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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-

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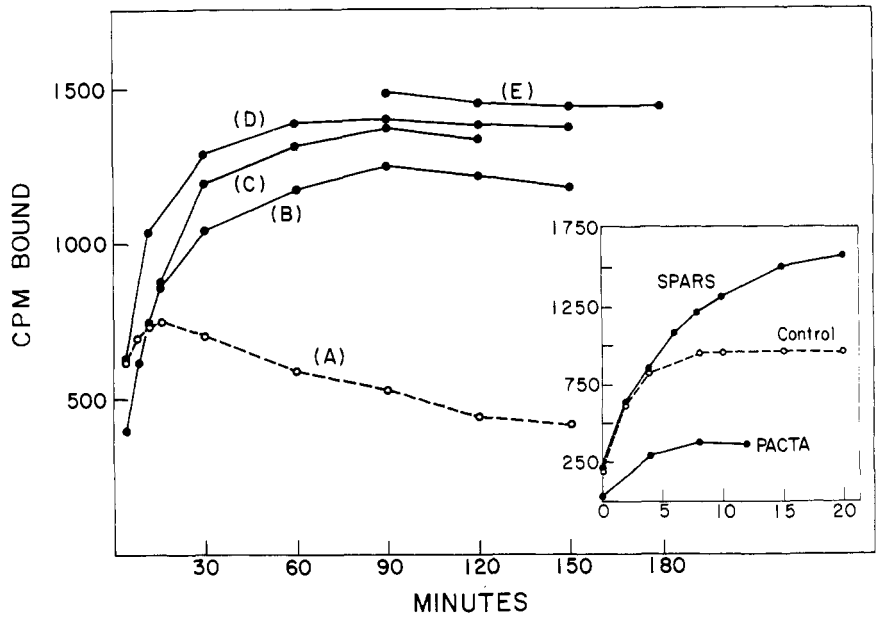

FIGURE 1: Sparsomycin stimulation of binding of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA to ribosomes. The incubation mixture contained the following in a volume of 0.5 ml: 50 mM Tris-HCl (pH 7.4), 160 mM NH_4Cl, 10 mM dithiothreitol, 20 μg of poly U, 0.24 mM GTP, 4.8 mM magnesium acetate, 6.8 A_{260} units ribosomes, 80 μg of ribosomal wash protein, and 1.85×10^4 cpm or 20.5 $\mu\mu$moles of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA. After the indicated times at 25°, 50-μl aliquots were removed and binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes was determined by the procedure of Nirenberg and Leder (1964) on Millipore filters as indicated in Materials and Methods. Curve A: control; curve B: sparsomycin (10^{-5} M) and pactamycin (10^{-5} M) at zero time; curve C: sparsomycin (10^{-5} M) added at 15 min; curve D: sparsomycin (10^{-5} M) at zero time; curve E: sparsomycin (10^{-5} M) at zero time and pactamycin at 90 min. Insert: same as above except that 100-μl aliquots were removed.

lize binding to the peptidyl site on the ribosomes may result from its interaction with the peptidyl transferase on the 50S ribosomal subunit.

Materials and Methods

Preparation of ribosomes, ribosomal subunits, initiation factors and [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA; sucrose density gradient centrifugation; and determination of acid-insoluble radioactivity are described elsewhere (Cohen *et al.*, 1969a,b). 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 1. 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 was determined in a Packard scintillation spectrometer.

Contamination of 30S ribosomes by 50S ribosomes and of 50S ribosomes by 30S ribosomes was less than 3%.

The nature of the radioactive material bound to ribosomes in the presence or absence of sparsomycin was analyzed by the procedure of Lucas-Lenard and Lipmann (1967) as described in a previous paper (Cohen *et al.*, 1969a). All the bound radioactivity was found to be *N*-acetyl-L-phenylalanyl-tRNA; no phenylalanyl-tRNA or oligopeptides were detected.

Erythromycin was obtained from Eli Lilly and Co.; sparsomycin, gougerotin, and chloramphenicol were gifts, respectively, of the Upjohn Co., Takeda Chemical Industries, and Park Davis and Co. Other antibiotics and materials were obtained as described in a previous paper (Cohen *et al.*, 1969a).

Results

Stimulation of N-Acetyl-L-phenylalanyl-tRNA Binding to Ribosomes by Sparsomycin. As shown in Figure 1 (curve A and control in insert), the binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes reaches its maximum by about 10 min, is maintained at this level for another 10 min, and then gradually declines over the next several hours. If sparsomycin has been included in the reaction, however, binding continues to increase up to 1 hr and remains fixed at this level until at least 2.5 hr (curve D). As is evident in the insert to Figure 1, the initial rate of binding is not increased by sparsomycin but increasing binding continues for a longer time and is maintained at this high level. These results suggest that sparsomycin exerts its effect on binding by stabilizing the initiation complex, consisting of ribosome, *N*-acetyl-L-phenylalanyl-tRNA, and poly U. Comparison of the maximum level of binding in the control (at 15 min) with the highest determined binding in the presence of sparsomycin (90 min) shows the latter to be higher by a factor of almost 2. If the addition of sparsomycin is delayed until the control has reached its peak (15 min) the stimulation observed is almost equal to that found when sparsomycin is present from

the start (curve C). The inhibition of binding due to pactamycin (insert of Figure 1) is largely overcome when both pactamycin and sparsomycin are present at zero time (curve B) and the release by pactamycin of *N*-acetyl-L-phenylalanyl-tRNA bound in the presence of sparsomycin is completely blocked (curve E). Similar results to these with *N*-acetyl-L-phenylalanyl-tRNA and poly U have been obtained with *N*-formyl-methionyl-tRNA and poly (AUG) (A. E. Herner and I. H. Goldberg, 1968, unpublished data).

