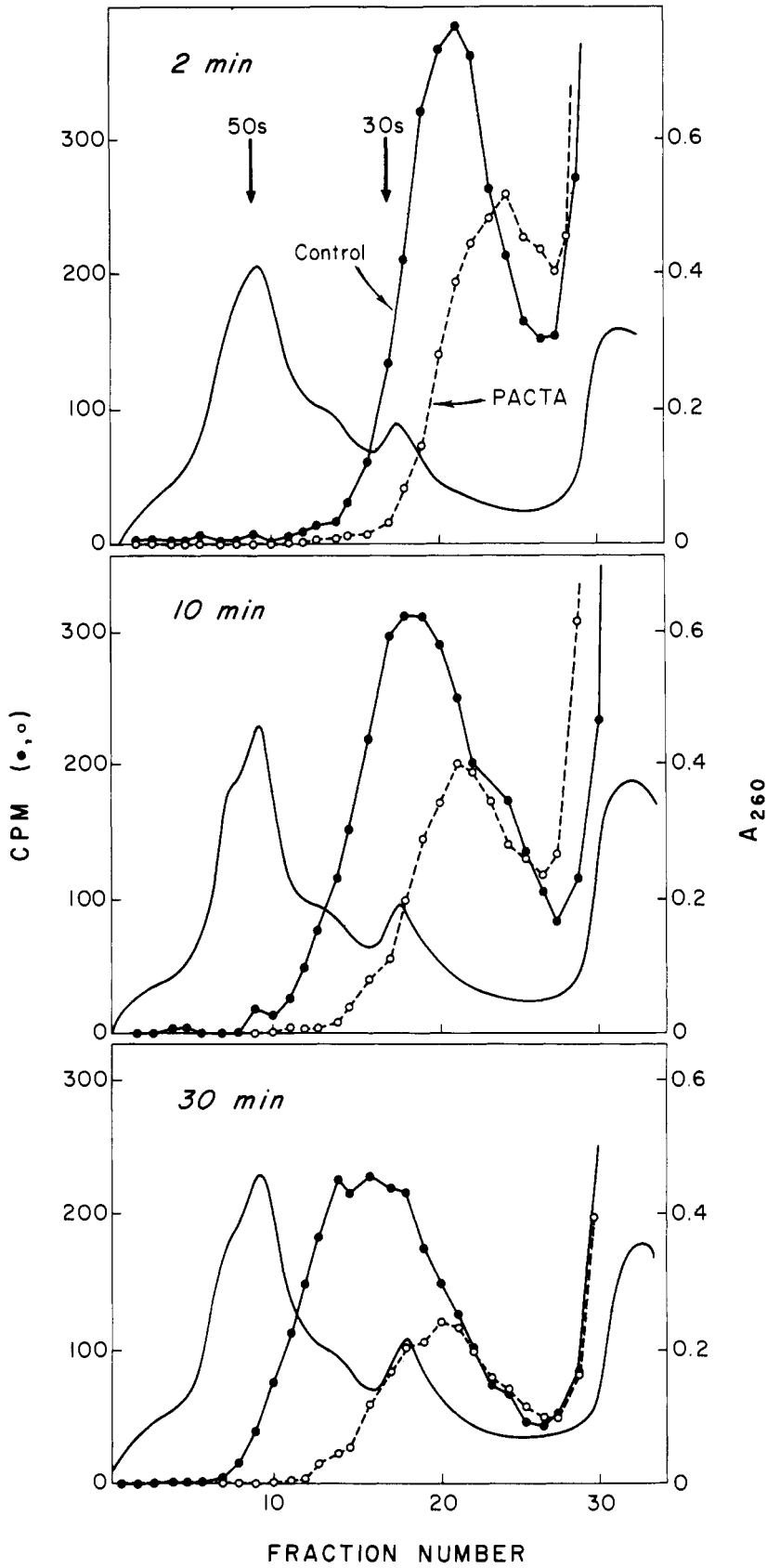

FIGURE 10: Effect of pactamycin on binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes at 5 mM Mg^{2+} and 160 mM NH_4^+. Incubation conditions and analysis were identical with those in Figure 8, except that (1) the Mg^{2+} concentrations in the incubation mixtures and sucrose gradients were 5 mM, (2) incubation volume was 0.2 ml, (3) incubation was for 2, 10, and 30 min, (4) a 0.15-ml aliquot was applied to each gradient, and (5) gradients were centrifuged for 3.5 hr. The A_{260} pattern shown (control) was identical with that with pactamycin present.

