# Sparsomycin Inhibition of Polypeptide Synthesis Promoted by Synthetic and Natural Polynucleotides*

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ABSTRACT: Sparsomycin inhibits polypeptide synthesis promoted by synthetic polynucleotides, MS2 ribonucleic acid (RNA), and endogenous messenger ribonucleic acid (mRNA). The sensitivity of polypeptide formation to sparsomycin depends on the base composition of the synthetic polynucleotide, increasing markedly upon inclusion of cytidylate or guanylate in a uridylate-containing copolymer. Sparsomycin blocks polypeptide synthesis at a stage after binding of aminoacyl-sRNA and mRNA to ribosomes. The inhibition cannot be overcome by increasing the concentrations of polyuridylic acid (poly U), soluble ribonucleic acid (sRNA), or ribosomes. While sparsomycin resembles chloramphenicol in some ways, it is clear that its basic action is different. Sparsomycin does not interfere with the binding of [$^{14}$C]chloramphenicol to ribosomes and is highly effective against protein synthesis in mammalian cell-free systems. Polypeptide synthesis promoted by endogenous mRNA is relatively sensitive to sparsomycin and chloramphenicol, when account is taken of the antibiotic-resistant fraction due to the soluble, $NH_2$-terminal end addition reaction.

Sparsomycin, a sulfur-containing antibiotic of unknown structure produced in the fermentation broth of *Streptomyces sparsogenes* (Argoudelis and Herr, 1962), is a potent inhibitor of the growth of many organisms, including bacteria, mammalian cells, yeasts, and molds (Owen *et al.*, 1962). It is thought to be identical with almarcetin, an amino acid containing antibiotic produced by a subspecies of *Streptomyces albus* (Bachler *et al.*, 1964). Sparsomycin inhibits protein synthesis in *Escherichia coli* (Slechta, 1965) and L cells (Goldberg and Mitsugi, 1966) while RNA synthesis continues. Sparsomycin is a highly effective inhibitor of polypeptide synthesis by extracts of *E. coli* (Goldberg and Mitsugi, 1966) and rabbit reticulocytes (Colombo *et al.*, 1966). Inhibition is exerted at a stage beyond the formation of aminoacyl-sRNA. Polypeptide synthesis promoted by various synthetic polynucleotides is blocked by the antibiotic at levels below $10^{-7}$ M, the degree of inhibition being related to the base composition of the synthetic mRNA (Goldberg and Mitsugi, 1966). From experiments on the interaction of aminoacyl-sRNA with ribosomes and others involving the puromycin-induced release of polypeptide (Goldberg and Mitsugi, 1967), it appears that sparsomycin exerts its effect at the level of the peptide bond forming step itself. This paper will describe experiments using sparsomycin with various polynucleotides (synthetic, endogenous, and MS2 RNA) as messengers and will compare sparsomycin with other antibiotics which also inhibit polypeptide synthesis after formation of the ternary complex active in protein synthesis.

## Materials and Methods

*E. coli* strain B crude extracts (S30), washed ribosomes, and high-speed supernatant (S105) were prepared and assayed for polypeptide synthesis as described by Nirenberg (1964) except that ammonium acetate replaced KCl. When incorporation of free amino acid into polypeptide was followed, the standard reaction mixture contained the following in a volume of 0.25 ml: 0.1 M Tris–HCl (pH 7.8), 0.014 M magnesium acetate, 0.05 M ammonium acetate, 0.001 M ATP,[1] 0.0003 M GTP, 0.0075 M phosphoenolpyruvate, 4 $\mu$g of pyruvate kinase, 0.006 M $\beta$-mercaptoethanol, 5 m$\mu$moles each of 19 nonlabeled amino acids and one [$^{14}$C]amino acid, 10 $\mu$g of synthetic polynucleotide, 200 $\mu$g of *E. coli* sRNA (stripped), and 0.05 ml of S30. The standard reaction mixture used when starting with added [$^{14}$C]phenylalanyl-sRNA contained the following in a volume of 0.25 ml: 0.05 M Tris–HCl (pH 7.4), 0.16 M $NH_4Cl$, 0.01 M magnesium acetate, 0.006 M $\beta$-mercaptoethanol, 0.009 M phosphoenolpyruvate, 10 $\mu$g of pyruvate kinase, 0.0004 M GTP, 10 $\mu$g of polynucleotide, 67 $\mu$g of [$^{14}$C]phenylalanyl-sRNA (6200 cpm, 330 $\mu$c/$\mu$mole of [$^{14}$C]phenylalanine), and 0.05 ml of S30. Polypeptide synthesis was measured by the incorporation of [$^{14}$C]amino acid into hot 10% TCA-precipitable material isolated and washed on Millipore filters. In each case the radioactivity incor-

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[1] Abbreviations used: GTP, guanosine triphosphate; TCA, trichloroacetic acid; UDP, uridine diphosphate; ATP, adenosine triphosphate; CTC, chlortetracycline.