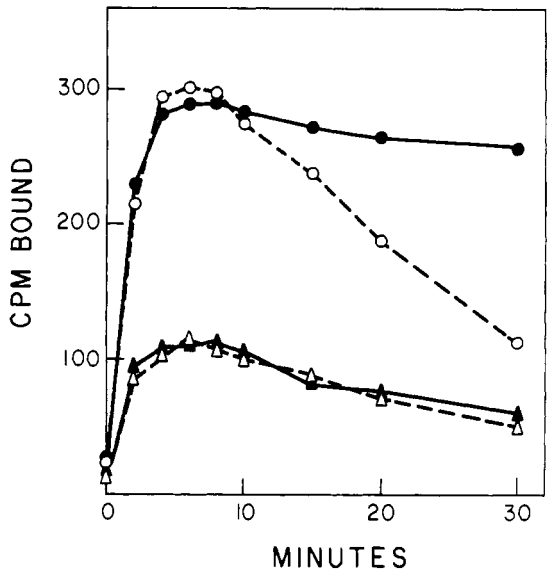

FIGURE 2: Effect of sparsomycin on binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes with 5'-guanylylmethylenediphosphonate. Binding was followed as described in Figure 1 except where indicated 5'-guanylylmethylenediphosphonate (0.26 mM) was substituted for GTP and sparsomycin was at 10^{-4} M. (△ — — △) No nucleotide or sparsomycin; (▲—▲) no nucleotide, with sparsomycin; (○ — — ○) 5'-guanylylmethylenediphosphonate, no sparsomycin; (● — ●) 5'-guanylylmethylenediphosphonate plus sparsomycin.

Requirement for GTP or 5'-Guanylylmethylenediphosphonate for the Sparsomycin Effect. As has been shown by Lucas-Lenard and Lipmann (1967), the binding of *N*-acetyl-L-phenylalanyl-tRNA to salt-washed ribosomes at 4.8 mM Mg^{2+} is dependent upon the addition of factors eluted from the ribosomes by salt treatment and of GTP. In the presence of the GTP analog, 5'-guanylylmethylenediphosphonate, maximal binding is about 30% of that found with GTP and binding is considerably less stable than with GTP (Figure 2; compare with Figure 1). While sparsomycin also stabilizes the *N*-acetyl-L-phenylalanyl-tRNA bound in the presence of 5'-guanylylmethylenediphosphonate, there is no further increase in over-all binding after the first 5 min. In the absence of added nucleoside triphosphate, sparsomycin does not influence *N*-acetyl-L-phenylalanyl-tRNA binding to ribosomes.

Release of Bound N-Acetyl-L-phenylalanyl-tRNA Is not Due to Deacylation. The question arises as to whether the decrease in radioactivity bound to the ribosomes which occurs during incubation results from splitting of the *N*-acetylphenylalanine from its tRNA on the ribosome. Such a reaction, should it take place, might result from an esterase action of the peptidyl transferase. Inhibition of this reaction by sparsomycin might then be related to its interference with the function of this enzyme. Deacylation, however, is not directly involved in the release of bound *N*-acetyl-L-phenylalanyl-tRNA. Binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes is not required for deacylation, as measured by the conversion of radioactivity into an acid-soluble form; loss of trichloroacetic acid precipitable radioactivity occurs in the absence of poly U, ribosomes, or GTP and this is not affected by sparsomycin. Since deacylation is diminished markedly by omission of the crude factor preparation, it appears that deacylation occurs in the soluble fraction mainly after release of *N*-acetyl-L-phenylalanyl-tRNA from the ribosomes, perhaps due to the peptidyl-tRNA-deacylating enzyme described by Cuzin *et al.* (1967) and de Groot *et al.* (1968).

This conclusion is supported by the experiment shown in Figure 3. *N*-Acetyl-L-phenylalanyl-tRNA was bound to ribosomes at 10 mM Mg^{2+} in the absence of factors (Lucas-Lenard and Lipmann, 1967); the labeled ribosomes were reisolated by centrifugation and incubated in medium containing mM Mg^{2+} with and without factors. Sparsomycin completely blocks the release of radioactivity from the ribosomes whether factors are present or not (Figure 3A). In the absence of sparsomycin, release from ribosomes is only slightly faster with factors present. As expected, by blocking release sparsomycin also prevents deacylation (Figure 3B). When sparsomycin is omitted, deacylation is more extensive in the presence of factors but is always considerably less than can account for the amount of *N*-acetyl-L-phenylalanyl-tRNA released from the ribosomes. It is likely, therefore, that the initiation complex dissociates at 5 mM Mg^{2+} releasing *N*-acetyl-L-phenylalanyl-tRNA which is then enzymatically split to give *N*-acetylphenylalanine and tRNA. Similar results are obtained with ribosomes to which *N*-acetyl-L-phenylalanyl-tRNA has been bound in the presence of factors.

Relation of the Sparsomycin Effect to Ribosome Concentration. In Figure 4 binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes is compared under conditions of maximal binding in the absence (at 15 min) and in the presence (at 90 min) of sparsomycin. Both curves remain linear over the same concentrations of ribosomes so that the per cent stimulation by sparsomycin remains fixed at almost 200%. Over the linear portion of the curves it can be calculated that there are about 1 molecule of *N*-acetyl-L-phenylalanyl-tRNA bound for every 18 ribosomes in the absence of sparsomycin and 1 for 6 ribosomes in its presence.

Effect of Mg^{2+} and NH_4^+ Concentrations. The rate at which bound *N*-acetyl-L-phenylalanyl-tRNA is released from ribosomes varies directly with the NH_4^+ concentration and inversely with the Mg^{2+} concentration (Figure 5). Complete protection from release is afforded