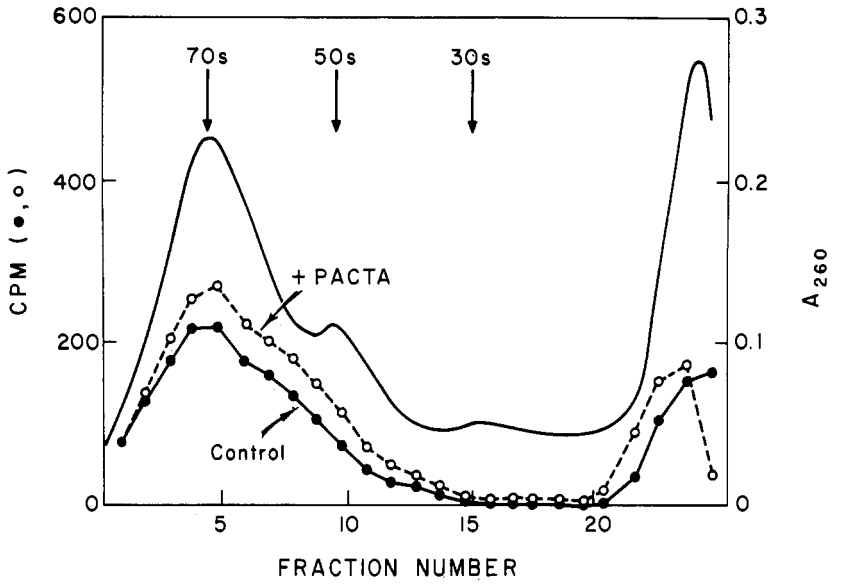

FIGURE 11: Effect of pactamycin on binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes at 20 mM Mg^{2+}. Incubation conditions and analysis were identical with those in Figure 8 except that (1) the Mg^{2+} concentration in the incubation mixtures and sucrose gradients was 20 mM, (2) incubation was for 5 min, and (3) gradients were centrifuged for 3 hr. The A_{260} pattern shown (control) was identical with that with pactamycin present.

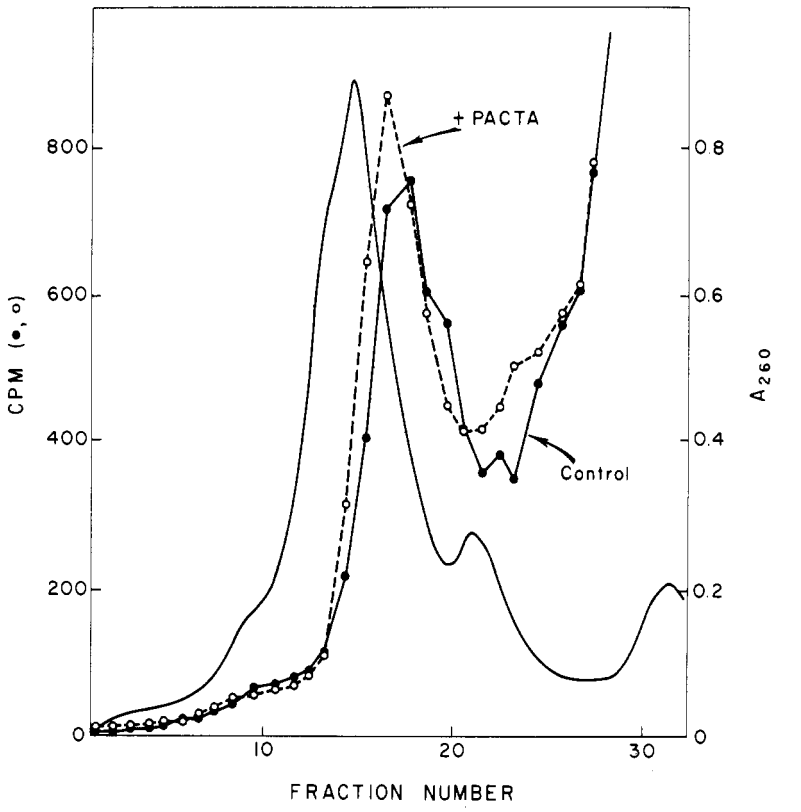

FIGURE 12: Effect of pactamycin on binding of [^{3}H]poly U to ribosomes in the absence of GTP and initiation factors. The standard reaction mixture was incubated for 20 min at 0° with or without 5×10^{-5} M pactamycin. Components of the mixture and conditions for gradient centrifugation and analysis are described in Materials and Methods. Gradients were centrifuged for 3.25 hr. The A_{260} pattern shown (control) was identical with that with pactamycin present.

