Table VI that was hydrolyzed to the greatest extent should include those sequences most rapidly hydrolyzed.

Acknowledgment

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References

Alexander, M., Heppel, L. A., and Hurwitz, J. (1961), *J. Biol. Chem. 236*, 3014.

DeMeuron-Landolt, M., and Privat deGarilhe, M. (1964), *Biochim. Biophys. Acta 91*, 433.

Hilmoe, R. J. (1960), *J. Biol. Chem. 235*, 2117.

Hoard, D. E., and Ott, D. G. (1965), *J. Am. Chem. Soc. 87*, 1785.

Josse, J., Kaiser, A. D., and Kornberg, A. (1961), *J. Biol. Chem. 236*, 864.

Kato, K., Gonçalves, J. M., Houts, G. E., and Bollum, F. J. (1967), *J. Biol. Chem. 242*, 2780.

Khorana, H. G., and Vizsolyi, J. P. (1961), *J. Am. Chem. Soc. 83*, 675.

Laskowski, M., Sr. (1967), *Advan. Enzymol. 29*, 165.

Lehman, I. R., Bessman, M. J., Simms, E. S., and Kornberg, A. (1958), *J. Biol. Chem. 233*, 163.

Markham, R., and Smith, J. D. (1952), *Biochem. J. 52*, 552.

Ratliff, R. L., Hoard, D. E., Ott, D. G., and Hayes, F. N. (1967), *Biochemistry 6*, 851.

Richardson, C. C., and Kornberg, A. (1964), *J. Biol. Chem. 239*, 242.

Roberts, W. K., Dekker, C. A., Rushizky, G. W., and Knight, C. A. (1962), *Biochim. Biophys. Acta 55*, 664.

Yoneda, M., and Bollum, F. J. (1965), *J. Biol. Chem. 240*, 3385.

Localization of Sparsomycin Action to the Peptide-Bond-Forming Step*

J. Jayaraman† and I. H. Goldberg

ABSTRACT: Sparsomycin, a sulfur-containing antibiotic, which inhibits the puromycin-induced release of peptide from ribosomes, is a highly effective inhibitor of the single addition of [^{14}C]lysine residues onto polylysyl transfer ribonucleic acid (tRNA) bound to ribosomes. This latter reaction is inhibited 50% by 10^{-7} M sparsomycin.

Antibiotics such as erythromycin, chlortetracycline, and puromycin are somewhat less effective inhibitors; chloramphenicol and gougerotin are much less effective. Inhibition can not be reversed by increasing the concentration of lysyl-tRNA, polylysyl-tRNA, or ribosomes. The degree of inhibition is constant throughout the course of the addition reaction. These results provide further evidence for the action of sparsomycin on peptide-bond formation itself.

Previous work from this laboratory (Goldberg and Mitsugi, 1966, 1967a,b) has shown that sparsomycin, a sulfur-containing antibiotic, is a potent inhibitor of *in vitro* polypeptide synthesis and that its inhibitory action is probably exerted at or close to the peptide-bond-forming step. Sparsomycin, at very low levels, blocks the puromycin-induced release of polylysine from polylysyl-tRNA bound to *Escherichia coli* ribosomes. The formation of polylsylpuromycin, which takes place at 0.01 M Mg^{2+} in the absence of added GTP[1] and other factors, serves as a model for the formation of a single peptide bond. Sparsomycin neither causes the deacylation of polylysyl-tRNA nor affects its binding to ribosomes but acts as a competitive inhibitor of puromycin in this reaction. In order to obtain further evidence in support of the contention that sparsomycin acts on peptide-bond formation and not on the other reactions involved in peptide chain elongation, we have studied its effect on the limited addition of lysine units to polylysyl-tRNA bound to ribosomes. This reaction, which has been characterized by Gottesman

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[1] Abbreviations used: GTP, guanosine triphosphate, TCA, trichloroacetic acid.

and Lipmann (1967) and shown to involve most probably the addition of one lysine per reacting peptide chain, is extremely sensitive to inhibition by sparsomycin.