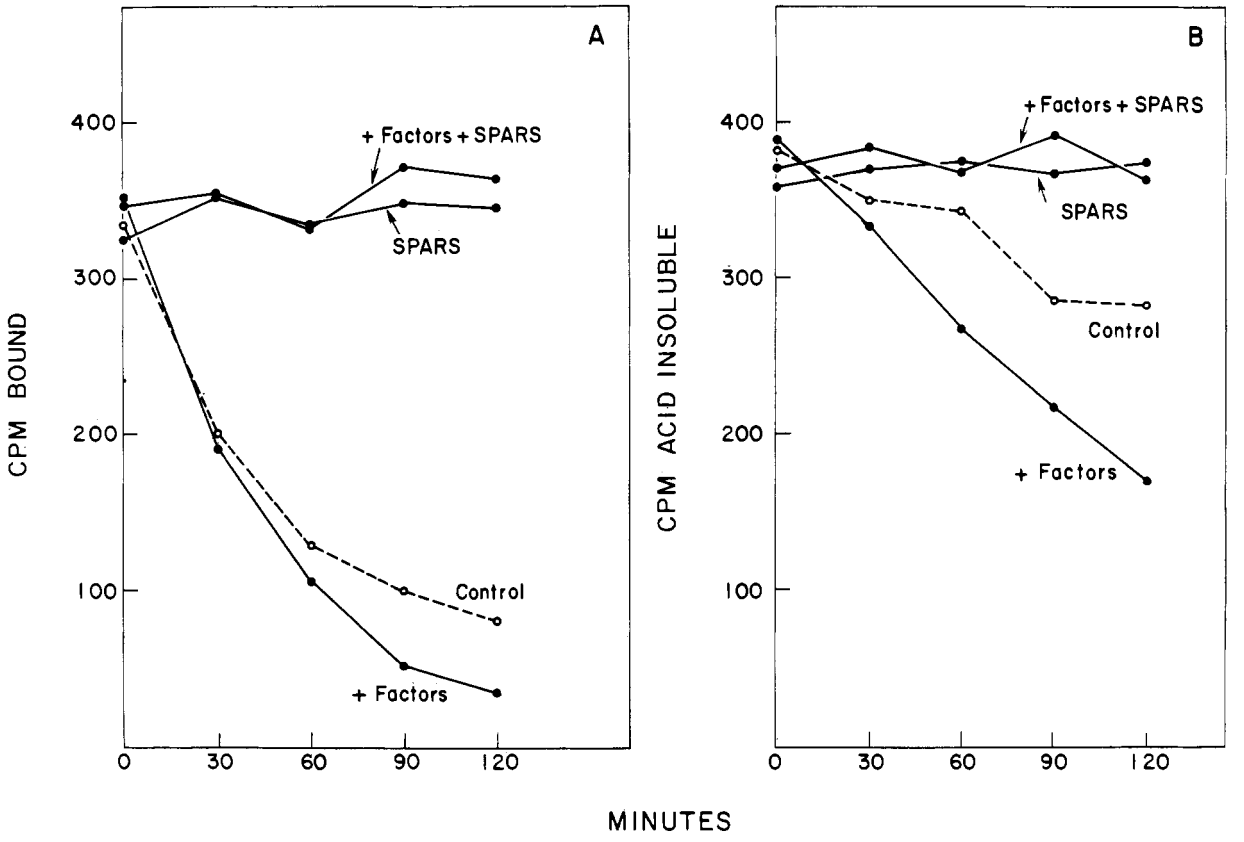

FIGURE 3: Effect of sparsomycin and initiation factors on the release of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA bound to ribosomes. [^{14}C]*N*-Acetyl-L-phenylalanyl-tRNA was bound to ribosomes in the absence of initiation factors at 10 mM Mg^{2+} for 40 min at 35°. Conditions, otherwise, were as described in Figure 1. The labeled ribosomes were isolated by centrifugation at 65,000 rpm for 90 min. The pellet was suspended in 10 mM Tris-HCl (pH 7.4) and 10 mM magnesium acetate. Release from 3.6 A_{260} units of ribosomes (4.5×10^3 cpm)/250 μl of incubation mixture was studied at 30° in 10 mM Tris-HCl (pH 7.4) at 5 mM Mg^{2+} with sparsomycin (5×10^{-5} M) and initiation factors (29 μg) added where indicated. At the designated time points 20-μl aliquots were assayed by the filter technique for residual binding of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA to ribosomes and for radioactivity insoluble in cold 5% trichloroacetic acid.

TABLE I: Sparsomycin Inhibition of 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
Sparsomycin	706	635	−10	675	733	+8	883	907	+3

[a] Ribosomes were prelabeled with [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA as described in the legend to Figure 1 (15 min, 25° for 4.8 mM Mg^{2+}; 30 min, 30° for 10.8 and 18.8 mM Mg^{2+}). Initiation factors were present only in the incubation at 4.8 mM Mg^{2+}. Where indicated sparsomycin was at 10^{-4} M. Puromycin (6×10^{-4} M) was then added and incubation was continued for 5 min. Samples (0.1 ml) were then assayed for ribosomal-bound radioactivity by the Millipore filter technique of Nirenberg and Leder (1964).

by sparsomycin at all Mg^{2+} and NH_4^+ concentrations, but at 10 mM Mg^{2+} and 50 mM NH_4^+ where the complex is relatively stable by itself, no additional effect can be attributed to sparsomycin until 60-min incubation. The ability of sparsomycin to inhibit the transfer of the *N*-acetylphenylalanyl moiety to puromycin is also inde-

pendent of the cation concentrations, whether or not initiation factors are present (Table I), although the puromycin reaction is markedly reduced at very high Mg^{2+} concentrations (Lucas-Lenard and Lipmann, 1967; Table I).

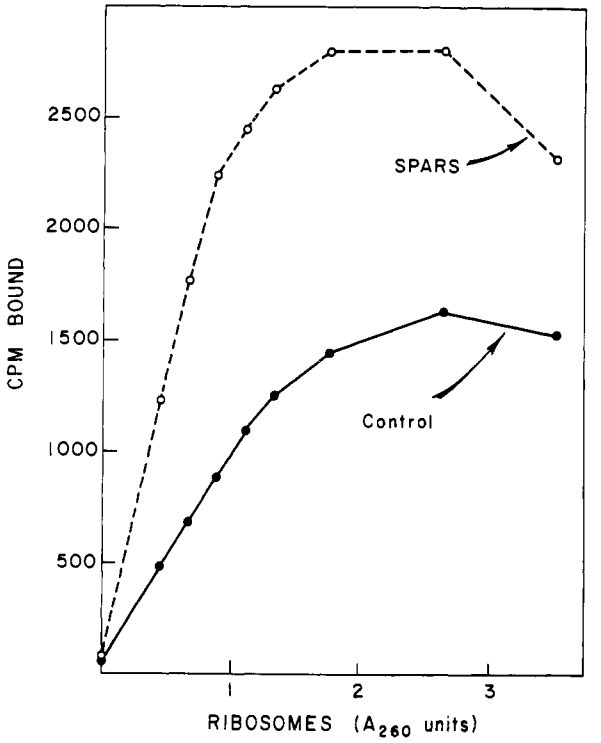

FIGURE 4: Relation of ribosome concentration to *N*-acetyl-L-phenylalanyl-tRNA binding with and without sparsomycin. Incubations are performed at varying ribosome concentrations in a volume of 100 μl. Other constituents are present at concentrations indicated in Figure 1. Incubations are for 90 min with sparsomycin and 15 min for the control.