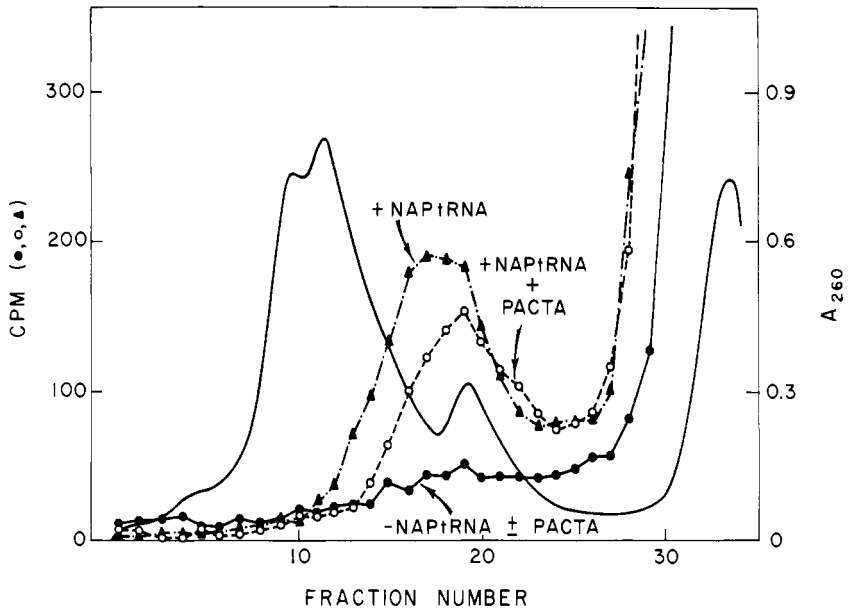

FIGURE 13: Effect of pactamycin on binding of [^{3}H]poly U to ribosomes in the presence of GTP and initiation factors: requirement for *N*-acetyl-L-phenylalanyl-tRNA. The standard reaction mixture containing 0.24 mM GTP and 32 μg of ribosomal wash protein was incubated for 2 min at 25° with or without 25.6 μg of [^{12}C]*N*-acetyl-L-phenylalanyl-tRNA and/or 5×10^{-5} M pactamycin. Conditions for assay and analysis are described in Materials and Methods. Gradients were centrifuged for 4 hr. The A_{260} pattern shown (control) was identical with that with pactamycin present.

at 5 mM Mg^{2+} and 160 mM NH_4^+. Unlike the *N*-acetyl-L-phenylalanyl-tRNA-independent binding of poly U, the *N*-acetyl-L-phenylalanyl-tRNA-dependent binding is reduced by pactamycin and the antibiotic causes the bound poly U to sediment more slowly.

The stimulation of poly U binding by *N*-acetyl-L-phenylalanyl-tRNA is specific, since neither stripped tRNA nor unacetylated, charged tRNA have any significant stimulatory effect (Figure 14). Further, this stimulation depends upon the presence of both factors and GTP. The binding which occurs in the absence of factors is not stimulated by *N*-acetyl-L-phenylalanyl-tRNA and/or GTP. Figure 15 shows that in the presence of factors *N*-acetyl-L-phenylalanyl-tRNA stimulates binding in the absence of GTP, but the stimulation is much more marked when GTP is present.

Discussion

The extent to which pactamycin inhibits polyphenylalanine synthesis or the binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes depends upon the concentration of Mg^{2+}. We have obtained similar results on the synthesis of polypeptides directed by other synthetic or natural mRNAs and on the binding of polylysyl-tRNA or *N*-formylmethionyl-tRNA to ribosomes (Cohen and Goldberg, 1967, and in preparation). The effect of pactamycin on the binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes can be correlated with the intrinsic stability of the ternary complex at the particular Mg^{2+} and NH_4^+ concentrations. This relationship may also account for the greater susceptibility to pactamycin of *N*-acetyl-L-phenylalanyl-tRNA bound in the presence of 5′-guanylylmethylenediphosphonate than that bound with GTP (Figure 3 and Herner *et al.*, 1969). Under the conditions described, the sensitivity to pactamycin of polyphenylalanine formation and *N*-acetyl-L-phenylalanyl-tRNA binding are parallel. It is possible, however, that even when the binding is not quantitatively affected, the altered initiation complex functions poorly in the subsequent steps involved in protein synthesis. Peptide-bond formation presumably remains intact as measured by the puromycin reaction, but steps involving relative motion of the subunits, as in translocation, might be affected.