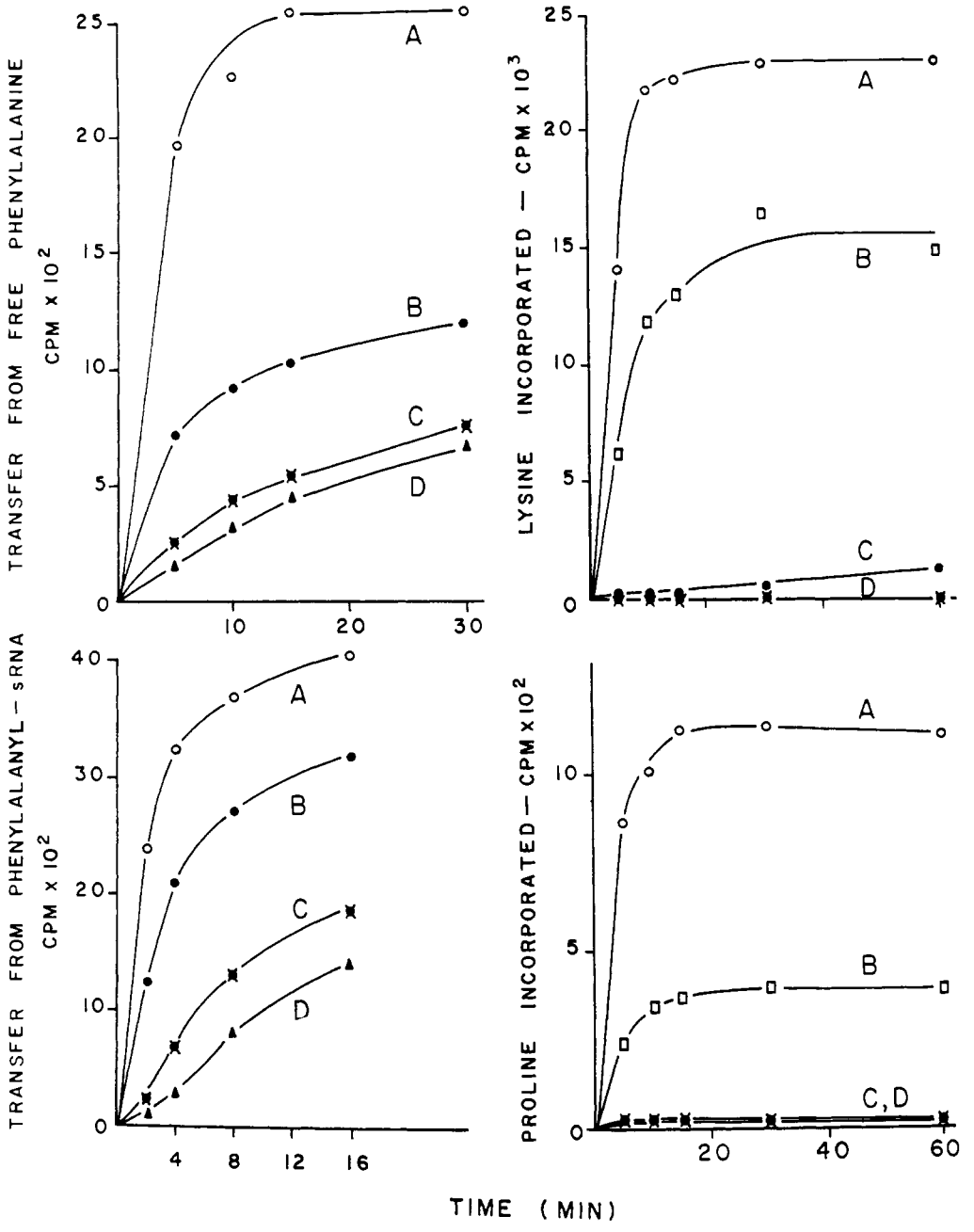


FIGURE 1: Time course of sparsomycin inhibition of polypeptide synthesis. (left top) The following were incubated at 35° in a volume of 0.5 ml: 0.1 M Tris–HCl (pH 7.8), 0.014 M magnesium acetate, 0.05 ammonium acetate, 0.001 M ATP, 0.0003 M GTP, 0.006 M β-mercaptoethanol, 0.0075 M phosphoenolpyruvate, 8 μg of pyruvate kinase, 10 mμmoles each of 19 nonlabeled amino acids and [$^{14}$C]phenylalanine (30 μc/μmole), 40 μg of poly U, 400 μg of sRNA, and 0.1 ml of S30. Sparsomycin was added as follows: (A) none, (B) $2.64 \times 10^{-6}$ M, (C) $2.64 \times 10^{-5}$ M, and (D) $2.64 \times 10^{-4}$ M. At the indicated times, 0.1-ml samples were removed and assayed for [$^{14}$C]phenylalanine incorporation into polypeptide. (left bottom) The standard reaction mixture containing 67 μg of [$^{14}$C]phenylalanyl-sRNA (6200 cpm) was incubated for the indicated time. Sparsomycin was added as indicated above. (right) The following were incubated without (A) or with sparsomycin ($2.64 \times 10^{-7}$ M (B), $2.64 \times 10^{-6}$ M (C), or $2.64 \times 10^{-5}$ M (D)) in a final volume of 0.6 ml at 37°: 0.1 M Tris–HCl (pH 7.8), 0.014 M magnesium acetate, 0.05 M ammonium acetate, 0.001 M ATP, 0.0003 M GTP, 0.0075 M phosphoenolpyruvate, 8 μg of pyruvate kinase, 0.006 M β-mercaptoethanol, 48 μg of poly C or poly A, 480 μg of sRNA, 12 mμmoles each of 19 nonlabeled amino acids, and [$^{14}$C]proline (bottom) or [$^{14}$C]lysine (top) (165 μc/μmole), and 0.12 ml of S30. Aliquots (0.1 ml) were removed and assayed as above.

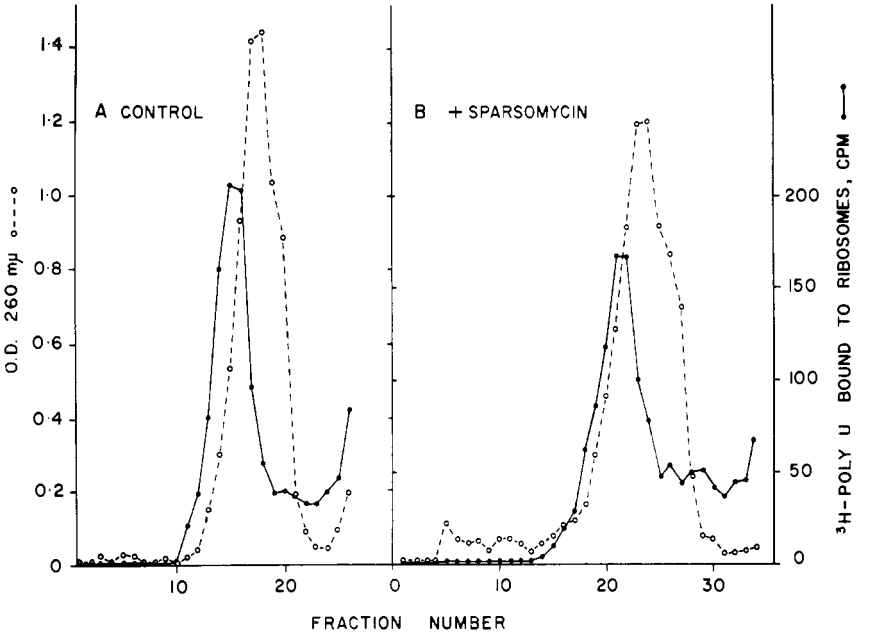


FIGURE 2: Absence of sparsomycin effect on binding of poly U to ribosomes. The following were incubated in a volume of 0.2 ml: 0.05 M Tris–HCl (pH 7.4), 0.012 M $MgCl_2$, 0.16 M $NH_4Cl$, 8 μg of [$^3$H]poly U (5.85 × $10^6$ cpm/mg), 834 μg of ribosomes, and sparsomycin (A) none, (B) 2.64 × $10^{-4}$ M. All components except poly U were incubated for 10 min at 30°, after which poly U was added, and the mixture was allowed to remain at 0° for 20 min. The sample was placed on a 5-ml linear sucrose gradient (5–20%) containing 0.05 M Tris (pH 7.4), 0.012 M $MgCl_2$, and 0.16 M $NH_4Cl$. Sparsomycin (2.64 × $10^{-4}$ M) was present in the gradient in expt B. After centrifugation in an SW 30 Spinco rotor at 39,000 rpm for 60 min, three-drop fractions were collected from the bottom of each tube for determination of cold TCA-precipitable radioactivity (●—●) and $OD_{260}$ (O—O).

TABLE I: Sparsomycin Inhibition of Phenylalanine Incorporation Stimulated by Various Polynucleotides.[a]

| Polynucleotide | Phenylalanine Incorporation (cpm) Complete | +Sparsomycin | % Inhibn |
|---|---|---|---|
| Poly U | 10,045 | 6,991 | 30 |
| Poly AU (1:5)[b] | 2,747 | 1,712 | 38 |
| Poly GU (1:40) | 20,978 | 9,332 | 56 |
| Poly CU (1:8) | 15,296 | 5,331 | 65 |
| Poly CU (1:5) | 20,518 | 6,383 | 69 |

[a] [$^{14}$C]Phenylalanine (5 mμmoles, 25 μc/μmole) was added to the standard reaction mixture. Sparsomycin (5.28 × $10^{-7}$ M) was added as indicated. After incubation for 15 min at 35°, the reaction was assayed for incorporation of radioactivity into polypeptide as described in Methods. In the absence of added polynucleotide 173 cpm was incorporated. [b] Indicates the input ratios of the two nucleotides in this and subsequent tables and figures.

porated into polypeptide in the absence of added polynucleotide messenger (with and without antibiotic) was subtracted from that in its presence. Polylysine formation was followed as described by Gardner *et al.* (1962). Binding of [$^{14}$C]phenylalanyl-sRNA to ribosomes was determined by the method of Nirenberg and Leder (1964). [$^{14}$C]Phenylalanyl-sRNA and [$^{14}$C]lysyl-sRNA were prepared as described by Nathans and Lipmann (1961). The molecular weight of sparsomycin is 379 (Argoudelis and Herr, 1962).