Requirement for the 50S Ribosomal Subunit for the Sparsomycin Effect. *N*-Acetyl-L-phenylalanyl-tRNA binds to the 30S ribosomal subunit at 10 mM Mg^{2+} with factors and GTP present (Cohen *et al.*, 1969b); the small amount of *N*-acetyl-L-phenylalanyl-tRNA found with the 50S ribosomal subunit can be accounted for by the slight contamination with 30S subunits (Figure 6). Sparsomycin causes slight stimulation of binding to either the 30S or 50S subunit preparation. When 30S and 50S subunits are added together, however, sparsomycin has four times the stimulatory effect as it does on either subunit preparation alone. Further, we have examined the binding of *N*-acetyl-L-phenylalanyl-tRNA to 30S ribosomes repurified by recentrifugation and have found absolutely no stimulation of binding by sparsomycin after as long as 90-min incubation.

We also have examined the effect of sparsomycin on the release of *N*-acetyl-L-phenylalanyl-tRNA from prelabeled, reisolated 30S ribosomes in the presence and absence of 50S subunits (Table II). When these 30S subunits are reincubated without added 50S subunits,

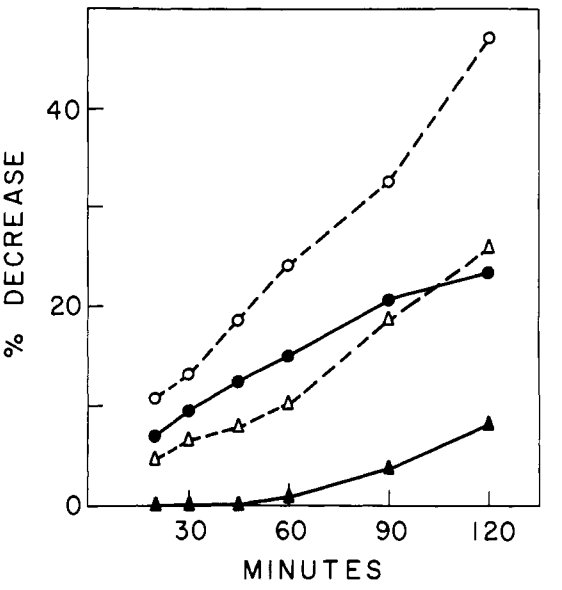

FIGURE 5: Relation of Mg^{2+} and NH_4^+ concentrations to the binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes. Binding of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA to ribosomes was followed at the indicated Mg^{2+} and NH_4^+ concentrations with and without sparsomycin (10^{-5} M) as described in Figure 1. The binding at each of the designated time points was compared with that in which sparsomycin was included in the incubation. The percentage decrease of bound radioactivity at each combination of Mg^{2+} and NH_4^+ level is calculated from the difference between the incubation containing and lacking sparsomycin. The former is taken as 100% binding. (○---○) 5 mM Mg^{2+} and 160 NH_4^+; (●—●), 5 mM Mg^{2+} and 50 mM NH_4^+; (△---△) 10 mM Mg^{2+} and 160 mM NH_4^+; (▲—▲) 10 mM Mg^{2+} and 50 mM NH_4^+.

sparsomycin has no effect on the release of ribosome bound radioactivity. In the presence of 50S subunits, however, sparsomycin exerts a significant stabilizing effect. When aliquots from this experiment were precipitated with trichloroacetic acid, it was evident that deacylation did not accompany the release.

Effect of Sparsomycin on 70S Ribosome Formation. Since the binding of *N*-acetyl-L-phenylalanyl-tRNA to 30S ribosomes is stabilized by sparsomycin only in the presence of added 50S subunits, it is possible that sparsomycin acts by promoting the formation of 70S ribosomes to which *N*-acetyl-L-phenylalanyl-tRNA binds more firmly. Such a possibility is supported by the experiments described above in which the concentrations of Mg^{2+} and NH_4^+ were varied. 70S ribosome formation is promoted by increasing Mg^{2+} and decreasing NH_4^+ concentrations; the stability of *N*-acetyl-L-phenylalanyl-tRNA binding is similarly affected.

When isolated 30S ribosomes to which [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA has been bound are incubated with 50S subunits in the presence of sparsomycin, the formation of labeled 70S ribosomes is increased (Figure 7). In the absence of sparsomycin most of the radioactivity is found in the 50–60S region which, at 10 mM Mg^{2+} and 160 mM NH_4^+, may consist of 30S–50S initiation complexes in which the subunits are not tightly coupled and actually separating during sedimentation (Cohen *et al.*, 1969a). Sparsomycin counteracts

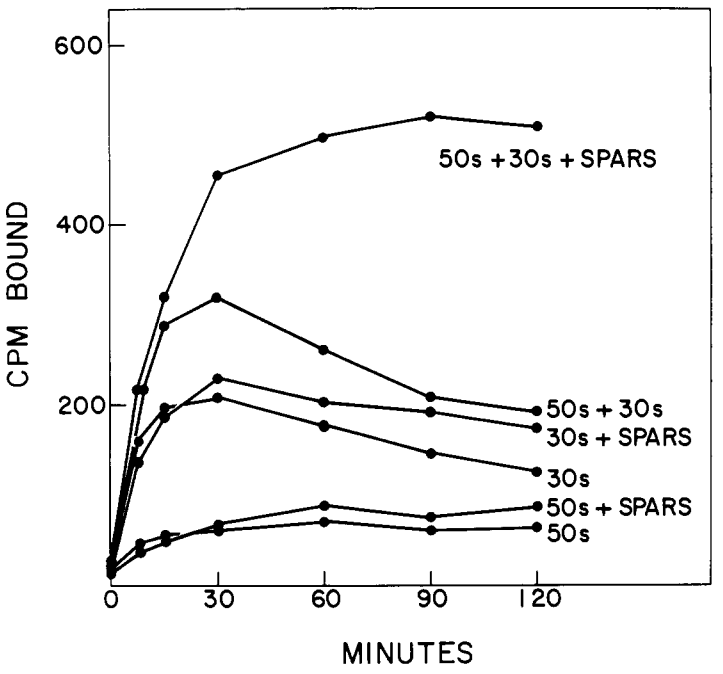

FIGURE 6: Relation of sparsomycin effect to the 30S and 50S subunits. The reaction mixture was as described in Figure 1 except that the volume of 200 μl contained 10 mM magnesium acetate, 0.438 A_{260} unit of 30S ribosomes and/or 0.92 A_{260} unit of 50S ribosomes, and 5×10^{-5} M sparsomycin as indicated. Samples (25 μl) were removed at the times shown and assayed for binding by the Millipore filter technique of Nirenberg and Leder (1964).