The stability of the whole initiation complex is decreased by pactamycin, for the binding of poly U which is dependent upon the attachment of *N*-acetyl-L-phenylalanyl-tRNA is similarly affected. On the other hand, poly U bound to ribosomes in the absence of other components is insensitive to the antibiotic. While the factor-dependent binding of poly U to ribosomes resembles that recently described (Revel and Gros, 1967; Revel *et al.*, 1968; Brown and Doty, 1968), the requirement for concomitant binding of *N*-acetyl-L-phenylalanyl-tRNA is different and suggests that there is an inherent stability in the complete initiation complex which is lost when the initiator tRNA is absent. The latter may be especially important in the labile poly U–*N*-acetyl-L-phenylalanyl-tRNA–ribosome system and be highly susceptible to changes in Mg^{2+} concentration. Concomitant binding of *N*-acetyl-L-phenylalanyl-tRNA may protect the bound poly U from degradation by nucleases in the factor preparation, perhaps by inducing conformational changes in the ternary complex. The

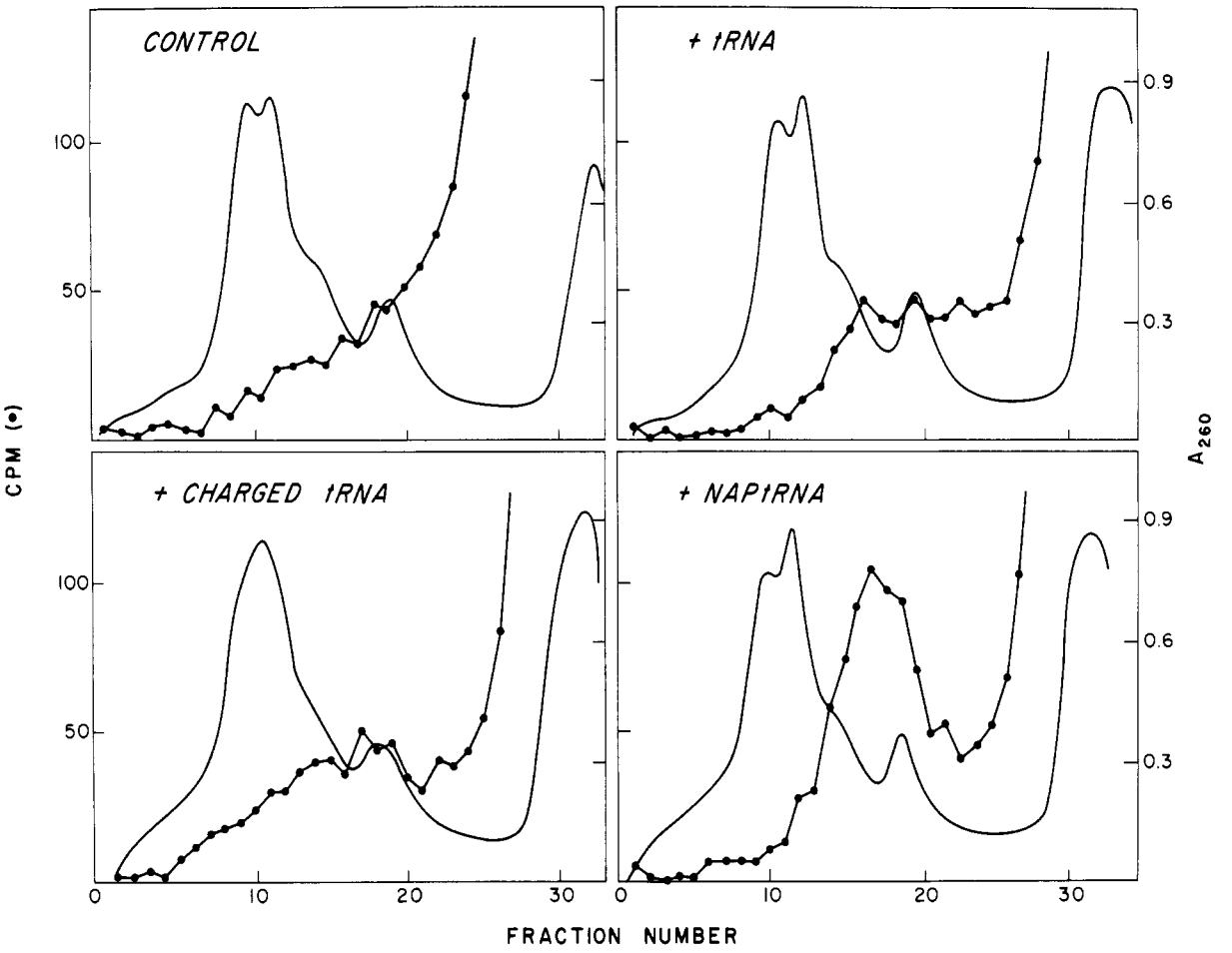

FIGURE 14: Specificity of *N*-acetyl-L-phenylalanyl-tRNA stimulation of binding of [^{3}H]poly U. The standard reaction mixture containing 0.24 mM GTP and 32 μg of ribosomal wash protein was incubated at 25° with the following additions as indicated: (A) none; (B) 30 μg of stripped *E. coli* tRNA; (C) 28.8 μg of *E. coli* tRNA charged with a mixture of 20 nonlabeled amino acids; (D) 25.6 μg of [^{12}C]*N*-acetyl-L-phenylalanyl-tRNA. Conditions for assay and analysis are described in Materials and Methods. Gradients were centrifuged for 4 hr.

need for all three components to form a complex stable to heat-induced dissociation has been reported by McLaughlin *et al.* (1966). The requirement for tRNA in the binding of mRNA resembles that recently reported by Iwasaki *et al.* (1968) except that in their work crude, uncharged tRNA (but not purified phenylalanyl-tRNA) was as effective as *N*-formyl-methionyl-tRNA in stimulating the factor-dependent binding of Q_β phage RNA to ribosomes. Further, in their system *binding* of initiator-tRNA appeared not to be required for mRNA binding, since the omission of one of the factors required for the former did not affect the latter.

