Inhibition by Pactamycin of the Initiation of Protein Synthesis. Effect on the 30S Ribosomal Subunit*

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

ABSTRACT: Pactamycin inhibits the guanosine triphosphate and factor-dependent binding of *N*-acetylphenylalanyl transfer ribonucleic acid to 30S ribosomal subunits. Inhibition is markedly decreased by high Mg^{2+} and low NH_4^+ levels, where binding is most stable.

Similarly pactamycin causes the release of prebound *N*-acetylphenylalanyl transfer ribonucleic acid at cation concentrations which favor release. Streptomycin at low concentrations and chlortetracycline at high concentrations also interfere with the binding reaction. Pactamycin inhibits binding to the 30S ribosomal subunit only, and does not affect the *increased* binding due to the addition of 50S subunits. Zonal centrifugation analyses of *N*-acetylphenylalanyl transfer ribonucleic acid bound to 30S subunits alone or with added 50S subunits show that (1) the factor-dependent binding to 30S subunits is relatively sensitive to inhibition by pactamycin, while the factor-independent binding is relatively resistant, unless the subunits are pretreated with pactamycin or 30S subunits which are prelabeled with *N*-acetylphenylalanyl transfer ribonucleic acid are incubated with pactamycin at low Mg^{2+} (5 mM), and (2) in the presence of 50S subunits the complexes bearing *N*-acetylphenylalanyl transfer ribonucleic acid sediment more slowly when pactamycin is present than when it is omitted. Spermidine (10 mM), which favors the formation of 70S ribosomes, overcomes the pactamycin effect on *N*-acetylphenylalanyl transfer ribonucleic acid binding. By both zonal centrifugation and precipitation of ribosomes with ethanol it has been found that [^{3}H]pactamycin binds specifically to 30S ribosomal subunits at 0° and is readily released by addition of unlabeled pactamycin. One molecule of pactamycin is bound for every three of the 30S subunits.

In the preceding paper (Cohen *et al.*, 1969a), it was shown that as a result of the interaction of pactamycin with *Escherichia coli* ribosomes to which *N*-acetyl-L-phenylalanyl-tRNA is bound, the stability of the initiation complex is decreased. Since the first steps in polypeptide synthesis involve the mRNA-dependent binding of initiator-tRNA to 30S ribosomal subunits (Nomura and Lowry, 1967; Ghosh and Khorana, 1967; Schlessinger *et al.*, 1967; Kaempfer *et al.*, 1968; Ohta *et al.*, 1967; Hille *et al.*, 1967), it was of interest to study the possible interaction of the antibiotic with the isolated 30S subunit. Evidence is presented that the initiation complex on the 30S ribosomal subunit is specifically affected by pactamycin.

Materials and Methods

Measurement of Binding of [^{14}C]*N-Acetyl-L-phenylalanyl-tRNA to Ribosomes.* The standard reaction mixture for measuring binding of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA to ribosomes contained the following: 50 mM Tris-HCl (pH 7.4), 160 mM NH_4Cl, 10 mM dithiothreitol, 40 μg of poly U/ml, 0.24 mM GTP, 41 $\mu\mu$moles of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA/ml (37,000 cpm), 160 μg of ribosomal wash fluid/ml as initiation factors, and the indicated amounts of magnesium acetate and A_{260} units of ribosomes. Reaction mixture volume was varied from 0.1 to 0.25 ml as indicated in each figure. Unless otherwise noted, incubation was carried out at 25° and reactions were initiated by the addition of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA followed immediately by magnesium acetate.

Incubation was 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 present in the incubation mixtures. The samples were immediately passed through Millipore filters as described by Nirenberg and Leder (1964). Radioactivity was determined in a Packard scintillation spectrometer at a counting efficiency of 88% for ^{14}C.

Preparation of Ribosomal Subunits. Ribosomes were prepared by the method of Ohta *et al.* (1967). Ribosomal subunits were prepared as follows. Ribosomes were dialyzed overnight against 10 mM Tris-HCl (pH 7.4)–0.5 mM magnesium acetate. Approximately 180A_{260} units of the dialyzed ribosomes was applied to a 30-ml linear sucrose gradient (5–20%) containing 10 mM Tris-HCl (pH 7.4) and 0.5 mM magnesium acetate. Gradients were centrifuged for 15 hr at 20,000 rpm in the SW25.1 rotor at 4°. Fractions comprising the descending portions of the 30S peak or the ascending portion of the 50S peak were pooled, adjusted to

* From the Department of Medicine, Harvard Medical School, and the Beth Israel Hospital, Boston, Massachusetts 02215. *Received October 3, 1968.* This work was supported by grants from the National Institutes of Health, U. S. Public Health Service (GM 12573 and CA 10736), and the American Cancer Society.

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TABLE I: Relation of NH_4^+ to pactamycin Inhibition of Binding of *N*-Acetyl-L-Phenylalanyl-tRNA to 30S Subunits at 10 mM Mg^{2+}.[a]

NH^+ (mM[4])	[^{14}C]*N*-Acetyl-L-phenylalanyl-tRNA Bound (cpm)			% Inhibition by Pactamycin
	Control	+Pactamycin	−Factors	
0	178	174	46	2
50	280	254	94	9
160	290	210	66	28

[a] The standard reaction mixture (0.1 ml) contained 0.19 A_{260} unit of repurified 30S ribosomes, 10 mM magnesium acetate, and 5×10^{-5} M pactamycin as indicated. The NH_4^+ concentration was varied as shown. Incubation was for 20 min. Samples were assayed for ribosomal-bound radioactivity as described in Materials and Methods.

10 mM Mg^{2+}, dialyzed against 10 mM Tris-HCl (pH 7.4)–10 mM magnesium acetate for several hours to remove the sucrose, and centrifuged at 50,000 rpm in the 50 Ti Spinco rotor for at least 12 hr at 4°. The resulting pellets were resuspended in 10 mM Tris-HCl (pH 7.4)–10 mM magnesium acetate and stored at 0°.

In some of the preparations a portion of the ribosomal subunits did not form a pellet but was located in a small volume of buffer immediately above the pellet. When this occurred the pellets and overlying buffer layers were homogenized together. Incomplete pelleting was largely overcome by maintaining the M^{2+} at 10 mM during the centrifugation.

