SPARSOMYCIN, AN INHIBITOR OF AMINOACYL TRANSFER TO POLYPEPTIDE*

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Sparsomycin ($C_{13}H_{21}N_3O_6S_2$), a sulfur-containing antibiotic of unknown structure (Argoudelis and Herr, 1962), has been found to inhibit the growth of gram-negative and gram-positive bacteria, human epidermoid carcinoma cells in culture, a variety of transplantable neoplasms, and fungi (Owen et al., 1962). Against Escherichia coli this agent is initially bacteriostatic and slowly bactericidal (Slechta, 1963). In E. coli protein synthesis has been reported to be inhibited more readily than RNA synthesis (Slechta, 1963, 1965). Recently this agent has been reported to alter the lethal action of ionizing irradiation on E. coli (Pittillo et al., 1965).

We have found sparsomycin to be an effective inhibitor of polypeptide synthesis in the E. coli cell-free amino acid incorporating system and have localized its action to a point between the formation of the active ternary complex (aminoacyl-sRNA, messenger RNA and ribosome) and peptide bond synthesis. Further, this agent, like chloramphenicol, inhibits the initial rate of the puromycin-induced release of polypeptide and its effectiveness against polypeptide synthesis increases as the content of nucleotides other than uridylate increases in the synthetic messenger RNA.

Materials and Methods

Preparation of E. coli B S30, washed ribosomes and high speed supernate (S105) was carried out and assayed as described by Nirenberg (1964) except that ammonium acetate replaced KCl. C^{14}-phenylalanyl-sRNA was prepared by the method of

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Nathans and Lipmann (1961). H^3-polyuridylate (10 uc/umole) was synthesized by polymerization of H^3UDP (Schwarz Bioresearch, Inc. Orangeburg, N.Y.) with polynucleotide phosphorylase from E. coli (Thang et al., 1965) (a gift of Dr. E. Reich). Binding of H^3-polyuridylate to ribosomes was determined by the sucrose density gradient centrifugation technique of Spyrides and Lipmann (1962).

Polypeptide synthesis was measured by the incorporation of C^{14}-amino acid into hot trichloracetic acid (TCA)-precipitable material isolated and washed on Millipore filters (Nirenberg, 1964). Polylysine formation was assayed as described by Gardner et al. (1962). Binding of C^{14}-phenylalanyl-sRNA to ribosomes was determined by the method of Nirenberg and Leder (1964). The puromycin-induced release of polyphenylalanine from polyphenylalanyl-sRNA on ribosomes was followed by zone centrifugation (Traut & Monro, 1964). Mouse fibroblasts (L cells) were grown in suspension culture in Eagles' medium. Protein and nucleic acid synthesis were measured by the incorporation of C^{14}-uridine into RNA, H^3-thymidine into DNA and H^3-leucine into protein. Radioactivity was measured in a Beckman Low Beta gas-flow counter (C^{14}) or in a Packard Liquid Scintillation Spectrometer (H^3). Counting efficiencies for C^{14} and H^3 were 20%. E. coli sRNA was purchased from General Biochemicals Co., Chagrin Falls, Ohio; synthetic polynucleotides from Miles Chemical Co., Elkhart, Indiana; radioactive amino acids from New England Nuclear Co., Boston, Massachusetts. Sparsomycin was obtained through the generosity of Dr. C. G. Smith of the Upjohn Co., Kalamazoo, Michigan.

Results

Sparsomycin is an effective inhibitor of protein synthesis in L cells grown in suspension culture (Fig. 1). When incubation with labeled precursor in the presence of sparsomycin is for one hour, RNA synthesis is inhibited a maximum of 25 per cent while protein synthesis is inhibited by 95 per cent. With increasing times of incubation there is further inhibition of RNA synthesis. DNA synthesis has been found to be intermediate to protein and RNA in sensitivity to sparsomycin.

In the E. coli cell-free protein synthesizing system of Nirenberg, sparsomycin at low concentrations inhibits polypeptide synthesis from free amino acid (Fig. 2).