TABLE II: Effect of Sparsomycin and 50S Subunits on the Stability of *N*-Acetyl-L-phenylalanyl-tRNA Prebound to 30S Subunits.[a]

Additions	% Release
None	39
Sparsomycin	36
50 S	29
Sparsomycin + 50 S	19

[a] 30S ribosomes (8.8 A_{260} units) were incubated with the following in a volume of 3.0 ml for 40 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 (1.1×10^5). The mixture was diluted to 12 ml with 10 mM Tris-HCl (pH 7.4), containing 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 0.05 ml of buffer containing 10 mM Tris-HCl (pH 7.4) and 10 mM magnesium acetate; 1 A_{260} unit of the labeled 30S ribosomes (containing 3525 cpm) was reincubated at 25° with 50 mM Tris-HCl (pH 7.4), 10 mM magnesium acetate, 160 mM NH_4Cl, 10 mM dithiothreitol, and 1.91 A_{260} units of 50S ribosomes and 5×10^{-5} M sparsomycin as indicated in a volume of 0.4 ml. At zero time and 60 min 0.18-ml aliquots were removed and assayed for ribosomal-bound radioactivity as described. At the start of the reaction there were 1400 cpm bound to ribosomes.

the effect of the high NH_4^+ concentration by bringing the two subunits together in a compact structure. This effect of sparsomycin holds only for the 30S ribosomes to which *N*-acetyl-L-phenylalanyl-tRNA is bound, since the optical density pattern does not change significantly. This action of sparsomycin is readily reversible since sparsomycin must be added to the gradient to get the effect. Qualitatively similar results are found at other Mg^{2+} and NH_4^+ concentrations as shown in Figure 8. In these experiments incubation of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA with 30S and 50S ribosomes is (A) at 5 mM Mg^{2+} and 160 mM NH_4^+ or (B) at 5mM Mg^{2+} and 50 mM NH_4^+ in the presence of factors. In the absence of sparsomycin, the bound radioactivity in A is mainly in the 30S region but with sparsomycin it has moved mainly to the 50–60S region of the gradient. At the lower NH_4^+ concentration (B) the radioactivity shifts in the presence of sparsomycin from the 50–60S region to the 70S region. In all cases sparsomycin produces more rapidly sedimenting particles which are radioactive.

Sparsomycin Concentration Curve and Effect of Other Antibiotics. As can be seen in Figure 9, the sparsomycin stimulation of binding is half-maximal at about 10^{-7} M and is complete by 10^{-6} M, concentrations much too low to explain the effect on the basis of a cationic action of sparsomycin in the same sense as with Mg^{2+}. Other antibiotics which interfere with the function of the 50S ribosome (Monro and Vazquez, 1967; Jayaraman and Goldberg, 1968) were also studied for a stimulatory effect on *N*-acetyl-L-phenylalanyl-tRNA binding to ribosomes (Figure 9). At much higher concentrations than required for sparsomycin, both gougerotin and chloramphenicol have qualitatively similar but lesser actions. In the case of chloramphenicol this result was somewhat variable and occasionally at late times actually decreased binding (Figure 10). It is of interest, as shown in Figure 10, that either gougerotin or chloramphenicol, under conditions where each stimulates