Depending upon the mono- and divalent cation concentrations the bound [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA and the peaks of $A_{260\,m\mu}$ sediment on sucrose gradients between 30 and 70 S. In some cases these intermediate peaks may represent less compact 30S–50S ("open" 70S) ribosomal complexes such as have been postulated to be produced by various artificial means (Suzuka, 1967; Tamaoki and Miyazawa, 1967; Miyazawa *et al.*, 1968). On the other hand, especially in the case of radioactive peaks between 30 and 50 S, it appears likely that they do not represent discrete structures but, rather, result from the dissociation and reassociation of 70S ribosomes during sedimentation. Pactamycin, by further weakening the attraction between the subunits, results in less compact structures bearing *N*-acetyl-L-phenylalanyl-tRNA which dissociate into their subunits at an earlier point in sedimentation (Figure 8). At 5 mM Mg^{2+} and 160 mM NH_4^+, where there is little tendency for 30S and 50S subunits to stay together, pactamycin actually decreases *N*-acetyl-L-phenylalanyl-tRNA binding to the 30S subunit (Figure 10). The instability of binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes induced by pactamycin must result from an effect more fundamental than one solely affecting the ability of 30S and 50S subunits to form tight 30S–50S complexes (70S ribosomes), since the antibiotic complexes with isolated 30S ribosomes and affects the binding of *N*-acetyl-L-phenylalanyl-tRNA to these subunits (Cohen *et al.*, 1969). The structure of the 30S initiation complex itself must be altered in a way which affects both the binding of *N*-acetyl-L-phenylalanyl-tRNA and its ability to form a stable particle with the 50S subunit.

Several lines of evidence suggest that pactamycin inhibits polypeptide synthesis by affecting the formation