[$^3$H]Poly U (10 μc/μmole) was synthesized by polymerization of [$^3$H]UDP (Schwarz Bioresearch, Inc.) with *E. coli* polynucleotide phosphorylase (Thang *et al.*, 1965) (a gift of Dr. E. Reich). The binding of [$^3$H]poly U to ribosomes was determined by sucrose density-gradient centrifugation (Spyrides and Lipmann, 1962). The binding of [$^{14}$C]chloramphenicol to ribosomes was determined by a modification of the methods of Vazquez (1966a) and Wolfe and Hahn (1965). Radioactivity was determined in a Packard scintillation spectrometer or in a Beckman low β gas-flow counter with an ultrathin window.

Labeled protein for $NH_2$-terminal analysis was prepared as follows: the reaction mixture (5 ml) con-

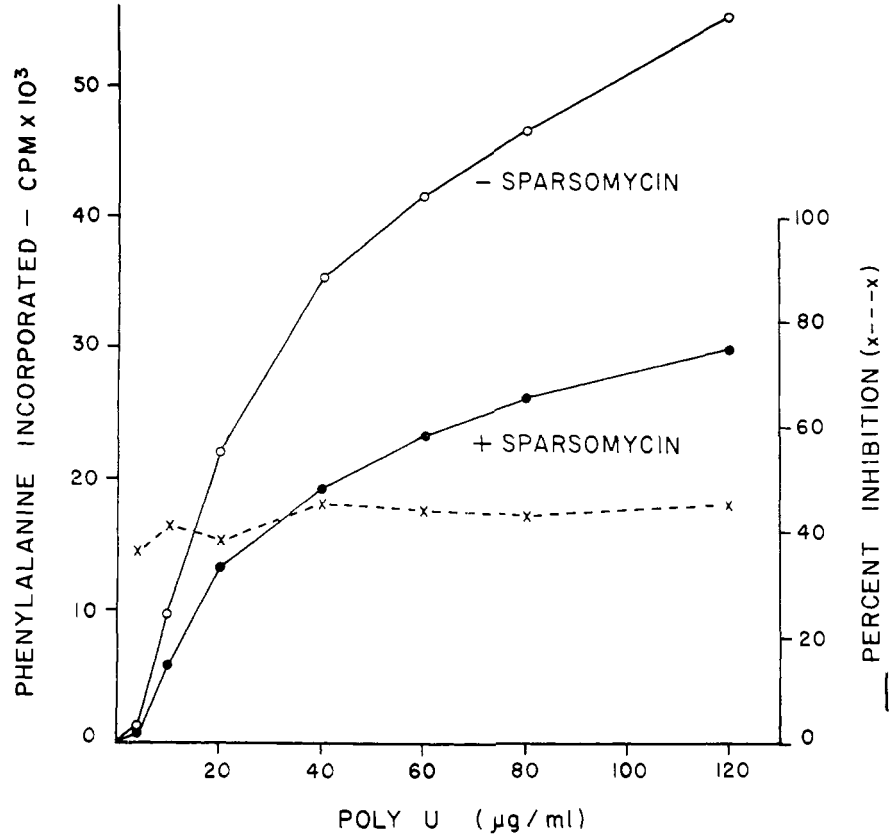


FIGURE 3: Effect of concentration of poly U on sparsomycin inhibition. The standard reaction mixture with or without sparsomycin ($1.58 \times 10^{-6}$ M) contained 5 mμmoles of [$^{14}$C]phenylalanine (165 μc/μmole). Poly U was varied as indicated. After incubation for 15 min at 35°, incorporation into polyphenylalanine was measured as described in Methods. In the absence of added poly U, 1198 cpm were incorporated.

TABLE II: Effect of Sparsomycin on Incorporation of Amino Acids Stimulated by Poly CU (1:5).[a]

| [$^{14}$C]Amino Acid | Sparsomycin | Cpm Inc | % Inhibn |
|---|---|---|---|
| Leucine | – | 31,252 | |
| | + | 8,337 | 73 |
| Proline | – | 9,461 | |
| | + | 2,505 | 73 |
| Serine | – | 27,019 | |
| | + | 7,798 | 71 |

[a] The standard reaction mixture contained 5 mμmoles of [$^{14}$C]leucine, [$^{14}$C]proline, or [$^{14}$C]serine (100 μc/μmole each), 10 μg of poly CU (1:5), and sparsomycin ($5.28 \times 10^{-7}$ M) as indicated. After incubation for 15 min at 35°, the reaction was assayed for incorporation of radioactivity into polypeptide as described in Methods. In the absence of added synthetic polynucleotide 1179 cpm of [$^{14}$C]leucine, 79 cpm of [$^{14}$C]proline, and 422 cpm of [$^{14}$C]serine were incorporated.

tained 0.1 M Tris–HCl (pH 7.8), 0.01 M magnesium acetate, 0.05 M ammonium acetate, 0.001 M ATP, 0.0003 M GTP, 0.0075 M phosphoenolpyruvate, 80 μg of pyruvate kinase, 0.006 M β-mercaptoethanol, 0.2 μmole each of 19 nonlabeled amino acids and [$^{14}$C]-leucine (231 μc/μmole) or [$^{14}$C]valine (208 μc/μmole), 1 mg of sRNA, and 1 ml of S30 (nonpreincubated). After incubation for 40 min at 37°, the reaction was stopped with 5 ml of 10% TCA. The precipitate was washed twice with 5% TCA, dissolved in 1 M $NH_4OH$, reprecipitated by addition of an equal volume of 10% TCA, washed with 5% TCA, and suspended in 2.5 ml of 5% TCA. After resolution in $NH_4OH$, reprecipitation, and washing with 5% TCA, the precipitate was extracted with ethanol–ether (1:1) and finally with ether, as described by Kaji *et al.* (1965b). Dinitrophenylation and 6 N HCl hydrolysis of the product were carried out by the method of Kaji *et al.* (1965b). The hydrolysate was extracted with ether, and aliquots of both aqueous and ether layers were plated in planchets for counting in the gas-flow counter.

MS2 phage was a gift of Dr. D. Nathans. MS2 RNA was extracted from purified virus in 0.1 M sodium