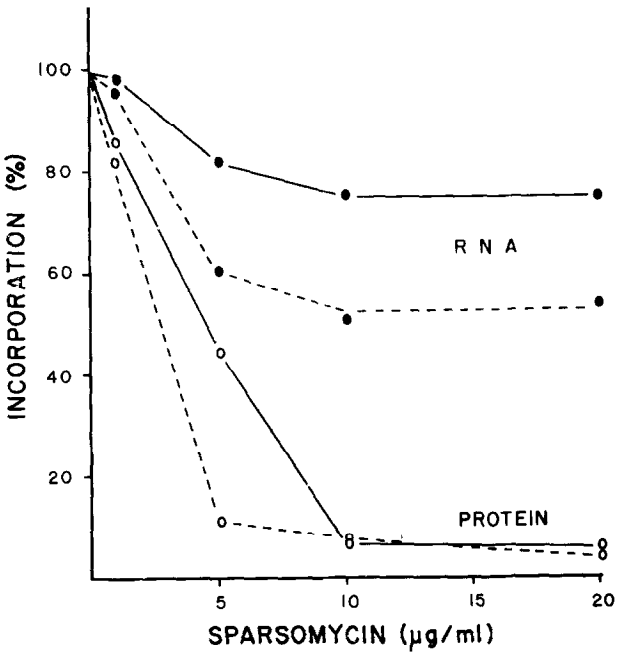

Fig. 1 Effect of sparsomycin on protein and RNA synthesis in L cells. L cells (1.8×10^6 in 4.5 ml Eagles' medium with 10% fetal calf serum) were preincubated for 15 minutes at 37° with the indicated amounts of sparsomycin. 2-C^{14}-uridine (0.1 uc, 8 m umoles) and H^3-L-leucine (2 uc, 0.4 m umoles) were added and incubation continued for either 60 minutes (solid line) or 120 minutes (dotted line). Duplicate 1 ml aliquots were then removed, centrifuged at 1000 RPM for 5 minutes, and the cells washed twice with cold phosphate buffered saline before collection on Millipore filters and washing with 10 ml of 5% TCA. Incorporation of H^3-leucine (open circles) and C^{14}-uridine (closed circles) were measured simultaneously in the Packard Scintillation Spectrometer in the presence of a toluene scintillator mixture. In the absence of antibiotic 1615 and 3365 cpm were incorporated into RNA at 60 and 120 minutes respectively; 2120 and 3867 cpm were incorporated into protein at 60 and 120 minutes respectively.

Polyphenylalanine synthesis directed by poly U is not as sensitive as polylysine synthesis directed by poly A and the latter is less sensitive than polyproline synthesis directed by poly C. The 50 per cent inhibition point with sparsomycin (MW, 379) is about 1.6×10^{-6}M for poly U, 2.6×10^{-7}M for poly A and 1.3×10^{-7}M for poly C in a 5 minute incubation. Since it has been found that sparsomycin affects the initial rate of polypeptide synthesis, the inhibition produced by a given concentration of antibiotic depends on the duration of incubation. In a series of experiments, similar to those reported by Kucan and Lipmann (1964), in which synthetic polynucleotide copolymers containing various amounts of adenylate,

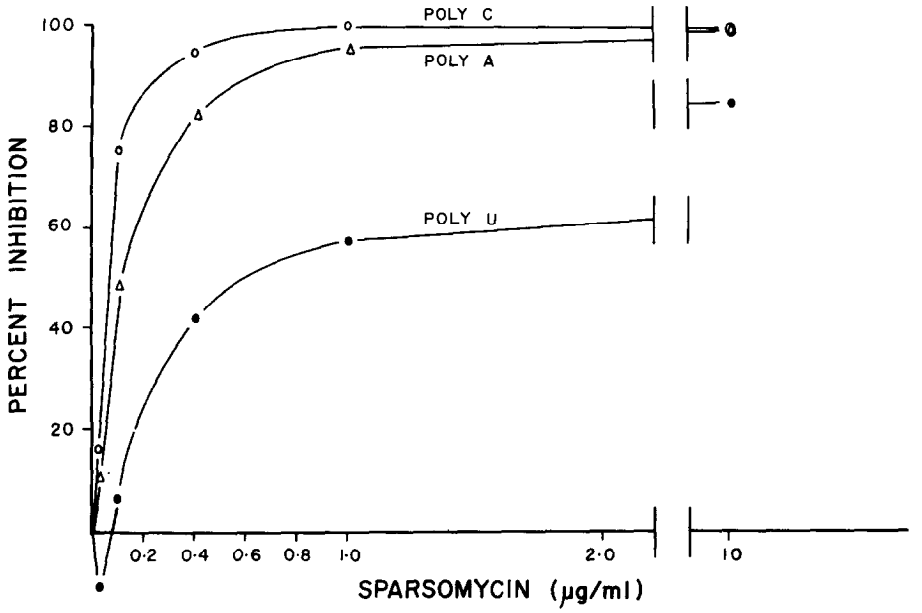

Fig. 2 Inhibition by sparsomycin of homopolynucleotide-promoted amino acid incorporation. The following were incubated in a volume of 0.25 ml: 0.1 M Tris HCl, pH 7.7; 0.012 M magnesium acetate; 0.05 M ammonium acetate; 0.001 M ATP; 0.0003 M GTP; 0.0075 M phosphoenolpyruvate; 4 ug pyruvate kinase; 50 m umoles each of the 19 non-labeled amino acids and one C^{14} labeled amino acid (100 uc/umole); 50 ug s-RNA; 0.004 M B-mercaptoethanol. Poly U, poly A or poly C (20 ug) and sparsomycin was added as indicated. Incubation was for 5 minutes at 35°. Incorporation of amino acid into polypeptide was measured as indicated in Methods.