Raising the Mg^{2+} concentration to 10 mM as soon as possible after separation of the subunits made it possible to keep them for at least 2 weeks without significant loss in activity. Contamination of 30S ribosomes by 50S ribosomes and of 50S ribosomes by 30S ribosomes was less than 3%. Repurified 30S ribosomes were prepared by subjecting the slightly contaminated 30S subunits to a second cycle of sucrose gradient centrifugation before adjusting the Mg^{2+} concentration to 10 mM and pelleting the ribosomes. These repurified 30S ribosomes were used in the experiment shown in Table I.

Sucrose Density Gradient Centrifugation Analyses. Linear (12-ml) sucrose gradients (5–20%) used in the analysis of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA and [^{3}H]pactamycin binding to ribosomes contained 10 mM Tris-HCl (pH 7.4) and the same concentration of NH_4Cl (160 mM) and magnesium acetate (5 or 10 mM) as present in the incubation mixture. After centrifugation at 41,000 rpm in a Spinco SW41 rotor at 4° for the times noted in the figures, drops were collected and assayed for radioactivity as previously described (Goldberg and Mitsugi, 1967). The counting efficiency for ^{3}H was 23%. Absorbance at 260 mμ was recorded automatically in the Gilford absorbance recorder.

Preparation of [^{3}H]Pactamycin. Pactamycin was catalytically exchanged with tritium (performed by Dr. R. O'Brien at the New England Nuclear Corp.). The product (4.7 Ci/mmole) was purified by two-dimensional thin-layer chromatography on an 8 × 8 in. 1000-μ silica gel G plate (Analtech) in the following solvent systems: first direction, ethyl acetate–acetone (2:1, v/v); second direction, butanol–water (84:16, v/v). Purity was verified on 2 × 8 in. 250-μ silica gel G plates in the following solvent systems: (a) butanol–10% formic acid (84:16, v/v); (b) butanol–methanol–10% citric acid (4:2:2, v/v). The [^{3}H]-pactamycin was diluted with unlabeled pactamycin to make a 10^{-5} M solution containing 1.5×10^6 cpm/ml.

Pactamycin Binding to Ribosomes. The conditions used for the binding of [^{3}H]pactamycin to ribosomes and subunits are described in the legends to the figures. [^{3}H]Pactamycin bound to ribosomes was determined in a procedure modified after Oleinick *et al.* (1968). Following incubation of a 0.1-ml sample in a 3-ml conical centrifuge tube, 1.5 ml of cold absolute alcohol containing NH_4Cl (0.04 M) was added to precipitate the ribosomes and the mixture was left at −20° overnight. After low-speed centrifugation for 10 min to collect the precipitated ribosomes, the supernatant was removed, and the ribosomes were resuspended in another 1.5 ml of the alcohol–NH_4Cl. The ribosomes were recentrifuged and the pellet was dissolved in 0.1 ml of 1 M NH_4OH. This solution and another 0.1 ml of 1 M NH_4OH used as a wash were transferred to a scintillation vial to determine radioactivity.

Materials. Pactamycin, chlortetracycline, and streptomycin were the gifts of the Upjohn Co., Lederle Laboratories, and Eli Lilly and Co., respectively. [^{14}C]*N*-Acetyl-L-phenylalanyl-tRNA (370 μCi/μmole) and initiation factors were prepared as previously described (Cohen *et al.*, 1969a).

Results

Effect of Mg^{2+} and NH_4^+ Concentrations on the Binding of N-Acetyl-L-phenylalanyl-tRNA to 30S Ribosomal Subunits and on Its Inhibition by Pactamycin. As shown in Figure 1, the poly U dependent binding of *N*-acetyl-L-phenylalanyl-tRNA to 30S ribosomes is a function of the Mg^{2+} concentration. It is very low at 5 mM Mg^{2+} and remains factor dependent at least up to 30 mM Mg^{2+}. Pactamycin inhibits binding at lower Mg^{2+} levels but, as the Mg^{2+} level is increased, the inhibition disappears. Free 50S ribosomal subunits fail to bind *N*-acetyl-L-phenylalanyl-tRNA at any Mg^{2+} concentration. The binding of *N*-acetyl-L-phenylalanyl-tRNA to 30S subunits and its inhibition

TABLE II: Effect of Mg^{2+}, NH_4^+, and Pactamycin on Prebound *N*-Acetyl-L-phenylalanyl-tRNA.[a]

		[^{14}C]*N*-Acetyl-L-Phenylalanyl-tRNA (cpm)					
		−Pactamycin		+Pactamycin		Release by Pactamycin	
Mg^{2+} (mM)	NH_4^+ (mM)	Bound	Released	Bound	Released	cpm	%
		313 (zero time)					
5	160	267	46	199	114	68	25
10	160	301	12	279	34	22	7
5	50	298	15	265	48	33	11
10	50	292	21	269	44	23	8

[a] Incubation conditions for prelabeling the 30S ribosomes (11.2 A_{260} units) and reisolation of these subunits are described in Figure 5. The 30S ribosomes (0.2 A_{260} units) containing 313 cpm were reincubated for 10 min at 25° (in a volume of 0.1 ml) with 50 mM Tris-HCl (pH 7.4) and the indicated concentrations of magnesium acetate and NH_4Cl with or without 5×10^{-5} M pactamycin. Samples were assayed for ribosomal-bound radioactivity as described in Materials and Methods.

TABLE III: Comparison of Effects of Pactamycin, Streptomycin, and Chlortetracycline on [^{14}C]*N*-Acetyl-L-phenylalanyl-tRNA Binding to 30S Ribosomal Subunits.[a]

Additions	[^{14}C]*N*-Acetyl-L-phenylalanyl-tRNA Bound (cpm)	% Inhibn
None	158	
Pactamycin	109	31
Streptomycin	25	84
Chlortetracycline	44	72

[a] The standard incubation mixture (25 μl) contained 10 mM magnesium acetate and 0.055 A_{260} unit of 30S ribosomes. Pactamycin and streptomycin were at 10^{-5} M; chlortetracycline was at 5×10^{-4} M. After 30 min at 25°, samples were assayed for ribosomal-bound radioactivity as described in Materials and Methods.

by pactamycin are also influenced by NH_4^+ concentration (Table I). At 10 mM Mg^{2+} significant pactamycin inhibition of binding to 30S subunits does not appear until levels of NH_4^+ over 50 mM are reached. A similar pattern of inhibition is found when 50S subunits are added to the 30S subunits in the binding reaction. These results resemble those with undissociated 30S–50S complexes reported in the previous paper (Cohen *et al.*, 1969a), except that at 5 mM Mg^{2+} the undissociated complexes bound *N*-acetyl-L-phenylalanyl-tRNA more efficiently than did the dissociated ribosomes.