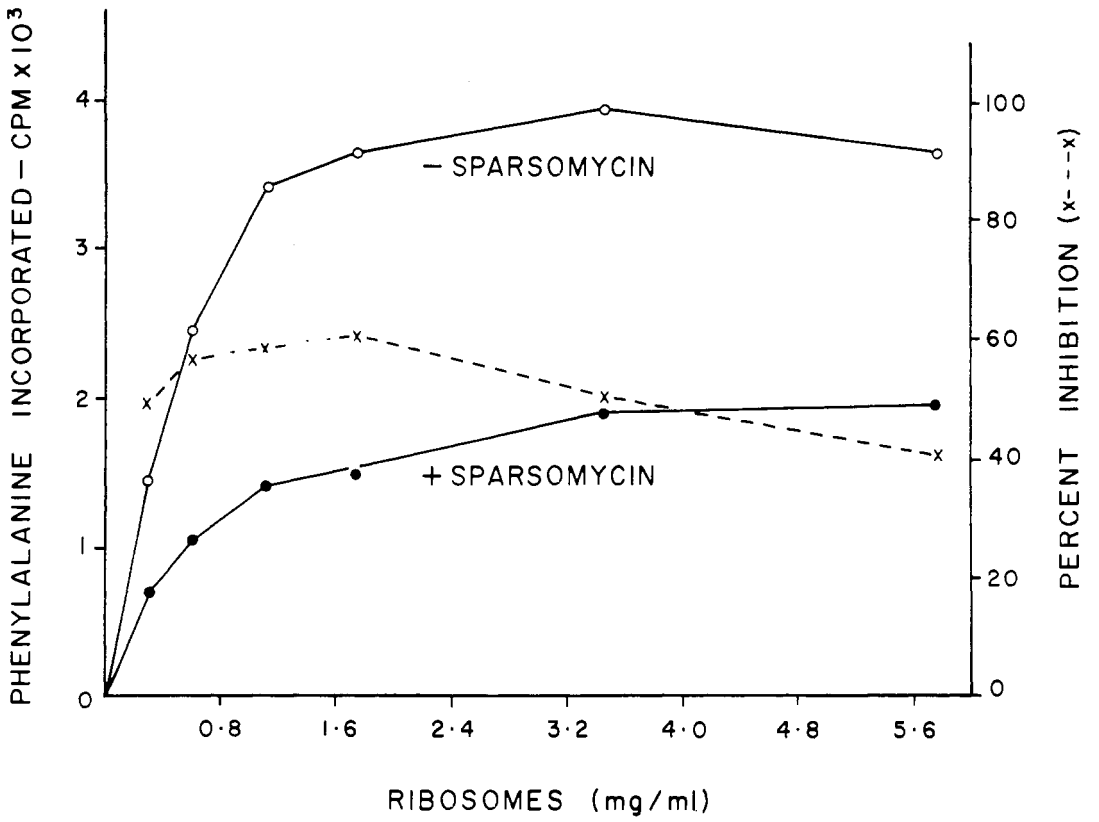


FIGURE 4: Effect of ribosome concentration on sparsomycin inhibition. The standard reaction mixture with or without sparsomycin ($2.64 \times 10^{-7}$ M) contained 5 mμmoles of [$^{14}$C]phenylalanine (25 μc/μmole), 10 μg of poly CU (1:5), 0.02 ml of S100, and the indicated amounts of washed ribosomes. After incubation for 15 min at 35°, the reaction was assayed for incorporation into polypeptide as described in Methods. Poly CU stimulated incorporation of [$^{14}$C]-phenylalanine was calculated by subtracting the incorporation found in the absence of poly CU at each concentration of ribosomes.

TABLE III: Effect of Gougerotin on Amino Acid Incorporation Promoted by Various Polynucleotides.[a]

| [$^{14}$C]Amino Acid | Polynucleotide | Incorporation (cmp) | | % Inhibn |
|---|---|---|---|---|
| | | Complete | +Gougerotin | |
| Phenylalanine | Poly U | 10,583 | 6,565 | 33 |
| Proline | Poly CU (1:1) | 19,716 | 5,893 | 70 |
| | Poly C | 704 | 140 | 80 |
| Lysine | Poly A | 19,711 | 15,370 | 22 |

[a] The standard reaction mixture contained 5 mμmoles of [$^{14}$C]phenylalanine (25 μc/μmole), [$^{14}$C]proline (100 μc/μmole), or [$^{14}$C]leucine (100 μc/μmole) and gougerotin ($4.26 \times 10^{-5}$ M) as indicated. After incubation at 35° for 5 min, the incorporation of [$^{14}$C]amino acid into polypeptide was assayed as described in Methods. In the absence of added polynucleotide, 86 cpm of [$^{14}$C]phenylalanine, 69 cpm of proline, or 1093 cpm of lysine were incorporated.

phosphate (pH 6.8) by two treatments with water-saturated phenol at 4°. The final aqueous layer was extracted six times with fresh ether and bubbled extensively with nitrogen to remove residual ether (Shimura *et al.*, 1965).

[$^{14}$C]Amino acids were obtained from the New England Nuclear Co. Synthetic polynucleotides were obtained from the Miles Chemical Co. *E. coli* sRNA (stripped) was purchased from General Biochemicals Co. [$^{14}$C]Chloramphenicol (8.5 mc/mmole, labeled in the terminal carbon of the dichloroacetamide side chain) was purchased from the California Corp. for Biochemical Research. Sparsomycin was a generous gift of Dr. C. G. Smith of the Upjohn Co.; gougerotin,

TABLE IV: Effect of Sparsomycin and Chlortetracycline on [$^{14}$C]Chloramphenicol Binding to Ribosomes.[a]

| | Total Cpm Associated with Ribosomes | Cpm Bound to Ribosomes | % Inhibn |
|---|---|---|---|
| Complete | 337 | 281 | – |
| +Sparsomycin | 325 | 269 | 4 |
| +Erythromycin | 78 | 22 | 92 |
| +[$^{12}$C]Chloramphenicol | 56 | – | – |

[a] The following were incubated in a volume of 2.5 ml: 0.01 M Tris–HCl (pH 7.4), 0.2 M KCl, 0.01 M $MgCl_2$, $2.6 \times 10^{-4}$ M sparsomycin, $2.5 \times 10^{-4}$ M erythromycin, or $3 \times 10^{-4}$ M [$^{12}$C]chloramphenicol as indicated, and 2.5 mg of washed ribosomes. After standing for 10 min at 4°, [$^{14}$C]chloramphenicol ($3 \times 10^{-6}$ M, 8.5 mc/mmole) was added to the reaction which was allowed to stand an additional 5 min in the cold (except for the tubes containing [$^{12}$C]chloramphenicol, to which [$^{14}$C]chloramphenicol was added just prior to centrifugation). The tubes were centrifuged at 50,000 rpm for 2 hr, decanted, and gently rinsed three times with cold buffer containing the above concentrations of Tris, KCl, and $MgCl_2$. The remaining pellets were dissolved in 0.5 ml of formic acid and 0.4-ml aliquots were transferred to vials. Absolute ethanol (4 ml) and toluene (10 ml) scintillation fluid were added, and radioactivity was determined in the liquid scintillation counter. The radioactivity found in the tubes to which [$^{12}$C]chloramphenicol had been added provided a correction for the [$^{14}$C]chloramphenicol trapped in the ribosomal pellet.