● ——— ● poly U promoted phenylalanine incorporation
Δ ——— Δ poly A promoted lysine incorporation
O ——— O poly C promoted proline incorporation

In the absence of sparsomycin incorporation of phenylalanine, lysine and proline are 34,023; 23,157 and 1,798 cpm respectively. Controls incubated without added homopolynucleotide were subtracted from the experimental values.

cytidylate or guanylate in addition to uridylate are used, the degree of inhibition by sparsomycin varies with the base composition of the polynucleotide messenger and not with the particular labeled amino acid being incorporated. Thus sparsomycin inhibition increases as the uridylate content of the copolymer decreases. Further, polypeptide synthesis directed by uridylate copolymers containing cytidylate is more sensitive to sparsomycin than that directed by uridylate copolymers having the same content of adenylate. These results resemble those found by Kucan and Lipmann (1964) and Speyer et al. (1963) with chloramphenicol.

Sparsomycin blocks protein synthesis beyond the esterification of sRNA. As

shown in Table I sparsomycin does not affect the formation of aminoacyl-sRNA with phenylalanine, lysine or proline.

Table I

Lack of Effect of Sparsomycin on Charging of sRNA

Labeled Amino Acid	Sparsomycin (ug/ml)	Cold TCA Precipitable Radioactivity (CPM)
PHE	-	10,957
	100	10,629
LYS	-	12,069
	100	12,729
PRO	-	13,194
	100	13,507

The following were incubated in a volume of 0.25 ml: 0.1 M Tris HCl, pH 7.2, 0.01 M $MgCl_2$, 0.004 M B-mercaptoenthanol, 0.004 M ATP, 0.01 M phosphoenolpyruvate, 10 ug pyruvate kinase, 0.00002 M C^{14}-amino acid (100 uc/umole), 25 ug of sRNA and 50 ul S105. After incubation for 5 minutes at 35°, 3 ml of cold 10% TCA was added and the precipitate washed with cold 5% TCA on Millipore filters before counting. Over 90% of the radioactivity incorporated was solubilized by heating at 90° in 10% TCA for 15 minutes.

The antibiotic, however, prevents the transfer of phenylalanine from phenylalanyl-sRNA to polypeptide (Table II).

Table II

Inhibition by Sparsomycin of Polyphenylalanine Synthesis from Phenylalanyl-sRNA

Time (min)	Sparsomycin (ug/ml)	CPM	% Inhibition
2	-	2564	-
8	-	3711	-
2	1	1230	52
8	1	2703	27
2	10	272	89
8	10	1324	64
2	100	103	96
8	100	869	77

The following were incubated in a volume of 0.25 ml: 0.05 M Tris HCl, pH 7.4; 0.16 M NH_4Cl, pH 7.4; 0.01 M magnesium acetate; 0.006 M B-mercaptoethanol; 0.009 M phosphoenolpyruvate; 10 ug pyruvate kinase; 0.0004 M GTP; 10 ug poly U; 67 ug C^{14}-phenylalanyl-sRNA (6,200 cpm, 330 uc/umole C^{14}-phenylalanine); 50 ul S-30. After incubation at 30° for the indicated time, the reaction was stopped with cold 10% TCA and assayed for incorporation into protein as described in Methods.

Again, the initial rate is affected so that the percentage inhibition decreases during the incubation. As was found when starting with free amino acid, the sensitivity to sparsomycin of polypeptide formation from phenylalanyl-sRNA increases with the content of cytidylate in the uridylate-containing copolymer.

In other experiments it has been found that 1) sparsomycin does not inhibit the binding of phenylalanyl-sRNA to ribosomes in the presence of poly U, 2) sparsomycin does not inhibit the binding of H^3-poly U to ribosomes, 3) sparsomycin inhibition is not affected by the concentration of ribosomes, poly U or sRNA, 4) sparsomycin does not induce miscoding but streptomycin increases the inhibition due to sparsomycin, 5) sparsomycin blocks the puromycin-induced release of polyphenylalanine from polyphenylalanyl-sRNA on prelabeled ribosomes; the initial rate of release being affected.

Discussion

Sparsomycin like tetracycline, chloramphenicol and puromycin affects protein synthesis at a stage beyond the formation of aminoacyl-sRNA. The action of sparsomycin can be clearly differentiated from that of tetracycline which affects aminoacyl-sRNA binding to ribosomes (Suarez and Nathans, 1965; Hierowski, 1965) and puromycin which leads to release of incomplete peptides (see Goldberg, 1965). Sparsomycin, while a more effective inhibitor of cell-free polypeptide synthesis than chloramphenicol, resembles chloramphenicol in several ways (Kucan and Lipmann, 1964; Speyer, et al., 1963; Wolfe and Hahn, 1965), especially as relates to the sensitivity of synthesis directed by various synthetic polynuleotides. This particular feature is different from that reported for chlortetracycline (Hierowski, 1965) and is not found with puromycin (Speyer et al., 1963). Further studies to elucidate the site of action of sparsomycin are in progress. These experiments, including the effect, if any, on C^{14}-chloramphenicol binding to ribosomes, as well as details of the experiments outlined in this report will be described elsewhere.

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