Effect of Pactamycin, Mg^{2+}, and NH_4^+ on the Stability of Prebound N-Acetyl-L-phenylalanyl-tRNA. When 30S ribosomes to which *N*-acetyl-L-phenylalanyl-tRNA has been bound nonenzymatically (Lucas-Lenard and Haenni, 1968) are reisolated and reincubated at various Mg^{2+} and NH_4^+ levels, it is found that the *N*-acetyl-L-phenylalanyl-tRNA–ribosome complex is most stable at the higher Mg^{2+} and lower NH_4^+ concentrations (Table II), as observed for the 30–50S ribosomal complex (Herner *et al.*, 1969). Pactamycin enhances the spontaneous release of bound *N*-acetyl-L-phenylalanyl-tRNA and this labilizing effect is most pronounced at low Mg^{2+} and high NH_4^+ levels. The susceptibility to pactamycin, therefore, is inversely related to the stability of *N*-

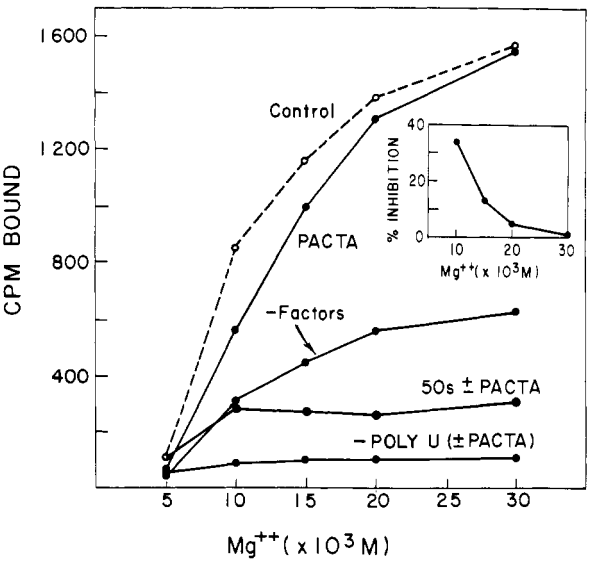

FIGURE 1: Mg^{2+} dependence of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA binding to ribosomal subunits. The standard reaction mixture (0.1 ml) contained 0.23 A_{260} unit of 30S ribosomes or 0.50 A_{260} unit of 50S ribosomes. The Mg^{2+} concentration was varied as indicated. Pactamycin when present was at 5×10^{-5} M. Binding was assayed as described in Materials and Methods.

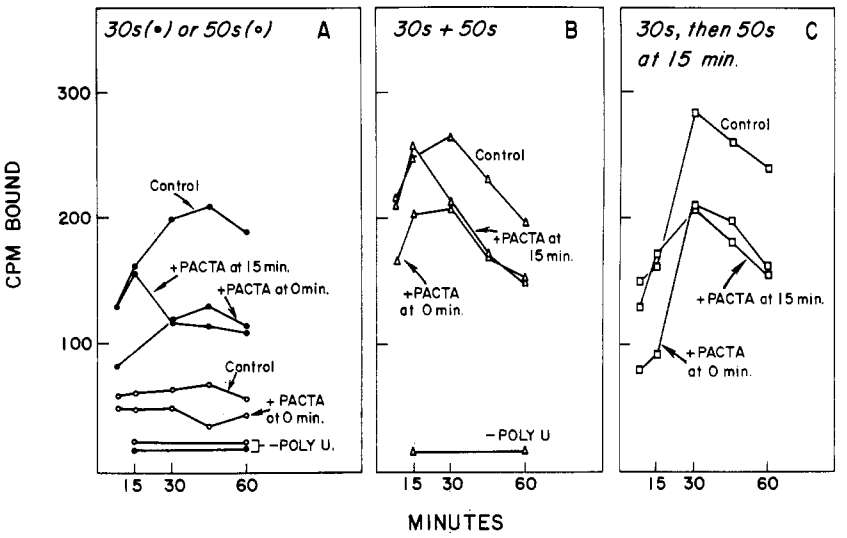

FIGURE 2: Effect of pactamycin on [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA to 30S subunits alone and on the binding stimulated by 50S subunits. The standard incubation mixture (0.15 ml) contained 10 mM magnesium acetate, 0.38 A_{260} unit of 30S ribosomes, 0.75 A_{260} unit of 50S ribosomes, and 5×10^{-5} M pactamycin as indicated. After 25-μl aliquots were removed at 8 and 15 min, 0.50 A_{260} unit of 50S ribosomes and/or 5 mμmoles of pactamycin were added in a volume of 10 μl. Samples (25 μl) were removed at the indicated times and assayed for ribosomal-bound radioactivity as described in Materials and Methods. Counts bound were corrected for the 10% dilution after the 15-min samples were taken.

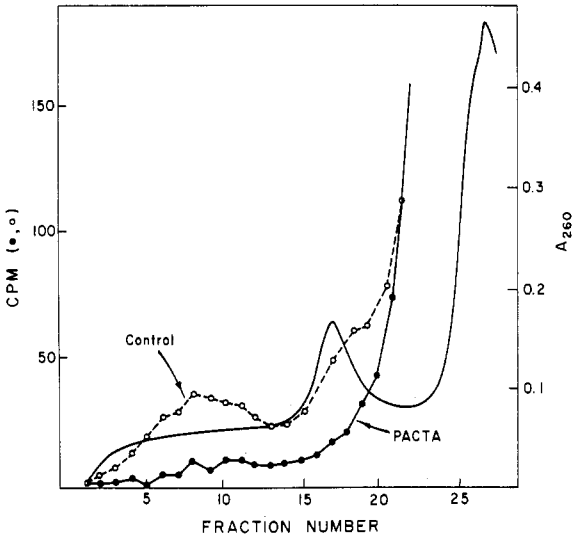

FIGURE 3: Effect of pactamycin on the factor-dependent binding of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA to 30S subunits. The standard reaction mixture (0.25 ml) contained 10 mM magnesium acetate, 0.61 A_{260} unit of 30S ribosomes, and 4×10^{-5} M pactamycin as indicated. Incubation was carried out for 20 min. A 170-μl aliquot of the incubation mixture was applied to a 5–20% sucrose gradient and centrifuged in the SW41 Spinco rotor at 41,000 rpm for 3 hr at 4°. The gradients were analyzed for A_{260} and radioactivity as described in Material and Methods.