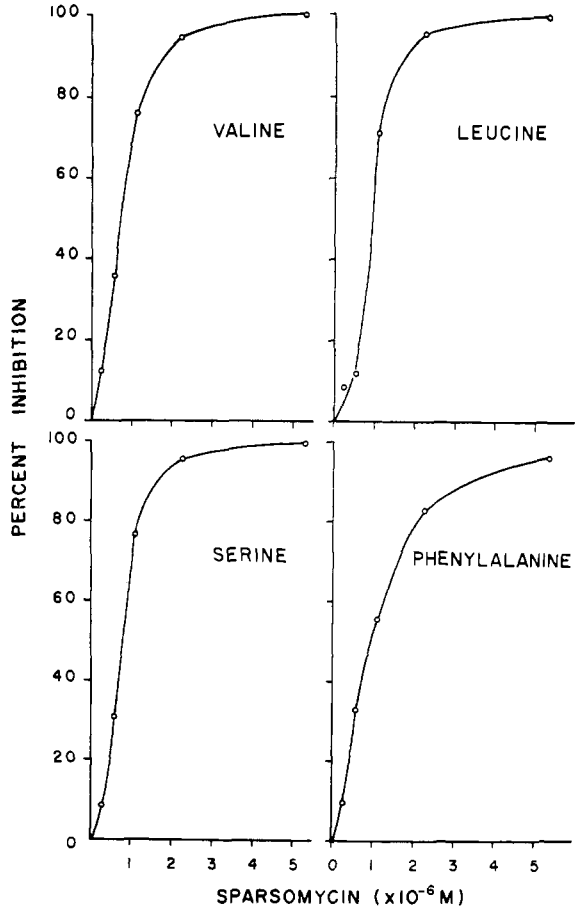


FIGURE 5: Effect of sparsomycin on MS2 RNA-promoted incorporation of amino acids. The following were incubated with the indicated amounts of sparsomycin in a final volume of 0.05 ml: 0.05 M Tris–HCl (pH 7.8), 0.015 M magnesium acetate, 0.003 M ATP, 0.05 M KCl, 0.0001 M GTP, 0 01 M phosphoenolpyruvate, 1.5 μg of pyruvate kinase, 0.01 M β-mercaptoethanol, 25 μg of sRNA, 1 mμmole each of 19 nonlabeled amino acids, and [$^{14}$C]phenylalanine (350 μc/μmole), [$^{14}$C]leucine (231 μc/μmole), [$^{14}$C]valine (208 μc/μmole), or [$^{14}$C]serine (115.5 μc/μmole), with or without 20 μg of MS2 RNA, and 0.01 ml of S30. After incubation for 30 min at 35°, the reaction was stopped and assayed as described in Methods. Incorporation stimulated by MS2 RNA in the absence of sparsomycin was 3314, 6617, 5665, and 5508 cpm for phenylalanine, valine, leucine, and serine, respectively.

a gift of Takeda Chemical Industries, Ltd., Osaka, Japan; and chloramphenicol, a gift of Parke, Davis and Co. Streptomycin sulfate and erythromycin were obtained from Eli Lilly and Co.; chlortetracycline was obtained from the Lederle Laboratories.

## Results

*Relation between Time Course and Sparsomycin Inhibition.* Throughout the time course poly C promoted incorporation of proline is most sensitive, poly A promoted incorporation of lysine next in sensitivity, and poly U promoted incorporation of phenylalanine least sensitive to inhibition by sparsomycin (Figure 1). These results resemble those of experiments in which the sparsomycin concentration was varied over a wide range at a fixed time of incubation (Goldberg and Mitsugi, 1966). This same spectrum of sensitivities was found for reactions starting with free amino acid or aminoacyl-sRNA.

*Sparsomycin Sensitivity of Copolymer-Promoted Incorporation of Amino Acids.* Polypeptide synthesis promoted by polynucleotide copolymers containing uridylate plus one of the other nucleotides is more sensitive to sparsomycin than that promoted by the uridylate homopolymer (Table I). The inclusion of guanylate or cytidylate confers greatest sensitivity. Similar results are found whether one starts with free phenylalanine or phenylalanyl-sRNA. As expected of a reaction dependent on all amino acids, the extent of sparsomycin inhibition of the incorporation of different labeled amino acids remains constant for any

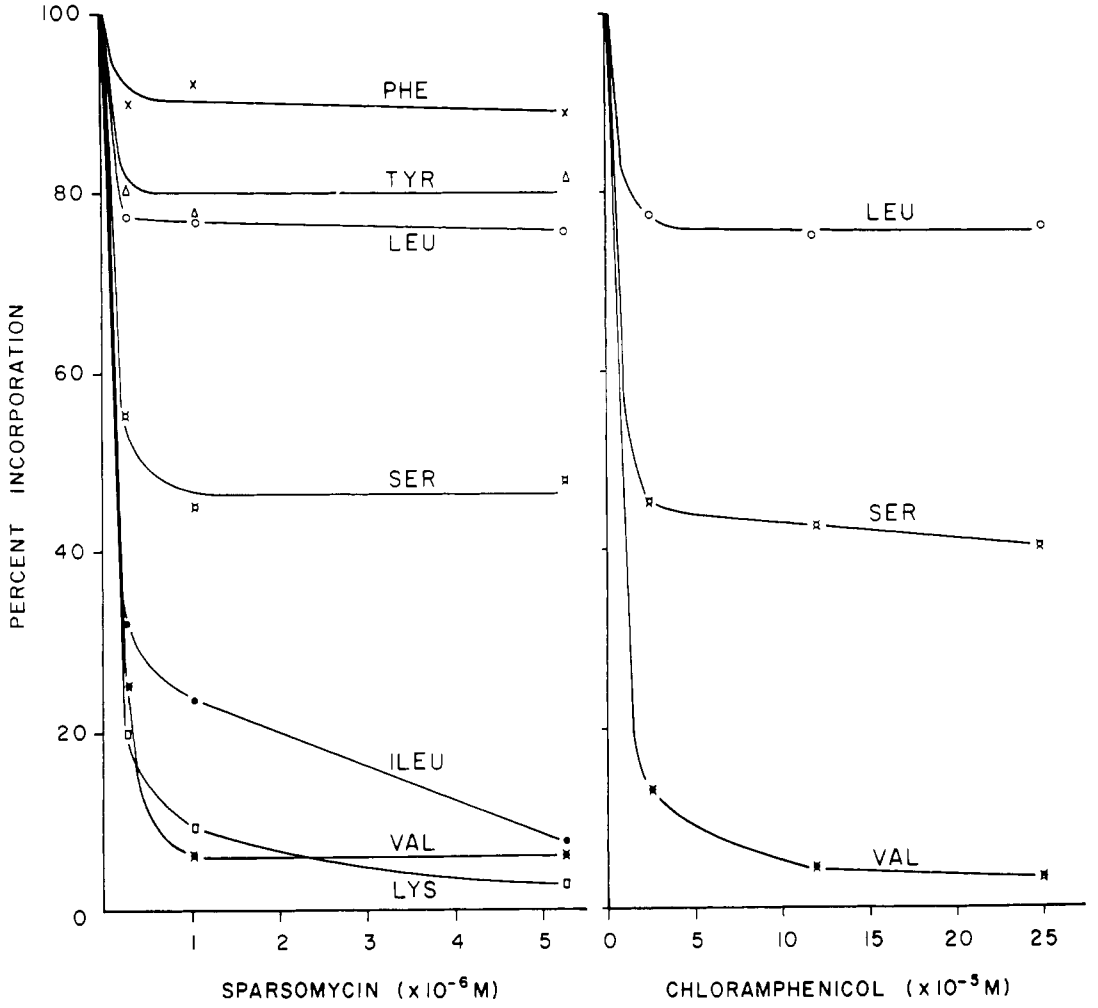


FIGURE 6: Effect of sparsomycin and chloramphenicol on endogenous protein synthesis. The standard reaction mixture contained 5 mμmoles of the indicated [$^{14}$C]amino acid (100 μc/μmole) and 0.05 ml of nonpreincubated S30. The concentration of sparsomycin and chloramphenicol was varied. After incubation fof 30 min at 35°, the reaction was assayed as described in Methods. In the absence of antibiotics, the incorporations of phenylalanine, tyrosine, leucine, serine, isoleucine, valine, and lysine were 1328, 2491, 915, 194, 395, and 419 cpm, respectively.

particular synthetic polynucleotide (Table II). Similar data have been obtained with other amino acids and other copolymers than cytidylate-containing ones.

These findings resemble those of Kućan and Lipmann (1964) and Speyer *et al.* (1963) with chloramphenicol. Further, Vazquez (1966b) has also reported the same sensitivity pattern of homopolymer-promoted polypeptide formation (poly C > poly A > poly U) for several other antibiotics which block the binding of chloramphenicol to ribosomes. On the other hand, the effectiveness of puromycin has been reported to be independent of the composition of the synthetic homopolynucleotide (Speyer *et al.*, 1963). It is of interest in this regard that we have found that gougerotin, which has been reported to compete with puromycin (Casjens and Morris, 1965), is most active against poly C promoted synthesis and somewhat less active against synthesis promoted by poly A than that promoted by poly U (Table III).