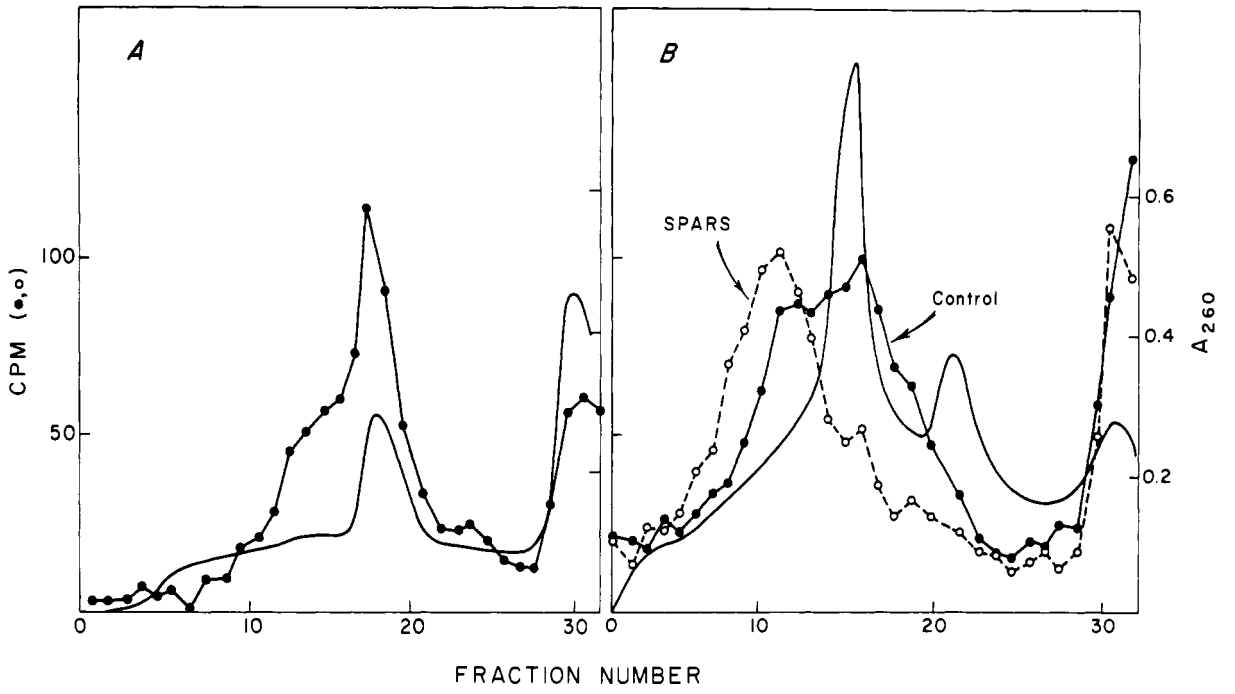

FIGURE 7: Effect of sparsomycin on the incubation of prelabeled 30S subunits with 50S subunits. Incubation conditions for prelabeling and reisolating 30S subunits (14.1 A_{260} units) were the same as those in Table II. The isolated 30S ribosomes (0.6 A_{260} unit) containing 1500 cpm were reincubated for 10 min at 25° (in 0.2 ml) with 50 mM Tris-HCl (pH 7.4), 10 mM magnesium acetate, 160 mM NH_4Cl, 0.24 mM GTP, 8 μg of poly U, 1.38 A_{260} units of 50S subunits (B), and 5×10^{-5} M sparsomycin as indicated. An aliquot (160 μl) of the incubation mixture was applied to a 5–20% sucrose gradient in the above Mg^{2+}, NH_4^+, and buffer and centrifuged at 4° in the SW41 rotor at 41,000 rpm for 3.5 hr in (A) and 3 hr in part B. The incubation mixture containing sparsomycin was layered onto a gradient containing 2×10^{-6} M sparsomycin. The gradients were analyzed for A_{260} and radioactivity as described elsewhere (Cohen *et al.*, 1959b). In part A 50S subunits were omitted from the second incubation.

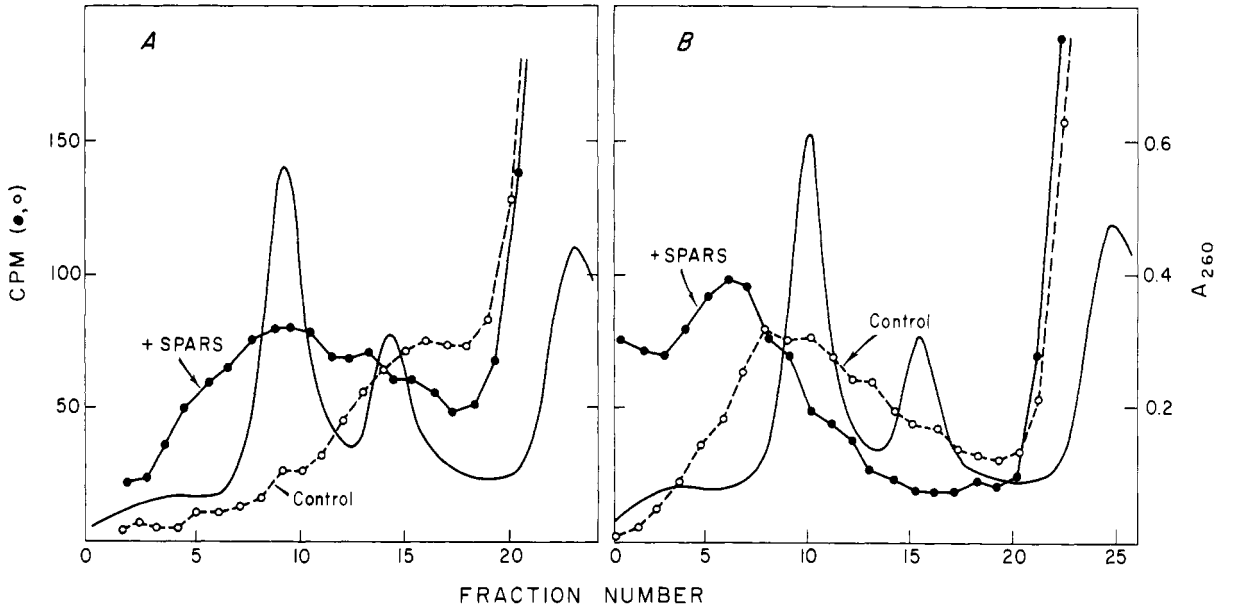

FIGURE 8: Centrifugation analysis of the effect of sparsomycin on the binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes. The following were incubated for 20 min at 25° in a volume of 0.2 ml: 50 mM Tris-HCl (pH 7.4), 5 mM magnesium acetate, 10 mM dithiothreitol, 8 μg of poly U, 0.24 mM GTP, 32 μg of ribosomal wash proteins, 1.8 A_{260} units of 30S ribosomes, 2.7 A_{260} units of 50S ribosomes, 8.2 $\mu\mu$moles [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA (7400 cpm), 5×10^{-5} M sparsomycin as indicated, and either 160 or 50 mM NH_4Cl (A and B, respectively). An aliquot (160 μl) of the incubation mixture was applied to a 5–20% sucrose gradient in the above Mg^{2+}, NH_4^+, and buffer and centrifuged in the SW41 Spinco rotor at 41,000 rpm for 3.25 hr at 4°. The incubation mixture containing sparsomycin was layered onto a gradient containing 2×10^{-6} M sparsomycin. The gradients were analyzed for A_{260} and radioactivity as described elsewhere (Cohen *et al.*, 1969b).

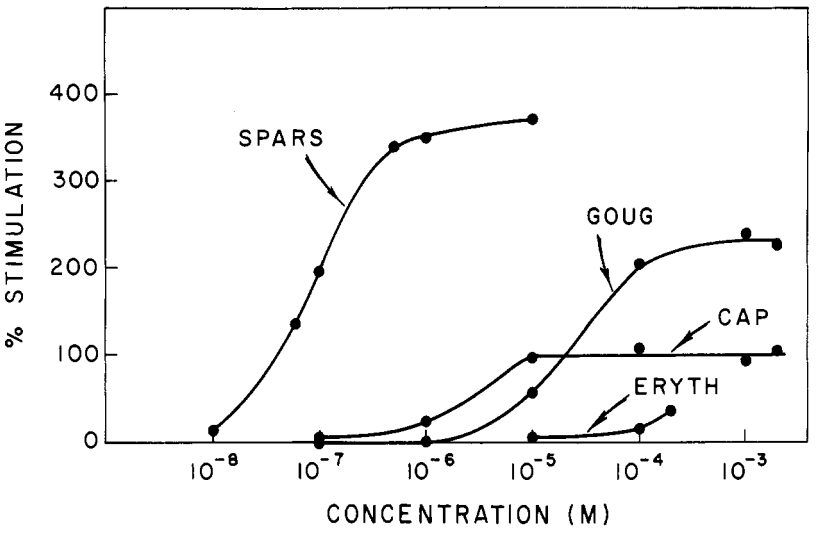

FIGURE 9: Concentration curves of antibiotics for stimulation of binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes. Incubations (100-μl volume) as described in Figure 1 were carried out for 90 min at the indicated concentrations of sparsomycin (SPARS), gougerotin (GOUG), chloramphenicol (CAP), or erythromycin (ERYTH), and in the absence of antibiotics (control). The per cent stimulation of binding in the control by the antibiotic was plotted on the ordinate.