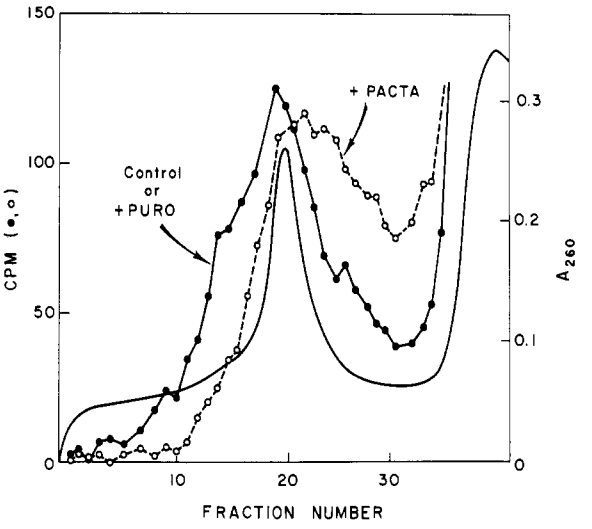

FIGURE 4: Effect of pactamycin on the factor-independent binding of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA to 30S ribosomes. In a volume of 0.2 ml, 30S ribosomes (1.41 A_{260} units) were preincubated for 5 min at 25° with 50 mM Tris-HCl (pH 7.4), 10 mM magnesium acetate, 160 mM NH_4Cl, 0.01 M dithiothreitol, 8 μg of poly U, 0.24 mM GTP, and 4×10^{-4} M puromycin or 10^{-4} M pactamycin as indicated. [^{14}C]*N*-Acetyl-L-phenylalanyl-tRNA (8.2 $\mu\mu$moles, 7400 cpm) was then added and incubation was continued for 10 min. A 175-μl aliquot of the incubation mixture was applied to a 5–2% sucrose gradient and centrifuged in the SW41 Spinco rotor at 41,000 rpm for 3.75 hr at 4°. The gradients were analyzed for A_{260} and radioactivity as described in Materials and Methods.

acetyl-L-phenylalanyl-tRNA binding in the absence of the antibiotic.

Comparison of Effects of Pactamycin, Streptomycin, and Chlortetracycline on N-Acetyl-L-phenylalanyl-tRNA Binding. In the previous paper (Cohen *et al.*, 1969a) streptomycin and chlortetracycline were shown to markedly inhibit binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes. Similarly these antibiotics interfere with the binding of *N*-acetyl-L-phenylalanyl-tRNA to 30S ribosomal subunits (Table III). While 10^{-6} M streptomycin inhibits maximally (about 80%), much higher

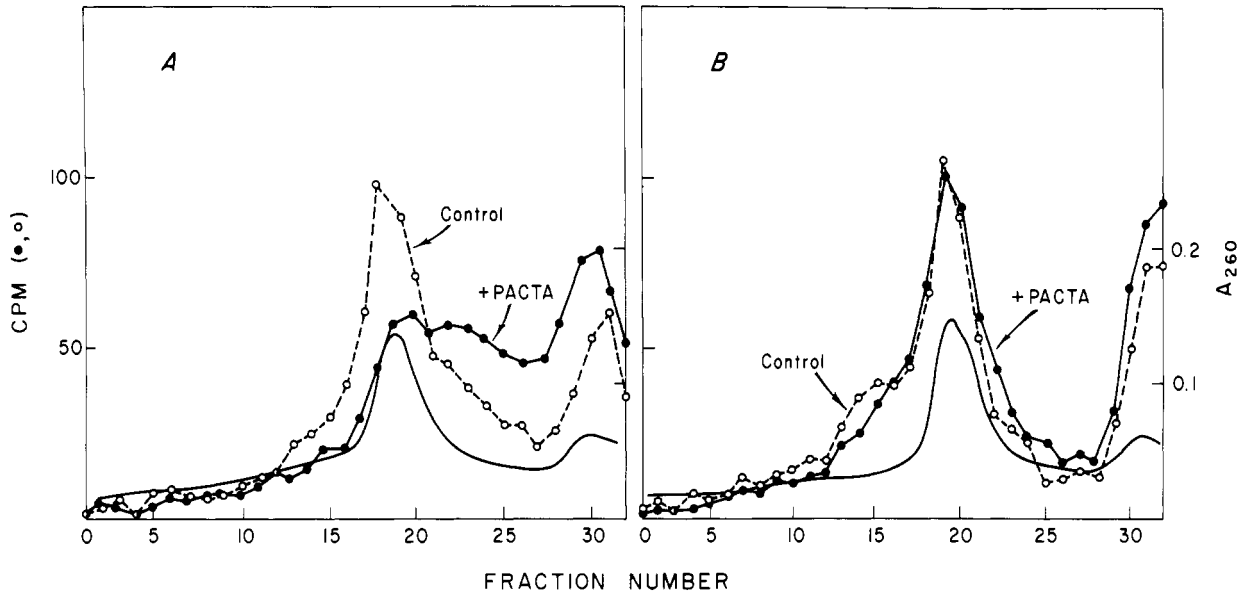

FIGURE 5: Effect of pactomycin on reincubation of prelabeled 30S ribosomes at 5 and 10 mM Mg^{2+}. 30S ribosomes (11.2 A_{260} units) were incubated with the following in a volume of 3.0 ml for 45 min at 35°: 50 mM Tris-HCl (pH 7.4), 160 mM NH_4Cl, 10 mM magnesium acetate, 10 mM dithiothreitol, 120 μg of poly U, 0.24 mM GTP, and 123 $\mu\mu$moles of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA (11.1 $\times$ 10^4 cpm). The mixture was diluted to 12 ml with 10 mM Tris-HCl (pH 7.4), 10 mM magnesium acetate, and 160 mM NH_4Cl, and centrifuged at 65,000 rpm for 3 hr at 4°. The resulting pellet was homogenized in 50 μl of buffer containing 10 mM Tris-HCl (pH 7.4) and 10 mM magnesium acetate. The isolated 30S ribosomes (0.6 A_{260} unit) containing 975 cpm were reincubated in a volume of 0.1 ml for 15 min at 0° (A) or 25° (B) with 50 mM Tris-HCl (pH 7.4), 160 mM NH_4Cl, and 5 or 10 mM magnesium acetate A and B, respectively) in the presence or absence of 5 $\times$ 10^{-5} M pactamycin. A 90-μl aliquot of the incubation mixture was applied to a 5–20% sucrose gradient and centrifuged in the SW41 Spinco rotor at 41,000 rpm for 3.50 hr at 4°. Gradients were analyzed for A_{260} and radioactivity as described in Materials and Methods.

concentrations of chlortetracycline ($>10^{-4}$) were needed for inhibition (see Cohen *et al.*, 1969a).