*Absence of Significant Effect of Sparsomycin on Binding of [$^{14}$C]Chloramphenicol to Ribosomes.* In view of the above-noted similarities of sparsomycin and chloramphenicol, the effect of sparsomycin on the binding of [$^{14}$C]chloramphenicol to ribosomes was studied. Whereas erythromycin ($2.5 \times 10^{-4}$ M) decreases binding of [$^{14}$C]chloramphenicol to ribosomes by 92%, sparsomycin ($2.64 \times 10^{-4}$ M) inhibits binding by only 4% (Table IV).

*Absence of Sparsomycin-Induced Misreading of the Code.* As seen in Table V, under conditions where streptomycin increases the incorporation of isoleucine promoted by poly U (Davies *et al.*, 1964), sparsomycin produced inhibition. Lowering the concentration of sparsomycin or varying the magnesium concentration

does not lead to detectable miscoding by sparsomycin. On the other hand, streptomycin appears to increase the sensitivity of the reaction to inhibition by sparsomycin (Table VI). The per cent inhibition by sparsomycin is the same for both the normally read and the misread amino acids.

TABLE V: Effect of Antibiotic of Poly U Directed Isoleucine Incorporation.[a]

| | [$^{14}$C]Isoleucine Inc (cpm) |
|---|---|
| Complete | 106 |
| +Sparsomycin | 59 |
| +Streptomycin | 1137 |

[a] The standard reaction mixture contained 5 mμmoles of [$^{14}$C]isoleucine (165 μc/μmole), sparsomycin (10 μg/ml), or streptomycin (4 μg/ml) as indicated, 10 μg of poly U, 216 μg of washed ribosomes, and 0.02 ml of S100 instead of S30. After incubation at 35° for 15 min, incorporation of [$^{14}$C]leucine into polypeptide was assayed as described in Methods.

*Lack of Sparsomycin Effect on Formation of the Ternary Complex.* While sparsomycin blocks the synthesis of polypeptide from aminoacyl-sRNA, it does not interfere with the formation of the ternary complex (aminoacyl-sRNA–mRNA–ribosome) active in protein synthesis. As shown in Table VII, a high level of sparsomycin ($2.6 \times 10^{-4}$ M) does not significantly affect polynucleotide-promoted binding of [$^{14}$C]aminoacyl-sRNA to ribosomes. This is true for both poly CU (1:5) and poly A stimulated binding; polypeptide synthesis promoted by these two polynucleotides are quite sensitive to sparsomycin (50% inhibition at about $3 \times 10^{-7}$ M). Further, decreasing the ribosome concentration so as to increase the ratio of aminoacyl-sRNA molecules to ribosomes from about 2 to 10 does not make the binding reaction sensitive to sparsomycin. Similarly, altering the magnesium concentration (0.01–0.02 M) with lysyl-sRNA and poly A does not confer sparsomycin sensitivity on the binding reaction, although the binding itself is less at the higher magnesium concentration. In these experiments comparable concentrations of CTC, as previously reported (Suarez and Nathans, 1965; Hierowski, 1965), cause significant inhibition of binding.

Similarly, as shown in Figure 2, sparsomycin does not interfere with the binding of [$^{3}$H]poly U to ribosomes and the formation of ribosomal aggregates. Consistent with these results are experiments in which it has been found that the degree of inhibition of polypeptide synthesis is independent of the time of addition of sparsomycin to the reaction.

*Relation of Sparsomycin Inhibition to mRNA, Ribosome, sRNA, or Magnesium Concentration.* The inhibition of polypeptide synthesis at a fixed level of sparsomycin is not decreased by increasing concentrations of poly U (Figure 3), ribosomes (Figure 4), or sRNA. While there appears to be some decrease in the sparsomycin effect when ribosomes are present in excess, this is not found when the ribosome concentration is rate limiting. Likewise, sparsomycin was not found to result in a shift in the concentration of magnesium optimal for polypeptide synthesis. In other experiments we have found that when sparsomycin is preincubated with S30 under conditions of protein synthesis, both ribosomes and soluble fraction are irreversibly inactivated (M. Yukioka and I. H. Goldberg, 1966, unpublished data).

*MS2 RNA as Messenger.* Figure 5 shows the relationship between the sensitivity to sparsomycin of polypeptide synthesis promoted by MS2 RNA and the amino acid being incorporated. In accord with the experience with synthetic polynucleotides, sensitivity to sparsomycin is a function of the RNA used as messenger and not of the particular labeled amino acid whose incorporation is being followed. Polypeptide formation directed by MS2 RNA is about one-half as susceptible to inhibition by sparsomycin as is polylysine synthesis due to poly A.

*Endogenous mRNA.* It appeared at first that when ribosomes were studied for amino acid incorporating ability due to endogenous mRNA somewhat different results were obtained than those described above. As shown in Figure 6, the incorporation of certain amino acids appears to be quite resistant to inhibition by high concentrations of sparsomycin. Further, inhibition levels off so that additional increases in concentration of sparsomycin do not result in increased inhibition.

Similar findings, with these same amino acids, were obtained with chloramphenicol. The results with leucine qualitatively resemble those of Kućan and Lipmann (1964) who commented on the apparent resistance of endogenous RNA-promoted polypeptide synthesis to the action of chloramphenicol. Our results suggested that for several of the amino acids (phenylalanine, tyrosine, leucine, and to a lesser extent serine), a large fraction of the total incorporation was completely resistant to the antibiotics. When other *E. coli* extracts which are more active in endogenous RNA-promoted polypeptide synthesis are used, a different pattern emerges. In such instances incorporations of these same amino acids are considerably more sensitive to the antibiotics (Figure 7). These results suggest that the antibiotic-resistant fraction might be independent of the ribosomes and due to the chloramphenicol-insensitive, soluble amino acid incorporation system described by Kaji *et al.* (1965a,b). In this system, leucine and phenylalanine are most efficiently transferred from aminoacyl-sRNA to the $NH_2$-terminal end of an acceptor protein. Support for such an explanation of the data reported here comes from (1) experiments in which ribosomes are omitted,

TABLE VI: Effect of Sparsomycin on Streptomycin-Induced Miscoding.[a]

| | Incorporation (cpm) | | Poly U Stimulated Incorporation | % Inhibn by Sparsomycin |
|---|---|---|---|---|
| | Without Poly U | With Poly U | | |
| | Phenylalanine Incorporation | | | |
| Complete | 758 | 29,162 | 28,404 | |
| +Sparsomycin | 718 | 21,305 | 20,587 | 27 |
| +SM | 819 | 19,391 | 18,572 | |
| +SM, +sparsomycin | 717 | 10,767 | 10,052 | 46 |
| | Isoleucine Incorporation | | | |
| Complete | – | 895 | – | |
| +SM | 331 | 7,953 | 7,622 | |
| +SM, +sparsomycin | 120 | 4,004 | 3,884 | 49 |

[a] The following were incubated with or without streptomycin (SM) (10 $\mu$g/ml) and sparsomycin ($5.28 \times 10^{-7}$ M) in a volume of 0.25 ml: 0.1 M Tris–HCl (pH 7.8), 0.018 M magnesium acetate, 0.05 M ammonium acetate, 0.001 M ATP, 0.0003 M GTP, 0.0075 M phosphoenolpyruvate, 4 $\mu$g of pyruvate kinase, 0.006 M $\beta$-mercaptoethanol, 5 m$\mu$moles each of 19 nonlabeled amino acids and [$^{14}$C]phenylalanine (104 $\mu$c/$\mu$mole) or [$^{14}$C]isoleucine (165 $\mu$c/$\mu$mole), 20 $\mu$g of poly U, 200 $\mu$g of sRNA, and 0.05 ml of S30. Incubation was for 15 min at 37°.