Materials and Methods

L-[^{14}C]Lysine was purchased from New England Nuclear Corp. Puromycin hydrochloride was a commercial product from Nutrition Biochemical Corp. Sparsomycin ($C_{13}H_{21}N_3O_6S_2$) and its photodegradation product ($C_{13}H_{21}N_3O_4S_2$) were gifts of Dr. Charles G. Smith of the Upjohn Co.; gougerotin, a gift of Takeda Chemical Industries, Ltd., Osaka, Japan; erythromycin, a gift of Eli Lilly and Co.; chlortetracycline, a gift of the Lederle Laboratories; and chloramphenicol, a gift of Parke Davis and Co. Ammonium chloride (0.5 M) washed ribosomes, [^{14}C]lysyl-tRNA, and unlabeled polylysyl-tRNA were prepared as previously described (Goldberg and Mitsugi, 1967a,b). The standard reaction for the limited addition of [^{14}C]lysine onto [^{12}C]-polylysyl-tRNA was measured as follows. Polylysyl-tRNA (0.68 ODU, equivalent to 5.68×10^{-5} μmole of polynucleotide) was incubated at 35° for 20 min in 0.1 M Tris-HCl (pH 7.2) to deacylate any contaminating lysyl-tRNA. To this were added ammonium acetate (0.1 M), magnesium acetate (0.01 M), polyadenylic acid (10 μg), antibiotic where indicated, and salt-washed ribosomes (2.5 ODU) in a total volume of 0.2 ml. Following incubation for 15 min at 35° to allow for completion of polylysyl-tRNA binding to ribosomes, [^{14}C]lysyl-tRNA (0.71 ODU, 5.9×10^{-5} μmole; 5.3×10^7 cpm/μmole) was added and incubation was continued for 15 min. The reaction was stopped with 0.1 ml of 1 N sodium hydroxide and incubation at 35° was continued for 20 min to strip labeled peptide and amino acid from tRNA. After cooling to 0°, the mixture was neutralized with 0.1 ml of 1 N HCl and 50 μl of carrier polylysine (1 mg/ml) was added. Lysine peptides were precipitated with 3 ml of freshly made cold 5% TCA–0.25% sodium tungstate (pH 2). After standing in ice for at least 1 hr, the content of each tube was filtered through a Millipore filter (0.45 μ) and the filter was washed three times with 3 ml of TCA–tungstate. The filter was dried and counted in a Packard scintillation spectrometer.

Results

A limited amount of [^{14}C]lysine from [^{14}C]lysyl-tRNA can be converted in the presence of polylysyl-tRNA, polyadenylic acid, Mg^{2+}, and salt-washed ribosomes into peptide which is precipitable by cold TCA–tungstate after treatment with alkali to split off tRNA (Table I). Cold TCA–tungstate has been shown to precipitate trilysine peptide partially and the tetramer and higher oligomers completely (Smith and Stahmann, 1963). Gottesman and Lipmann (1967) have analyzed the products of the enzymatic reaction and found the data consistent with the attachment of a single [^{14}C]-lysine residue per reacting polylysyl-tRNA molecule

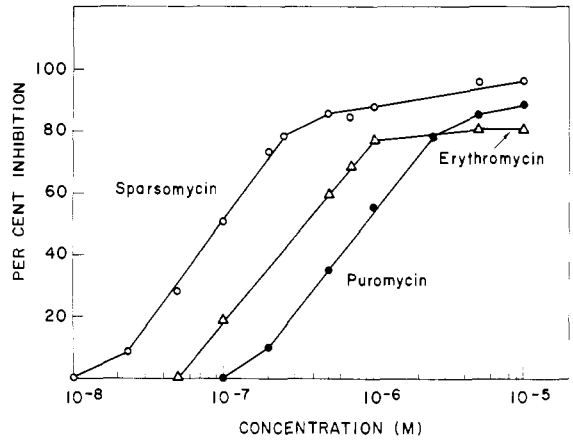

FIGURE 1: Relation of antibiotic concentration to inhibition of the lysine addition reaction. Incubation was carried out in a total volume of 0.2 ml containing [^{12}C]polylysyl-tRNA (7×10^{-5} μmole), ribosomes (6.4×10^{-5} μmole), and [^{14}C]lysyl-tRNA (7.6×10^{-5} μmole, 4038 cpm). The concentrations of sparsomycin, erythromycin, or puromycin was varied as indicated. The addition of [^{14}C]lysine onto ribosome-bound [^{12}C]polylysyl-tRNA was assayed as described in the text.

without specificity as to the chain length of the polylysine residue. Only if GTP and the supernatant transfer factors are added does chain elongation take place by the addition of multiple lysine units. As shown in Figure 1 and Table II the formation of a single peptide bond is extremely sensitive to the inhibitory action of sparsomycin. Erythromycin and puromycin are also effective inhibitors but somewhat less so than sparsomycin. As would be predicted from its ability to prevent messenger-specific binding of aminoacyl-tRNA to ribosomes, chlortetracycline inhibits the limited addition reaction while having little, if any, effect on the puromycin-induced release of polylysine (Goldberg and Mitsugi, 1967b; Gottesman and Lipmann, 1967) (Table II). Gougerotin and chloramphenicol are much less effective than the other antibiotics in inhibiting the

TABLE I: Properties of the Lysine Addition Reaction.[a]

	Cpm Incorp
Complete	385
Minus [^{12}C]polylysyl-tRNA	68
Minus poly A	51
Minus Mg^{2+}	66

[a] The incorporation of [^{14}C]lysine from [^{14}C]lysyl-tRNA into TCA–tungstate precipitable peptide was measured as described in the text.