Lack of Effect of Pactamycin on the Binding of N-Acetyl-L-phenylalanyl-tRNA Stimulated by Addition of 50S Subunits to 30S Subunits. Figure 2 shows the results of an experiment designed to determine if pactamycin only inhibits binding to the 30S subunit alone or if it also affects the additional binding observed when 50S subunits are added to the 30S subunits. As seen in Figure 2A, when pactamycin is added after binding has been allowed to proceed for 15 min, there is a release of bound radioactivity and an inhibition of further binding so that after 30 min the amount of *N*-acetyl-L-phenylalanyl-tRNA bound is the same as when pactamycin is added at zero time (40% inhibition). Binding to the 50S particle is low and can be accounted for by 30S subunit contamination. When both subunits are present from the start, the results are similar to those with 30S subunits alone except that the presence of 50S subunits stimulates binding about 25% and the inhibition due to pactamycin is less, 20–25% (Figure 2B).

When 50S subunits are added to 30S ribosomes which have been allowed to bind *N*-acetyl-L-phenylalanyl-tRNA for 15 min, stimulation of binding is observed (125 cpm) (Figure 2C). Allowing for the increased binding which would be expected between 15 and 30 min for the 30S subunits alone (40 cpm), the actual stimulation of binding during this time period due to the addition of 50S particles is 85 cpm. When pactamycin is present from the beginning of the incubation, binding to the 30S subunit again is inhibited about 40%; upon addition of 50S subunits at 15 min, the increase in binding is *exactly* the same as in the control. When both 50S particles and pactamycin are added at 15 min, the bound radioactivity increases to the same level as when the antibiotic is present at zero time. The stimulatory effect of 50S subunits in the presence of pactamycin can be calculated as the difference between the counts bound at 15 min after the delayed addition of 50S particles and pactamycin (207 cpm) and those bound at 15 min after the delayed addition of pactamycin alone to 30S subunits (119 cpm). When this difference (88 cpm) is compared with the stimulatory effect of 50S particles in the absence of pactamycin (85 cpm), it is clear that the antibiotic does not inhibit the binding of *N*-acetyl-L-phenylalanyl-tRNA stimulated by the addition of 50S subunits.

Analysis of Factor-Dependent N-Acetyl-L-phenylalanyl-tRNA Binding to 30S Subunits by Sucrose Gradient Centrifugation. When the binding of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA to 30S ribosomes is analyzed by sucrose gradient centrifugation at 10 mM Mg^{2+} and 160 mM NH_4^+, it is found that, in the presence of initiation factors, the small amount of radioactivity bound appears as a low broad peak in the 40–60S region with a shoulder extending from the 30S area to the top of the gradient (Figure 3). This trailing of radioactivity is not observed in the absence of poly U or 30S subunits, suggesting that the shoulder is formed by the release of radioactivity from the 30S ribosomes during centrifugation. The results shown in Figure 3 resemble those described by Revel *et al.* (1968) with *N*-formylmethionyl-tRNA. The nature of the material sedimenting in the 40–60S region is not clear; it is not due to direct binding to

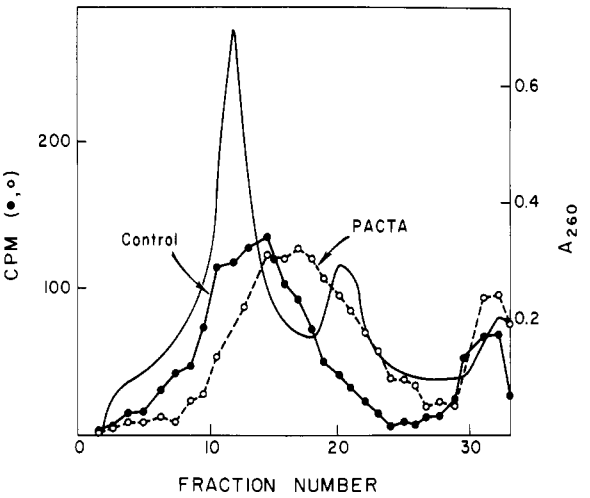

FIGURE 6: Effect of pactamycin on reincubation of prelabeled 30S ribosomes with 50S ribosomes. Incubation conditions for prelabeling the 30S ribosomes (14.1 A_{260} units) and reisolation of these subunits are described in Figure 5. 30S ribosomes (0.6 A_{260} unit) containing 1500 cpm were reincubated for 10 min at 25° (in a volume of 0.2 ml) with 50 mM Tris-HCl (pH 7.4), 10 mM magnesium acetate, 160 mM NH_4Cl, and 1.38 A_{260} units of 50S ribosomes in the presence or absence of 5×10^{-5} M pactamycin.

undetectable contaminating 50S subunits since the latter do not bind *N*-acetyl-L-phenylalanyl-tRNA as determined by centrifugation analysis. Further, none of the radioactivity along the gradient can be released by puromycin. It is possible that the more rapidly sedimenting material represents labeled multiples of 30S ribosomes bound to a single poly U molecule such as found by Pestka and Nirenberg (1966) with aminoacyl-tRNA bound to 30S ribosomes. Since the addition of poly U in great excess does not diminish the 40–50S peak singificantly, this explanation seems unlikely. Instead, this peak may represent aggregates of the whole initiation complex.

Pactamycin eliminates most of the bound *N*-acetyl-L-phenylalanyl-tRNA across the gradient (Figure 3). The small shoulder of radioactivity persisting at less than 30S probably represents some *N*-acetyl-L-phenylalanyl-tRNA released from the reversible complex during centrifugation. The almost complete release of bound radioactivity on the gradient by pactamycin contrasts with the filter assay and differs from the pattern of radioactivity observed at this same Mg^{2+} concentration (10 mM) with 30S–50S complexes (Cohen *et al.*, 1969a; see Figure 7A).