TABLE VII: Aminoacyl-sRNA Binding to Ribosomes.[a]

| [$^{14}$C]Aminoacyl-sRNA | Ribosomes ($\mu$g/0.1 ml) | Additions | Cpm Bound | % Inhibn |
|---|---|---|---|---|
| | | Experiment 1 | | |
| Phe-sRNA | 335 | Complete | 1539 | – |
| | | –Poly U | 120 | – |
| | | +Sparsomycin ($2.6 \times 10^{-4}$ M) | 1570 | 0 |
| | | +CTC ($2.0 \times 10^{-4}$ M) | 980 | 36 |
| | | Experiment 2 | | |
| Phe-sRNA | 170 | Complete | 1013 | – |
| | | –Poly CU (1:5) | 78 | – |
| | | +Sparsomycin ($2.6 \times 10^{-4}$ M) | 1085 | – |
| | | +CTC ($2.0 \times 10^{-4}$ M) | 527 | 48 |
| | 85 | Complete | 635 | – |
| | | –Poly CU (1:5) | 49 | – |
| | | +Sparsomycin ($2.6 \times 10^{-4}$ M) | 626 | 1 |
| | | +CTC ($2.0 \times 10^{-4}$ M) | 349 | 45 |
| | 34 | Complete | 281 | – |
| | | –Poly CU (1:5) | 18 | – |
| | | +Sparsomycin ($2.6 \times 10^{-4}$ M) | 275 | 2 |
| | | +CTC ($2.0 \times 10^{-4}$ M) | 189 | 33 |
| | | Experiment 3 | | |
| Lys-sRNA | 170 | Complete | 1089 | – |
| | | –Poly A | 170 | – |
| | | +Sparsomycin ($2.6 \times 10^{-4}$ M) | 1000 | 8 |
| | | +CTC ($2.0 \times 10^{-4}$ M) | 429 | 62 |

[a] Each 0.1-ml reaction mixture contained 0.05 M Tris–HCl (pH 7.4), 0.02 (expt 1 and 2) or 0.01 M (expt 3) magnesium acetate, 0.1 M ammonium acetate, 10 $\mu$g of polynucleotide (expt 1, poly U; expt 2, poly CU (1:5), expt 3, poly A), the indicated amounts of ribosomes, and antibiotics. The tubes were kept at 0° and 67 $\mu$g (6200 cpm) of [$^{14}$C]Phe-sRNA was added last to initiate binding in expt 1 and 50 $\mu$g (4660 cpm) in expt 2; 98 $\mu$g (10,200) cpm of [$^{14}$C]Lys-sRNA was added in expt 3. After incubation for 20 (expt 1) or 10 min (expt 2 and 3) at 24°, each reaction was assayed for binding to ribosomes by the Millipore filter technique of Nirenberg and Leder (1964).

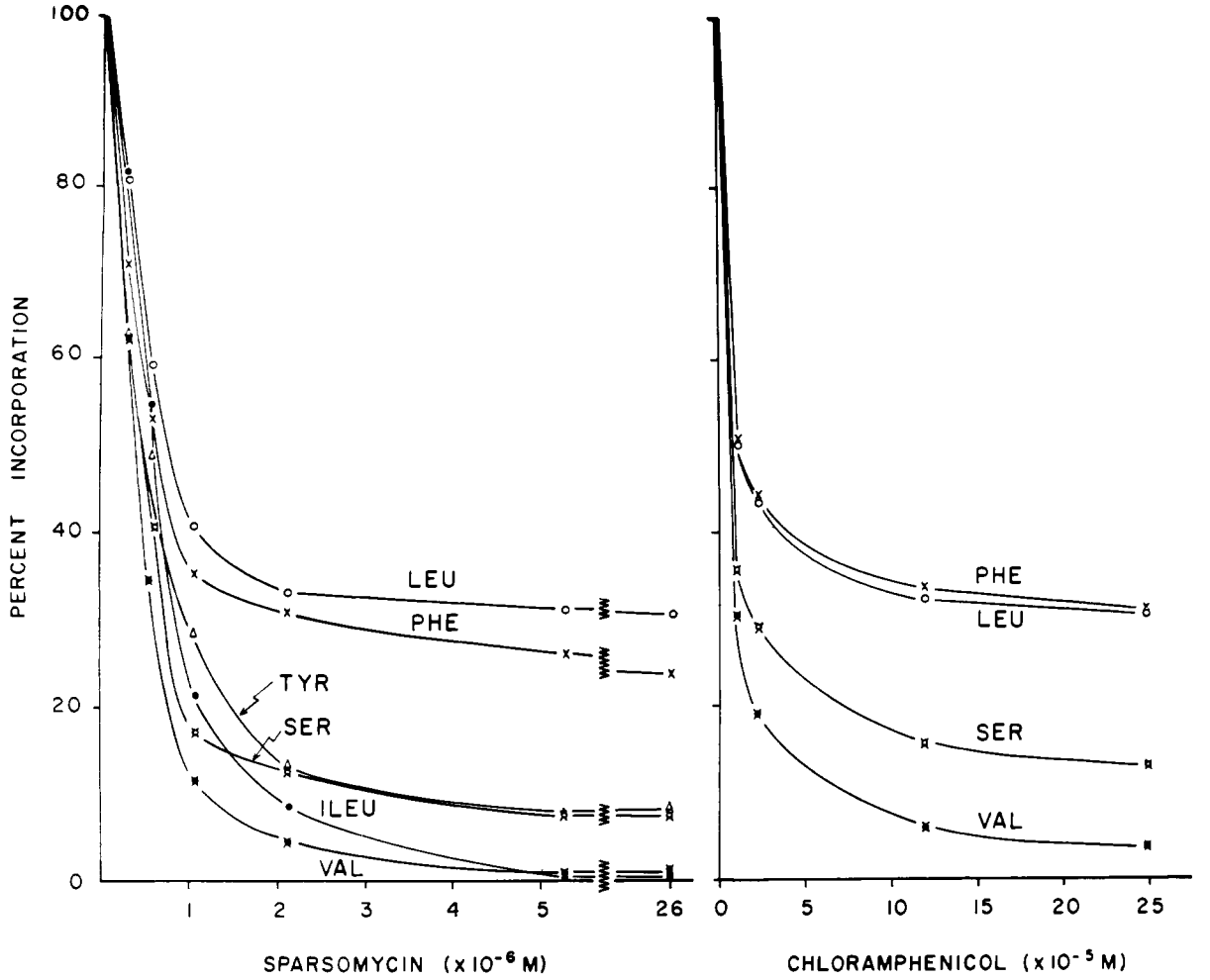


FIGURE 7: Effect of sparsomycin and chloramphenicol on endogenous protein synthesis. The conditions used were the same as described in Figure 6 except that the reaction was carried out on one-fifth the scale (0.05 ml), and 0.01 ml of S30 prepared from a different batch of *E. coli* was used. In the absence of antibiotics, the incorporations of leucine, phenylalanine, serine, tyrosine, isoleucine, and valine were 2722, 2104, 2961, 775, 1631, and 2029 cpm, respectively.

and (2) identification of the incorporated amino acid predominately at the amino end of a peptide chain for an amino acid whose incorporation is resistant to the antibiotics and predominately within the peptide for one sensitive to the antibiotics. Thus, 78% of the [$^{14}$C]-leucine incorporated into protein by the nonpreincubated S30 preparation used in Figure 6 is at the $NH_2$-terminal end, while only 23% of the [$^{14}$C]valine incorporated by the nonpreincubated S30 preparation used in Figure 7 is at the $NH_2$-terminal end. These results point out that endogenous polypeptide synthesis dependent on ribosomes is relatively sensitive to sparsomycin and chloramphenicol when compared to that promoted by exogenously added synthetic or natural RNAs. In the case of sparsomycin, results with polypeptide formation by the rat liver cell-free system lead to similar conclusions (T. L. Steck and I. H. Goldberg, 1966, unpublished data).