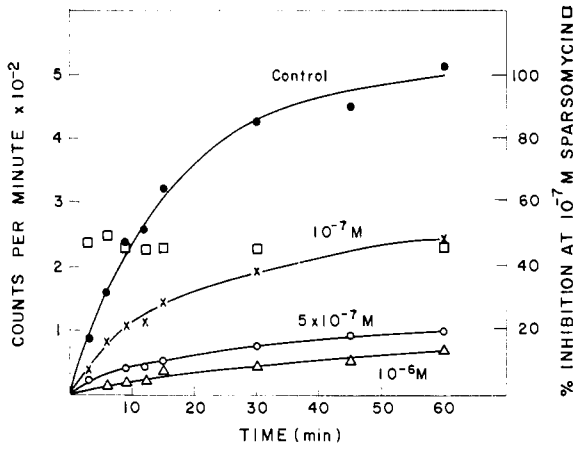

FIGURE 2: Time course of sparsomycin inhibition of the lysine addition reaction. The total reaction volume was 1.0 ml containing [^{12}C]polylysyl-tRNA (7.6 $\times$ 10^{-4} μmole), ribosomes (6.4 $\times$ 10^{-4} μmole), [^{14}C]-lysyl-tRNA (7.6 $\times$ 10^{-4} μmole), and sparsomycin as indicated. Starting at zero time, 100-μl aliquots were pipetted into 100 μl of 1 N NaOH for assay as described in the text. A zero-time control of 27 cpm was subtracted from the incorporation at each time point.

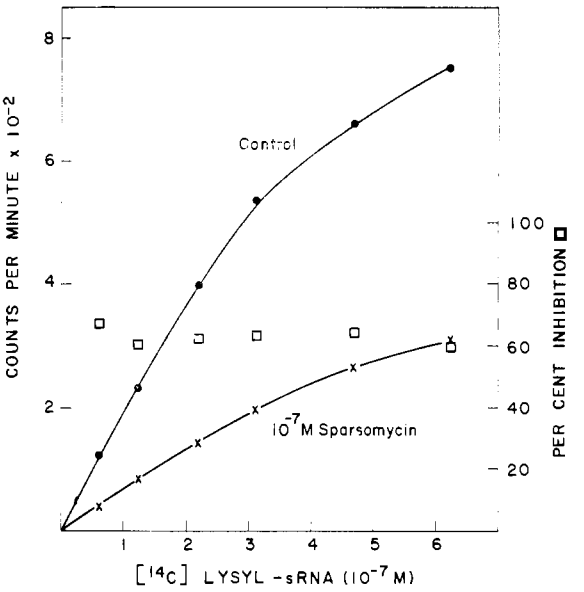

FIGURE 3: Relation of [^{14}C]lysyl-tRNA concentration to sparsomycin inhibition. The reaction mixture of 0.4 ml contained [^{12}C]polylysyl-tRNA (28 $\times$ 10^{-5} μmole), ribosomes (25.6 $\times$ 10^{-5} μmole), and [^{14}C]lysyl-tRNA and sparsomycin (10^{-7} M) as indicated. The addition of [^{14}C]lysine onto [^{12}C]polylysine was assayed as described in the text. A zero-time control at the highest concentration of [^{14}C]lysyl-tRNA was 42 cpm.

limited addition reaction (Gottesman and Lipmann, 1967) (Table II), and concentration curves with these antibiotics exhibit slopes, when plotted as in Figure 1, which are much less steep than those of sparsomycin, erythromycin, and puromycin. The latter results suggest a more complex action for chloramphenicol and gougerotin in this system. Sparsomycin photodegradation product which has about one-third the biological activity of the parent compound, is about 10% as active in the limited addition reaction. Sparsomycin inhibition remains constant throughout the course of the reaction (Figure 2), in contrast to the greater inhibition by sparsomycin of the puromycin-induced release of polylysine early in its course.

Since sparsomycin inhibits the puromycin reaction by competing with puromycin, an analog of aminoacyl-tRNA, it was of interest to see if increasing the con-

TABLE II: Antibiotic Concentration for 50% Inhibition.