Gradient Analysis of Binding to 30S Ribosomes in the Absence of Factors. At 10 mM Mg^{2+} without factors *N*-acetyl-L-phenylalanyl-tRNA binds in a relatively sharp peak at the 30S region with a shoulder or tailing in the 40–45S region of the gradient (Figure 4). When the 30S ribosomes are first treated with pactamycin and binding of *N*-acetyl-L-phenylalanyl-tRNA allowed to take place in the absence of factors, the peak of radioactivity becomes broader, sediments more slowly, and merges with unbound radioactivity at the top of the gradient. If pactamycin is added at the same time as the *N*-acetyl-L-phenylalanyl-tRNA, a smaller effect is noted on the pattern of the labeled 30S subunits. Puromycin, as expected, has no effect on the *N*-acetyl-L-phenylalanyl-tRNA bound to 30S subunits.

Gradient analysis of 30S ribosomes which have been prelabeled with *N*-acetyl-L-phenylalanyl-tRNA, reisolated, and then incubated at 5 or 10 mM Mg^{2+} with or without pactamycin is shown in Figure 5. At 5 mM Mg^{2+} (Figure 5A), radioactivity is released from the particles both during the incubation and during the centrifugation. At 10 mM Mg^{2+} (Figure 5B), the only effect of pactamycin is a slight decrease in radioactivity in the 40–45S region of the gradient.

If the prelabeled 30S ribosomes are reincubated with 50S particles at 10 mM Mg^{2+} in the absence of factors, the radioactivity now appears as a broad peak in the 45S region with considerable tailing into heavier and lighter regions (Figure 6). Pactamycin causes the peak to be shifted toward the top of the gradient with no significant decrease in the bound radioactivity. The gradient pattern of the labeled 30S subunits incubated under the same conditions but without added 50S subunits is identical with that shown for the control in Figure 5B.

It should be noted that in contrast to sparsomycin (Herner *et al.*, 1969), the changes in gradient patterns found with pactamycin do not require that the antibiotic be present in the gradient, indicating that pactamycin binds more firmly.

Gradient Analysis of Binding in the Presence of 50S Subunits and Factors. At 10 mM Mg^{2+} and 160 mM NH_4^+-labeled *N*-acetyl-L-phenylalanyl-tRNA appears as a broad peak in the 50–70S region with a shoulder in the 30S area of the gradient when both factors and 50S subunits are included with the 30S ribosomes (Figure 7A). The position of the radioactive peak is close to that seen with 30S ribosomes alone (Figure 3). The size of the peak, however, is always much greater when 50S subunits are present (in contrast to the 25% stimulation observed in the filter assay, see Figure 2). The complete release of bound radioactivity by puromycin implies that the 50S subunit is involved in this labeled complex. As expected from the work of Schlessinger *et al.* (1967) the bulk of the 50S and 30S subunits stay separate and only the 30S subunits bearing *N*-acetyl-L-phenylalanyl-tRNA join with the 50S subunits.

Pactamycin causes the radioactive peak to sediment more slowly but does not interfere with the release of bound radioactivity by puromycin. At 50 mM NH_4^+ and 5 or 10 mM Mg^{2+} similar but lesser shifts in radioactive peaks are observed whether or not there is a decrease in over-all binding. When 10 mM spermidine is included in the incubation mixture and in the gradient at 10 mM Mg^{2+} and 160 mM NH_4^+, an optical density peak appears in the 70S region; radioactivity is found under this peak as well as in the pellet, and pactamycin is without effect (Figure 7B).

Binding of [^{3}H]Pactamycin to 30S Subunits. The binding of [^{3}H]pactamycin to ribosomal subunits was examined by ethanolic precipitation of the ribosomes (Figure 8) and by sucrose density gradient centrifugation (Figure 9). Only the 30S subunits complex with

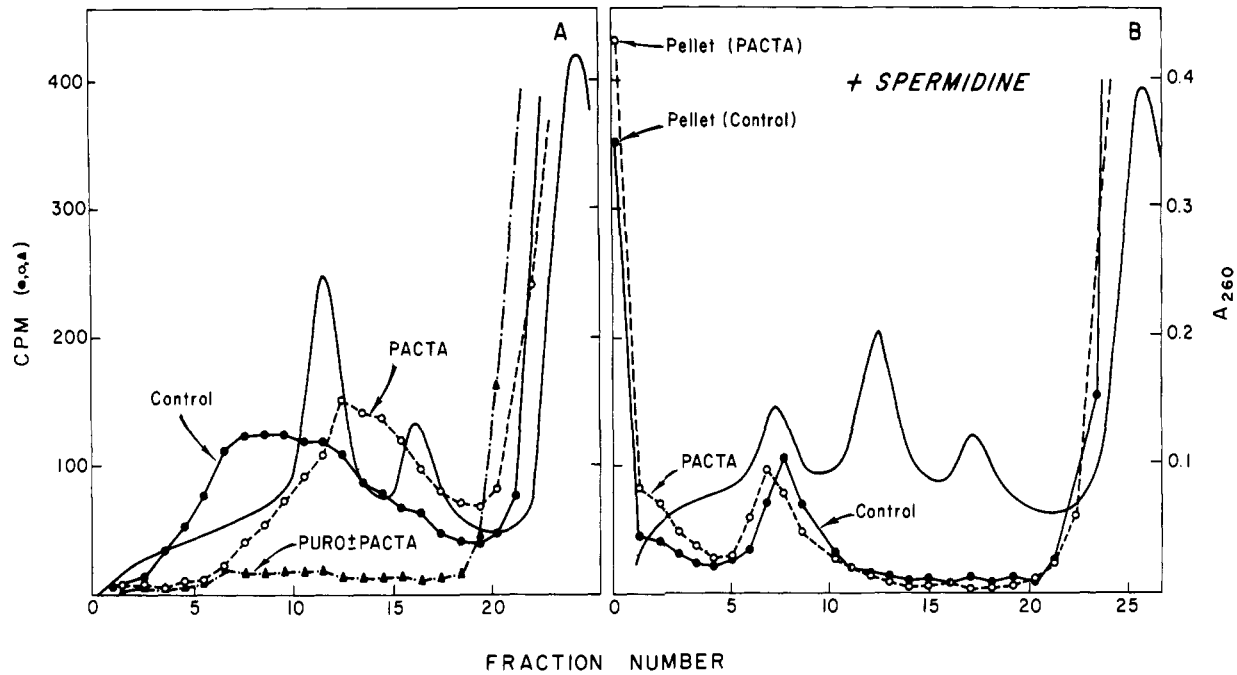

FIGURE 7: Effect of pactamycin on [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA binding to 30S plus 50S ribosomes in the presence and absence of spermidine. The standard reaction mixture (0.2 ml) contained 10 mM magnesium acetate, 0.45 A_{260} unit of 30S ribosomes, 0.90 A_{260} unit of 50S ribosomes, and pactamycin (5×10^{-5} M), puromycin (4×10^{-4} M), or 10 mM spermidine as indicated. Incubation was carried out for 20 min. A 175-μl aliquot of the incubation mixture was applied to a 5–20% sucrose gradient and centrifuged in the SW41 Spinco rotor at 41,000 rpm for 3 hr at 4°. The incubation mixture containing spermidine was layered onto a gradient containing 10 mM spermidine. The gradients were analyzed for A_{260} and radioactivity as described in Materials and Methods.