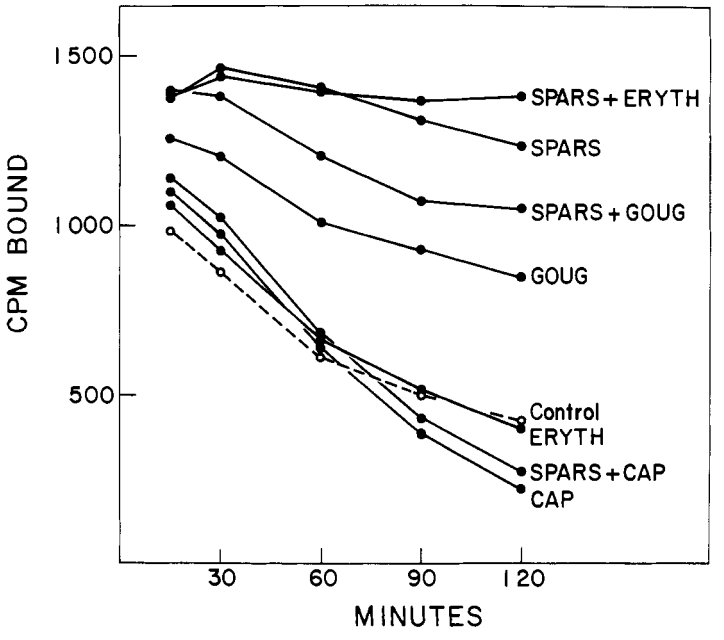

FIGURE 10: Time study of effect of other antibiotics on the sparsomycin stimulation of binding. Gougerotin (10^{-3} M) (GOUG), chloramphenicol (10^{-3} M) (CAP), and erythromycin (10^{-4} M) (ERYTH) alone and in combination with sparsomycin (10^{-6} M) (SPARS) were included as indicated in 0.3-ml incubations containing components listed in Figure 1. The binding of [^{14}C]*N*-acetyl-L-phenylalanyl-tRNA to ribosomes was measured by removal of 50-μl aliquots at the indicated times for the filter assay.

binding alone, inhibits the stimulatory effect of sparsomycin. This result is also shown in Figure 11 where the ability of sparsomycin (10^{-6} M) to stimulate binding was followed at different concentrations of gougerotin or chloramphenicol. For each point the control was represented by the amount of *N*-acetyl-L-phenylalanyl-tRNA bound at the particular concentration of gougerotin or chloramphenicol in the absence of sparsomycin.

Discussion

It is clear that sparsomycin at very low concentrations stabilizes the specific binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes. This is true for binding dependent upon factors and GTP or 5'-guanylylmethylenediphosphonate at 5 mM Mg^{2+}, as well as for *N*-acetyl-L-phenylalanyl-tRNA bound at 10 mM Mg^{2+} in the absence of factors and then reincubated at 5 mM Mg^{2+}. At the various cation concentrations used in this paper *N*-acetyl-L-phenylalanyl-tRNA is found by zonal centrifugation to be bound to complexes sedimenting more slowly than 70 S. It is assumed that such structures are either "open" forms of the 30S–50S complex or 70S ribosomes which reversibly dissociate and reassociate during sedimentation (see Cohen *et al.*, 1969a,b). Sparsomycin converts these complexes into more rapidly sedimenting particles, presumably by stabilizing the association between the subunits. Similar

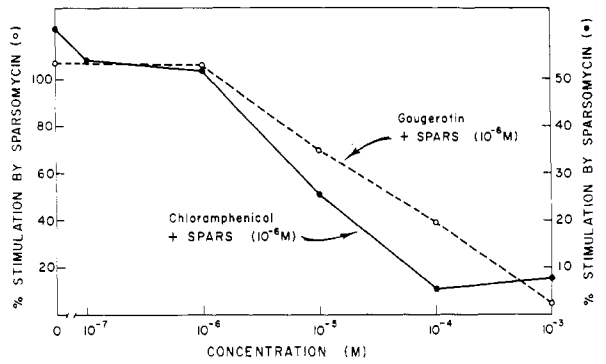

FIGURE 11: Concentration curves of gougerotin and chloramphenicol required to inhibit the sparsomycin stimulation of binding. On the ordinate is plotted the percent stimulation by sparsomycin (10^{-6} M) of the binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes in the presence of the indicated concentrations of gougerotin or chloramphenicol. Incubation conditions were as indicated in Figure 1 except that the volume was 100 μl. In the experiments with gougerotin incubation was for 60 min and with chloramphenicol for 30 min. In the presence of sparsomycin alone there were 2499 cpm bound at 60 min in the gougerotin experiments and 2573 cpm bound at 30 min in the chloramphenicol experiments.

effects can be produced by spermidine or high concentrations of Mg^{2+} (Cohen *et al.*, 1969a,b). If sparsomycin acts as a cation,[1] because of concentration considerations, it must be bound tightly to a crucial site on the ribosome.

There is another similarity between Mg^{2+} and sparsomycin. At very high concentrations of Mg^{2+} (18.8 mM; Suarez and Nathans, 1965; Lucas-Lenard and Lipmann, 1967; Table I), ribosome-bound *N*-acetyl-L-phenylalanyl-tRNA reacts very poorly with puromycin. It is possible that with sparsomycin or high Mg^{2+} the *N*-acetyl-L-phenylalanyl-tRNA is fixed in the peptidyl site and unable to undergo transfer of the *N*-acetylphenylalanyl group to the aminoacyl-tRNA in the acceptor site. Such an effect could be brought about by a change in the peptidyl site, direct inhibition of the peptide-bond-synthesizing enzyme, or a change in accessibility of the acceptor site. Since puromycin does not require binding to the acceptor site for its action, the last-mentioned possibility is eliminated unless the site encompasses more than its binding property. Further, the sparsomycin protection from the action of pactamycin can be related to one of these mechanisms.