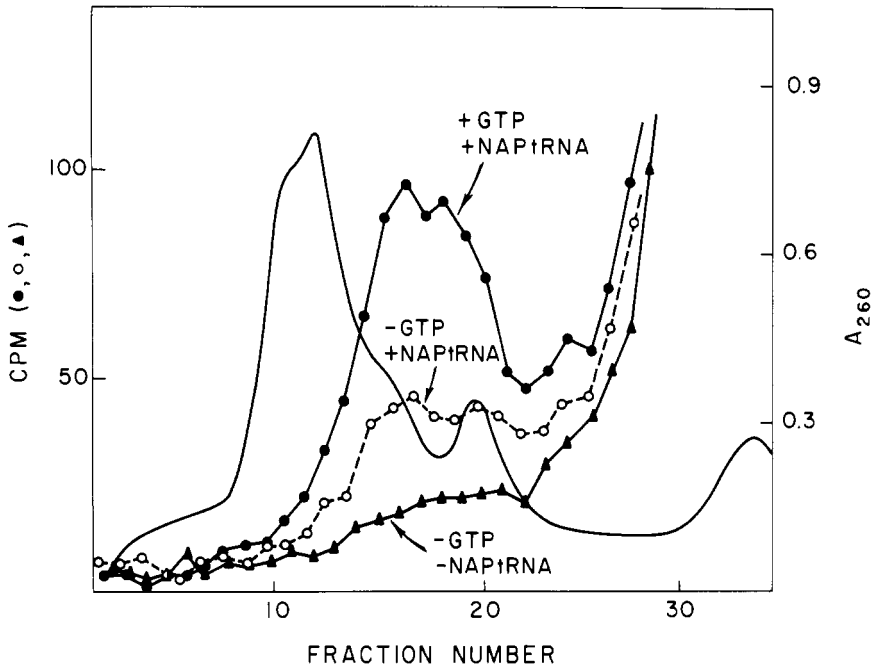

FIGURE 15: Requirement for GTP for *N*-acetyl-L-phenylalanyl-tRNA stimulation of [^{3}H]poly U binding. The standard reaction mixture contained, as indicated, 0.24 mM GTP and/or 25.6 μg of [^{12}C]*N*-acetyl-L-phenylalanyl-tRNA. Incubation was for 30 min at 25°. Assay and analysis conditions are described in Materials and Methods. Gradients were centrifuged for 4 hr.

of the initiation complex. (1) The experiments on polyphenylalanine synthesis at different Mg^{2+} concentrations in which either *N*-acetyl-L-phenylalanyl-tRNA or aminoacyl-tRNA are the initiators.[1] In each case, at high Mg^{2+} concentrations where initiation is by an "abnormal" mechanism and not rate limiting, the pactamycin effect on polypeptide formation is lost. These results explain the previously reported lack of inhibition of poly U dependent polyphenylalanine synthesis by pactamycin (Bhuyan, 1967). (2) The findings that the inhibition of polyphenylalanine formation by pactamycin is diminished by prior incubation with *N*-acetyl-L-phenylalanyl-tRNA and increased by prior incubation with antibiotic at Mg^{2+} levels where initiation is dependent upon *N*-acetyl-L-phenylalanyl-tRNA. (3) The delayed addition of pactamycin after completion of initiation has no effect on polypeptide elongation. (4) The experiments on the inhibition by pactamycin of the binding of initiator tRNA (*N*-acetyl-L-phenylalanyl-tRNA) and its mRNA to ribosomes at low Mg^{2+} concentrations by both filter and sucrose gradient analyses. (5) The alteration of the compact structure of the initiation complex as judged by centrifugation studies. (6) The report by Colombo *et al.* (1966) that pactamycin results in the degradation of reticulocyte polyribosomes to monomers suggesting that while the initiation complex may be fixed in an abnormal form, elongation and termination of the polypeptide chain remain intact. (7) The finding that pactamycin does not interfere with peptide-bond formation itself as indicated by the immunity of the puromycin reaction to its action. (8) Endogenous protein synthesis in which there is mainly completion of existing peptide chains is more resistant to the antibiotic than is that promoted by MS2 RNA (L. B. Cohen and I. H. Goldberg, manuscript in preparation).

Since pactamycin inhibits the binding of polylysyl-tRNA to ribosomes (Cohen and Goldberg, 1967), it is possible that with certain mRNAs the antibiotic can also release an elongating peptidyl-tRNA from the ribosome in addition to interfering with the initiation of polypeptide synthesis.

It is of considerable interest, although not studied in detail in this paper, that streptomycin is a highly effective inhibitor of *N*-acetyl-L-phenylalanyl-tRNA binding to ribosomes at low Mg^{2+} concentrations (Figure 6) and may also exert its effect on polypeptide synthesis by interfering with its initiation. A similar conclusion has been reached recently by Luzzatto *et al.* (1968).

Acknowledgment

We gratefully acknowledge the expert technical assistance of Miss Martha Trickey.

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[1] As expected, binding of aminoacyl-tRNA to ribosomes is partially inhibited by pactamycin at relatively low Mg^{2+} concentrations (L. B. Cohen and I. H. Goldberg, manuscript in preparation).

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