[^{3}H]pactamycin; the small amount of radioactivity associated with the 50S subunit preparation is due to contaminating 30S subunits. As expected, binding of [^{3}H]pactamycin to a preparation of dissociated ribosomes is about one-third that with the 30S subunits based on optical density. In other experiments it was shown that binding occurs rapidly at 0°.

A similar pattern is found by sucrose density gradient analysis where 50S subunits do not bind at all and the radioactivity peaks over the tail of the 30S subunit optical density profile, indicating that only a portion of the subunits is involved. The ready reversibility of the complex is indicated by the immediate release at 0° of radioactive pactamycin by an excess of unlabeled antibiotic.

A detailed analysis of the nature of the binding of pactamycin to 30S subunits is currently underway.

Discussion

At 5 mM Mg^{2+} in the presence of initiation factors *N*-acetyl-L-phenylalanyl-tRNA binds much more poorly to 30S subunits than to 30S–50S complexes (Cohen *et al.*, 1969a). At 10 mM Mg^{2+} binding to 30S subunits is much better although still sufficiently labile so as to result in dissociation during zonal centrifugation. It is of interest that a considerable portion of the *N*-acetyl-L-phenylalanyl-tRNA bound in the presence of initiation factors appear to be on aggregates of the 30S initiation complex, while in the absence of factors practically all the *N*-acetyl-L-phenylalanyl-tRNA is on 30S monomers. The inclusion of pactamycin in the former incubation increases the lability of binding strikingly (as measured by zonal centrifugation), while having little effect on the binding in the absence of factors, unless the 30S subunits are pretreated with the antibiotic or the Mg^{2+} concentration is lowered after binding. Addition of 50S subunits to both types of incubations results in an increase in the sedimentation of the bound *N*-acetyl-L-phenylalanyl-tRNA, presumably, by forming 30S–50S complexes. Since aggregates of *N*-acetyl-L-phenylalanyl-tRNA bearing 30S subunits do not occur in the absence of factors, it is probable that the labeled 50S particles formed on adding 50S subunits represent "open" or dissociating and associating forms of the 30S–50S complex (see Cohen *et al.*, 1969a). In both cases pactamycin results in more slowly sedimenting "particles" bearing *N*-acetyl-L-phenylalanyl-tRNA. Thus it appears that while the inclusion of 50S subunits in the incubation with factors has almost completely prevented the release by pactamycin of *N*-acetyl-L-phenylalanyl-tRNA from the 30S subunits, the 30S–50S complex so formed does not have its normal compact structure in the presence of the antibiotic. In the presence of spermidine or high Mg^{2+} concentrations, conditions which enhance the formation of 70S ribosomes, this effect of pactamycin is overcome.

That pactamycin specifically affects the 30S subunit is also indicated by the experiments in which the additional binding due to added 50S subunits is not affected by the antibiotic. At 10 mM Mg^{2+} addition of 50S subunits increases factor-dependent binding to 30S subunits by about 25%. It is not known whether

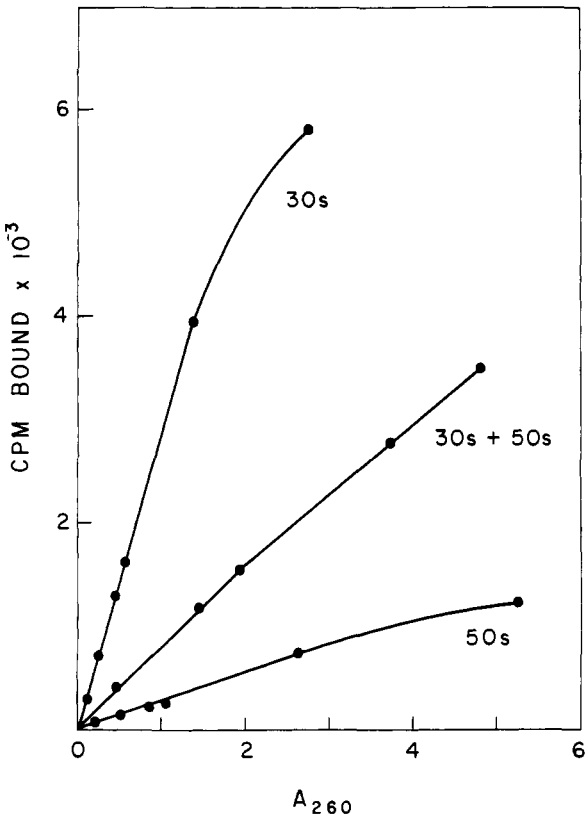

FIGURE 8: Binding of [^{3}H]pactamycin to dissociated ribosomes and ribosomal subunits. [^{3}H]Pactamycin (10^{-6} M, 1.5×10^4 cpm) was incubated with ribosomal subunits (30 or 50 S) or with dissociated ribosomes (dialyzed overnight against 0.5 mM Mg^{2+} and 10 mM Tris-HCl (pH 7.4)) in 50 mM Tris-HCl (pH 7.4), 10 mM dithiothreitol, 0.24 mM GTP, and 4 μg of poly U at 25° for 10 min in a total volume of 0.1 ml. The concentration of magnesium acetate was at 0.5 mM with the subunits and 5mM with the dissociated ribosomes. Ribosomes or subunits were precipitated and washed with alcohol–NH_4Cl, suspended in aqueous NH_4OH, and bound radioactivity was determined by liquid scintillation counting as described in Materials and Methods.