## Discussion

Sparsomycin is a potent inhibitor of cell-free peptide bond formation. It is more effective than chloramphenicol or gougerotin as an inhibitor of polypeptide synthesis and of the puromycin-induced release of lysine peptides from polylysyl-sRNA bound to ribosomes (Goldberg and Mitsugi, 1967). All three antibiotics appear to act after formation of the ternary complex and interfere ultimately with the peptide bond forming step itself.

Sparsomycin, unlike chloramphenicol, is a potent inhibitor of mammalian cell-free polypeptide synthesis, but resembles chloramphenicol in being more active against polypeptide formation promoted by synthetic polynucleotides low in uridylate content. The molecular basis for this latter finding is not yet apparent. In the case of chloramphenicol it has been

related to an effect of the antibiotic on the binding of the polynucleotide messenger to the ribosome (Weisberger and Wolfe, 1964). Polynucleotides high in uridylate have less secondary structure, bind more efficiently to ribosomes at ordinary temperatures, and presumably compete more effectively with chloramphenicol for binding. The data in support of such a mechanism for chloramphenicol action are not persuasive and are equally unconvincing in the case of sparsomycin. Additional evidence suggesting an effect of chloramphenicol on messenger interaction with ribosomes comes from experiments showing polypeptide formation promoted by previously bound (endogenous) mRNA to be more resistant to the antibiotic than that due to added natural or certain synthetic polynucleotides. We have found, however, that when the antibiotic-resistant, ribosome-independent addition of labeled amino acid onto the $NH_2$-terminal end of acceptor protein is taken into account, the sensitivities of polypeptide formation promoted by either type of messenger are comparable.

It is probable that sparsomycin has a different primary effect on protein synthesis than those of gougerotin and chloramphenicol. Gougerotin, unlike sparsomycin and chloramphenicol, is less effective against poly A promoted polylysine formation than against poly U promoted polyphenylalanine formation. Further, poly U promoted incorporation is very much more resistant to chloramphenicol, when compared with that promoted by the other two homopolynucleotides (Speyer *et al.*, 1963), than is the case with sparsomycin (2–3 logs *vs.* 1 log, respectively). Finally, sparsomycin and chloramphenicol do not share the same site of interaction with the protein-synthesizing system, since sparsomycin has little effect on chloramphenicol binding to ribosomes.

Another clear difference between the action of sparsomycin and that of chloramphenicol and gougerotin is revealed by experiments in which the oligolysines produced in a poly A dependent reaction are analyzed by carboxymethylcellulose or paper chromatography (M. Yukioka and I. H. Goldberg, in preparation). These data show that di- and trilysines accumulate in the presence of chloramphenicol, as reported by Julian (1965), and gougerotin, while formation of higher oligomers is inhibited. With added sparsomycin, however, synthesis of the di- and tripeptides is also blocked. Such a result might be expected of an agent, such as tetracycline, which interferes with formation of the ternary complex. Since sparsomycin has little, if any, effect, under various incubation conditions, on the polynucleotide-dependent binding of aminoacyl-sRNA to ribosomes, it appears that this antibiotic exerts its inhibitory effect on a subsequent reaction involved in the formation of the first peptide bond.

Puromycin has been reported to be equally effective against polypeptide formation promoted by poly U, poly C, and poly A (Speyer *et al.*, 1963). It is not clear how this finding is related to the action of sparsomycin, which does exhibit polynucleotide base composition specificity, yet competes competitively with puromycin in the peptide release reaction (Goldberg and Mitsugi, 1967). While it appears possible that allosteric effects may be important in the mechanism of action of some of these antibiotics, further speculation in the case of sparsomycin should be deferred until its chemical structure is known.

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# Inhibition by Sparsomycin and Other Antibiotics of the Puromycin-Induced Release of Polypeptide from Ribosomes*

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ABSTRACT: The antibiotic sparsomycin is a highly effective inhibitor of the puromycin-induced release of polypeptide (as polypeptidylpuromycin) from ribosomes. Sparsomycin blocks the puromycin-induced release of polyphenylalanine from prelabeled ribosomes, the initial rate being primarily affected. Similarly, sparsomycin, at very low concentrations ($10^{-6}$–$10^{-7}$ M), prevents the puromycin ($10^{-4}$ M) induced release of polylysine from polylysyl soluble ribonucleic acid (polylysyl-sRNA) bound to ribosomes in the presence of polyadenylic acid (poly A). The inhibition of the puromycin reaction by sparsomycin is not due to an effect of the antibiotic on the poly A dependent binding of polylysyl-sRNA to ribosomes. Gougerotin and chloramphenicol also failed to interfere with polylysyl-sRNA binding to ribosomes but inhibited the puromycin reaction at levels almost 2 logs greater than required with sparsomycin. Kinetic analysis reveals that sparsomycin is a competitive inhibitor of puromycin in this reaction, while gougerotin and chloramphenicol exhibit "mixed" types of inhibition kinetics. These data suggest that sparsomycin interferes with the peptide bond-forming step, either directly or by an allosteric mechanism.

As part of a study using antibiotics to help clarify the steps involved in nucleic acid (Goldberg and Reich, 1964) and protein synthesis, we have examined the mechanism of action of sparsomycin, a highly effective inhibitor of *in vitro* protein synthesis in *Escherichia coli* (Goldberg and Mitsugi, 1966, 1967). Sparsomycin resembles chloramphenicol in (1) being most effective against polypeptide synthesis promoted by synthetic polynucleotides with low uridylate content, and (2) blocking polypeptide formation at a point beyond the attachment of aminoacyl-sRNA to ribosomes. Sparsomycin, however, differs from chloramphenicol in being active at lower levels and being highly effective in mammalian systems *in vivo* (Goldberg and Mitsugi, 1966) and *in vitro* (Colombo *et al.*, 1966; T. L. Steck and I. H. Goldberg, 1966, unpublished data). Further, sparsomycin does not affect the binding of [$^{14}$C]chloramphenicol to ribosomes (Goldberg and Mitsugi, 1967). These results suggested that sparsomycin acts at or close to the peptide bond-forming reaction itself, but differs from chloramphenicol in precise interaction with the protein-synthesizing system.

Puromycin interferes with polypeptide chain elongation by acting as an analog of aminoacyl-sRNA and partaking in the peptide bond forming reaction, leading to the release of peptide as peptidylpuromycin from sRNA bound to ribosomes (Nathans, 1964). Accordingly, this reaction has proved to be a useful model for study of the formation of a single peptide bond (Nathans and Lipmann, 1961). We have followed peptide release due to puromycin using (1) zone centrifugation analysis of prelabeled ribosomes incubated with puromycin (Gilbert, 1963; Traut and Monro, 1964), and (2) the elegant, simplified procedure of Rychlik (1965a) employing polylysyl-sRNA, polyadenylic acid, and washed ribosomes. This paper will describe the effects of sparsomycin, gougerotin, and chloramphenicol on this reaction. Gougerotin, which is a dipeptidylpyrimidine nucleoside antibiotic (Fox *et al.*, 1964), also blocks protein synthesis beyond the stage of attachment of aminoacyl-sRNA (Clark and Chang, 1965), and has been reported to inhibit the puromycin reaction in a competitive fashion (Casjens and Morris, 1965). Like sparsomycin, it is effective against mammalian systems, but it is much less so, and is as potent an inhibitor of poly U promoted

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