Antibiotic	M
Sparsomycin	1×10^{-7}
Erythromycin	4×10^{-7}
Puromycin	1×10^{-6}
Sparsomycin photoproduct	1×10^{-6}
Chlortetracycline	2×10^{-6}
Chloramphenicol	2×10^{-5}
Gougerotin	6×10^{-5}

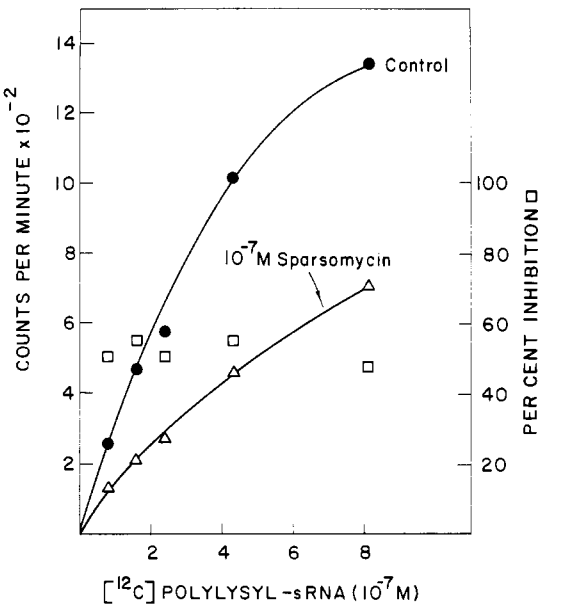

FIGURE 4: Relation of [^{12}C]polylysyl-tRNA concentration to sparsomycin inhibition. The reaction mixture of 0.4 ml contained [^{14}C]lysyl-tRNA (15 $\times$ 10^{-5} μmole), ribosomes (12 $\times$ 10^{-5} μmole), and [^{12}C]-polylysyl-tRNA and sparsomycin as indicated. The addition of [^{14}C]lysine onto [^{12}C]polylysine was assayed as described in the text. A zero-time control containing 1.6×10^{-7} M [^{12}C]polylysyl-tRNA had 73 cpm.

centration of lysyl-tRNA decreased the inhibition of the limited addition reaction by sparsomycin (Figure 3). No such decrease was observed. Such a result is not unexpected, however, since it is only that lysyl-tRNA which is *bound* to the acceptor site on the ribosome which is reactive for peptide-bond formation. Similar results have been obtained with increasing concentrations of polylysyl-tRNA (Figure 4) or ribosomes. With each of the three components, however, reciprocal plots of initial rates of reaction show kinetics in which the plot of the sparsomycin-inhibited reaction meets that of the uninhibited reaction on the ordinate. These results resemble those for chloramphenicol inhibition of polypeptide synthesis (Wolfe and Hahn, 1965) and probably are simply a reflection of the interaction of the antibiotic with the peptide-bond-synthesizing *complex* formed by these compounds.

Discussion

Several lines of evidence indicate that sparsomycin acts at or close to the actual site where peptide-bond synthesis takes place. Thus, both the puromycin reaction and the addition of a single amino acid onto the growing end of a preexisting peptide chain are readily inhibited by sparsomycin. Since sparsomycin does not interfere with the mRNA-specific binding of either aminoacyl-tRNA (Goldberg and Mitsugi, 1967a) or peptidyl-tRNA (Goldberg and Mitsugi, 1967b) to ribosomes, it appears that the step involved in formation of the peptide bond is inhibited by the antibiotic. Further, analysis of the oligopeptides synthesized in the presence of this antibiotic show that formation of the first peptide bond is sensitive to sparsomycin, while chloramphenicol and gougerotin exert a greater effect on chain elongation (M. Yukioka and I. H. Goldberg, unpublished data). Consistent with the conclusions arrived at here are the recent findings of Monro that sparsomycin inhibits the reaction between puromycin and the *N*-formylmethionylhexanucleotide fragment from *N*-formylmethionyl-tRNA which occurs in the presence of the 50S ribosomal subunit (R. E. Munro, personal communication). The formation of *N*-formylmethionylpuromycin in this reaction is analogous to the formation of the first peptide bond in protein synthesis.

References

Goldberg, I. H., and Mitsugi, K. (1965), *Biochem. Biophys. Res. Commun. 23*, 453.

Goldberg, I. H., and Mitsugi, K. (1967a), *Biochemistry 6*, 372.

Goldberg, I. H., and Mitsugi, K. (1967b), *Biochemistry 6*, 383.

Gottesman, M., and Lipmann, F. (1967), *J. Biol. Chem.* (in press).

Smith, J. D., and Stahmann, M. A. (1963), *Biochem. Biophys. Res. Commun. 13*, 251.

Wolfe, A. D., and Hahn, F. E. (1965), *Biochim. Biophys Acta 95*, 146.