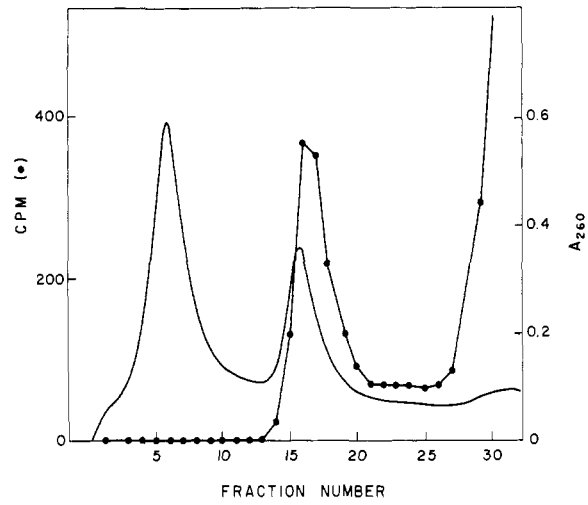

FIGURE 9: Sucrose gradient centrifugation analysis of binding of [^{3}H]pactamycin to ribosomal subunits. The following were incubated for 5 min at 25° in a volume of 0.1 ml: 50 mM Tris-HCl (pH 7.4), 10 mM dithiothreitol, 160 mM NH_4Cl, 5 mM magnesium acetate, 3.4 A_{260} units of ribosomes (dialyzed overnight against 0.5 mM magnesium acetate and 10 mM Tris-HCl (pH 7.4)), and 10^{-6} M [^{3}H]pactamycin (11,750 cpm). A 90-μl aliquot of the incubation mixture was applied to a 5–20% sucrose gradient and centrifuged in the SW41 Spinco rotor at 41,000 rpm for 4 hr at 4°. The gradient was analyzed for A_{260} and radioactivity as described in Materials and Methods.

this additional binding represents binding to a new site created by inclusion of the 50S subunit, as has been postulated for aminoacyl-tRNA (Suzuka *et al.*, 1966). While its insensitivity to pactamycin suggests that this may be so, other explanations are also possible. For example, it is possible that the increased binding of *N*-acetyl-L-phenylalanyl-tRNA with added 50S subunits is due to a protective effect against deacylation such as that described by Pestka (1967) for aminoacyl-tRNA.

Radioactive pactamycin binds specifically to 30S ribosomal subunits. The finding that the peak of radioactive pactamycin does not coincide exactly with the 30S A_{260} pattern on the sucrose gradient suggests that binding takes place only on certain 30S subunits. Further, it can be calculated from Figures 8 and 9 that one molecule of pactamycin is bound for about every three of the 30S subunits.

It is of interest that both streptomycin (Kaji and Tanaka, 1968) and pactamycin bind to the 30S ribosomal subunit and at low concentrations inhibit binding of *N*-acetyl-L-phenylalanyl-tRNA to these subunits. Evidence has been presented that both antibiotics interfere with polypeptide chain initiation (Luzzatto *et al.*, 1968; Cohen *et al.*, 1969a). Unlike streptomycin, however, pactamycin does not induce misreading of the mRNA at any Mg^{2+} concentration (L. B. Cohen and I. H. Goldberg, manuscript in preparation).

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Stabilization of *N*-Acetylphenylalanyl Transfer Ribonucleic Acid Binding to Ribosomes by Sparsomycin*

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

ABSTRACT: The over-all binding of *N*-acetylphenylalanyl transfer ribonucleic acid to ribosomes, which is dependent upon initiation factors and guanosine triphosphate, is markedly increased by sparsomycin (half-maximal effect at 10^{-7} M). Since the initial rate of binding is not affected, it appears that sparsomycin stabilizes the initiation complex. Binding with 5′-guanylylmethylenediphosphonate is less stable than that with guanosine triphosphate but is also completely stabilized by sparsomycin. Sparsomycin blocks the pactamycin-induced dissociation of the initiation complex; 1 molecule of *N*-acetylphenylalanyl transfer ribonucleic acid is bound for every 6 ribosomes in the presence of sparsomycin and for every 18 ribosomes in its absence. While the sparsomycin effect is most pronounced at low Mg^{2+} and high NH_4^+ concentrations where the initiation complex is more labile, stabilization of *N*-acetylphenylalanyl transfer ribonucleic acid binding to ribosomes and inhibition of the puromycin reaction by sparsomycin are independent of the cation concentrations. Stabilization of binding by sparsomycin requires the addition of the 50S ribosomal subunit to the 30S subunit and is associated with the formation of 70S ribosomes. Antibiotics such as gougerotin and chloramphenicol, which act on the 50S subunit, exhibit qualitatively similar actions, but at much higher concentrations, and compete with sparsomycin for this effect. Sparsomycin may act to fix the transfer ribonucleic acid in the peptidyl site either by changing the site or by interfering with the system (peptidyl transferase) which transfers it from this site for peptide-bond formation.

Sparsomycin, a sulfur-containing antibiotic, is a selective and potent inhibitor of polypeptide synthesis by cells and extracts of bacterial and mammalian origin (Slechta, 1965; Goldberg and Mitsugi, 1966, 1967a,b; Colombo *et al.*, 1966; Trakatellis, 1968). This antibiotic has been found to block peptide-bond formation at or close to the site of the peptide synthesis on the *Escherichia coli* 50S ribosomal subunit (Goldberg and Mitsugi, 1967b; Jayaraman and Goldberg, 1968; Monro and Vazquez, 1967; Lucas-Lenard and Haenni, 1968).

In the course of experiments on the mechanism of action of pactamycin, an antibiotic which interferes with the binding of peptidyl-tRNA to ribosomes (Cohen and Goldberg, 1967; Cohen *et al.*, 1969a,b), it was found, by contrast, that sparsomycin markedly stabilizes the binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes. While *N*-acetyl-L-phenylalanyl-tRNA binds to the 30S ribosomal subunit (Lucas-Lenard and Haenni, 1968; Cohen *et al.*, 1969b), stabilization of binding by sparsomycin requires the addition of the 50S subunit and leads to the formation of 70S ribosomes. Other antibiotics such as gougerotin and chloramphenicol exhibit qualitatively similar actions, but at much higher concentrations, and can be shown actually to compete with sparsomycin for this effect. It is suggested that the ability of sparsomycin to stabi-

* From the Department of Medicine, Harvard Medical School, and the Beth Israel Hospital, Boston, Massachusetts 02215. *Received October 3, 1968.* This work was supported by grants from the National Institutes of Health, U. S. Public Health Service (GM 12573 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).

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