The question arises as to whether the lability of binding of *N*-acetyl-L-phenylalanyl-tRNA to ribosomes is in some way related to normal peptide-bond formation. Under the influence of the peptidyl transferase on the 50S subunit the binding of *N*-acetyl-L-phenylalanyl-tRNA might be weakened in preparation for transfer to the aminoacyl-tRNA in the acceptor site. Inhibition of the peptidyl transferase by sparsomycin would then be accompanied by stabilization of the bound *N*-acetyl-L-phenylalanyl-tRNA.

[1] Sparsomycin has a $pK_{a'}$ of 8.67 in water (Argoudelis and Herr, 1962).

The need for the 50S subunit for the sparsomycin effect further suggests the possibility of involvement of the peptide-bond-forming apparatus in the action of the antibiotic. It is possible that sparsomycin prevents polypeptide chain elongation by abnormally fixing the 30S–50S complexes bearing initiator tRNA, as compact 70S ribosomes in which relative motion of the 30S and 50S subunits is prevented. The finding that sparsomycin inhibits the puromycin reaction by 50S subunits alone (Monro and Vazquez, 1967), however, indicates that its action is more fundamental than this.

Other inhibitors of 50S ribosome function such as gougerotin and chloramphenicol also stabilize *N*-acetyl-L-phenylalanyl-tRNA binding to some extent. The inhibition by these agents of the sparsomycin stimulation of binding suggests that they may share sites on the ribosome in common with sparsomycin. As noted before, however, sparsomycin does not interfere with the binding of [^{14}C]chloramphenicol to ribosomes (Goldberg and Mitsugi, 1967a). Erythromycin, an antibiotic acting on the 50S subunit (Wilhelm and Corcoran, 1967), which does not share a common binding site on the 50S subunit with chloramphenicol (Oleinick *et al.*, 1968), does not interfere with the sparsomycin effect on *N*-acetyl-L-phenylalanyl-tRNA binding, but does interfere with the binding and action of lincomycin (Chang and Weisblum, 1967).

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The Mercuric Bromide Rearrangement and 1-β-D-Ribofuranosyl-4,6-pyrimidinedione, an Isomer of Uridine*

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ABSTRACT: The conformation of the pyrimidine ring in a uridine isomer, 1-β-D-ribofuranosyl-4,6-pyrimidinedione (isouridine, IV), is opposite to the pyrimidine conformation of 1-β-D-ribofuranosyluracil. Condensation of 2 molar equiv of 2,3,5-tri-*O*-benzoyl-D-ribosyl chloride with 4,6-pyrimidinedionemercury (I) led to the formation of 4,6-di-*O*-(2′,3′,5′-tri-*O*-benzoyl-D-ribofuranoside)-4,-6-dioxypyrimidine (II). Compound II was converted into 1,4-di-(2′,3′,5′-tri-*O*-benzoyl-D-ribofuranosyl)-4-oxy-6-pyrimidinone (III) by the mercuric bromide rearrangement; the mild alkaline degradation of III gave isouridine (IV). The position of the glycosyl bond in isouridine and the β-D-configurational assignment were confirmed by the conversion of isouridine into 2′,3′-*O*-isopropylidene-5′,6-anhydro-(cyclo)isouridine.

The anhydro derivative shows a specific 1-alkyl-4-pyrimidinone intense absorption band near 240 mμ which supports the structure assigned to isouridine. The new ribonucleoside behaves as a substrate in the formation of ribose 1-phosphate and 4,6-pyrimidinedione by uridine phosphorylase.

All of the 2-, 4-, and 6-oxygen-substituted derivatives of ribofuranosylpyrimidine are now known compounds. Ukita and his collaborators (1964a,b) have reported the preparation of 1-β-D-ribofuranosylbarbituric acid (Ukita *et al.*, 1964b), 1-β-D-ribofuranosyl-2-pyrimidinone, and 1-β-D-ribofuranosyl-6-pyrimidinone (Funakoshi *et al.*, 1961). The synthesis of 1-β-D-ribofuranosyl-4-pyrimidinone was performed in this laboratory (Lee and Wigler, 1968), and tribenzoylribofuranosyl chloride with 4-ethoxy-2-pyrimidinone gave 3-β-D-ribofuranosyluracil (Scannell and Allen, 1960). We wish to report the synthesis of the final member of this class of substances 1-β-D-ribofuranosyl-4,6-pyrimidinedione (isouridine).

Several derivatives of isouridine have been prepared from an appropriate halogenoribose and 4,6-pyrimidinedione blocked with 4-alkoxy or 6-alkoxy groups (Prystas and Sorm, 1968). The formation of the *N*-glycosyl bond was readily accomplished, but attempts to remove the alkyl substituents from the pyrimidine moiety led to destruction of the nucleoside (Prystas, 1967). In a recent report, however, Prystas (1968) described the preparation of the 5-methyl derivative of isouridine.

The synthesis of an *N*-glycosyl derivative from the unmodified 4,6-pyrimidinedione depends upon an oxygen to nitrogen transglycosylation, the mercuric bromide rearrangement of an oxygen–glycosidic bond to a nitrogen–glycosyl bond. Wagner and Pischel (1961) found that when 2-*O*-(2′,3′,4′,6′-tetra-*O*-acetyl glucopyranoside)-2-oxypyridine was treated with $HgBr_2$ at 160° for 3 hr the *N*-glucosyl-2-pyridone was produced. The rearrangement has been confirmed by Ukita *et al.* (1964a) and by Prystas and Sorm (1968).

The isomeric pair uridine and isouridine are opposite with respect to the structural conformation of the pyrimidine ring. This isomeric pair of potential substrates may be used to elucidate the conformational requirements of enzymatic reactions. For example, Ward and Reich (1968) suggested that the conformational selectivity of pancreatic ribonuclease A is an important factor in the specificity of the enzyme.

Results

Condensation of 4,6-pyrimidinedionemercury (I)

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