Biochemical Pharmacology, Vol. 18, pp. 287–294. Pergamon Press. 1969. Printed in Great Britain

EFFECT OF STEFFIMYCIN ON CELL-FREE POLYPEPTIDE SYNTHESIS

FRITZ REUSSER

Department of Microbiology, The Upjohn Company, Kalamazoo, Mich., U.S.A.

(*Received* 9 *May* 1968; *accepted* 28 *June* 1968)

Abstract—Steffimycin interferes with amino acid incorporation mediated by synthetic polyribonucleotides in cell-free bacterial systems. This inhibition is a secondary activity of the antibiotic, which acts primarily as a suppressor of RNA synthesis in bacteria and bacterial cell-free systems. Inhibition of peptide biosynthesis is apparent only at a drug concentration higher than that necessary for inhibition of RNA synthesis in cell-free systems. The chelating properties of the antibiotic toward divalent cations do not appear to be responsible for inhibition of peptide biosynthesis. Reversal studies suggested that excess transfer RNA but not 30S fraction or polyuridylic acid reduces steffimycin inhibition of amino acid incorporation. Difference spectra obtained from antibiotic–transfer RNA mixtures indicate that steffimycin binds to transfer RNA. It is concluded that the binding of steffimycin to transfer RNA is the basis for its interference with amino acid incorporation.

STEFFIMYCIN (in earlier publications referred to as antibiotic U-20,661) is a potent inhibitor of RNA synthesis in both whole bacterial cells and cell-free systems.[1] However, concentrations approximately ten times greater (25–50 μg/ml) than those required for suppression of RNA synthesis (2·5–5 μg/ml) cause partial inhibition of polypeptide synthesis in cell-free *Escherichia coli* systems.

This paper describes the effects of steffimycin on polyribonucleotide-directed polypeptide biosynthesis in a cell-free *E. coli* system as well as on isolated reaction sequences occurring within such a system.

MATERIALS AND METHODS

The cell-free polyribonucleotide-directed phenylalanine and proline incorporation systems were prepared as described by Nirenberg.[2] The 30S fraction was used as an enzyme source. Difference spectra of antibiotic–transfer RNA (tRNA) mixtures were measured as described previously.[1]

E. coli soluble RNA (tRNA), stripped, was purchased from General Biochemicals. The enzyme source used to catalyze acylation of tRNA with one ^{14}C-amino acid and unlabeled amino acids was a dialyzed ribosomal supernatant solution (100S) prepared as described by Nirenberg.[2] The incubation mixtures contained the following ingredients in a total volume of 0·5 ml: 10 μmole Tris·HCl buffer, pH 7·4; 5 μmole mercaptoethanol; 5 μmole phosphoenol pyruvate (trisodium salt); 1·5 μmole ATP; 0·1 μmole each of 19 unlabeled amino acids minus either phenylalanine or proline; 0·1 μc of either proline-^{14}C or phenylalanine-^{14}C; 10 μg pyruvate kinase (Calbiochem); 100 μg tRNA and 100S fraction amounting to 275 μg protein. The samples were incubated at 37° for 15 min. Separate studies had indicated that the reaction was complete

after this time. The reaction was stopped by the addition of 0·5 ml of cold 10% TCA containing 0·5 mg celite per ml. The acid-insoluble product was collected on millipore filters (0·45 μ). The tubes and filters were washed with 3 × 2 ml of 5% cold TCA, followed by 3 × 2 ml of 70% cold ethanol. The filters were then dried and assayed for radioactivity.

Polyribonucleotide attachment to ribosomes was assessed by the filtration technique of Moore.[3] The ribosomes were prepared as described by Nirenberg,[2] but were washed three times. In the binding studies with poly U, 2·4 O.D.$_{260}$ units of ribosomes suspended in 3 ml of 0·005 M Tris·HCl buffer, pH 7·4, containing 0·01 M magnesium acetate (Tris buffer) were adsorbed to millipore filters (0·45 μ, type HA) with slow suction. The ribosomes adsorbed to the filters were washed three times with 3-ml portions of Tris buffer, followed by 1 ml of a 5 mg/ml solution of crystalline bovine serum albumin dissolved in Tris buffer to reduce nonspecific binding of poly U to the filters. The filters were again washed with 2 × 3-ml portions of Tris buffer. Finally, 3 ml of a ^{3}H-poly U solution containing 0·015 μc (10 μg) and the antibiotic in Tris buffer was passed through the filters. The filters were washed with 3 × 3-ml portions of Tris buffer, dried and counted by liquid scintillation spectrometry. In the binding studies with poly C, the reactants were mixed prior to filtration, since poly C shows only negligible self-adsorption to the filters. The samples contained 2·4 O.D.$_{260}$ units of ribosomes, 0·015 μc of ^{3}H-poly C (10 μg) and antibiotic in a total volume of 0·15 ml Tris buffer. The mixtures were incubated at room temperature for 20 min, chilled in ice, diluted to 3 ml with Tris buffer and filtered through 0·45 μ millipore filters with slow suction. The filters were washed and assayed for adsorbed radioactivity as described for poly U.

The reaction mixtures used for the preparation of prolyl-^{14}C–tRNA and phenylalanyl-^{14}C–tRNA were those used for assessing transfer activity except: tRNA, 2 mg/ml; proline-^{14}C or phenylalanine-^{14}C, 8 μc/ml; 100S fraction, 550 μg protein/ml. The mixtures were incubated at 30° for 30 min and made acidic by adding 0·25 ml of 2 M sodium acetate buffer. The samples were extracted twice with an equal volume of water-saturated phenol followed by several ether extractions. Residual ether was removed by gassing with N_2. The charged tRNA was precipitated with 2·2 volumes of cold absolute ethanol. The precipitate was redissolved in enough 0·01 M K-PO_4 buffer, pH 6·1 to yield a tRNA concentration of 2 mg/ml.

Formation of the ternary ^{14}C-amino acyl–sRNA polyribonucleotide–ribosome complexes was determined by the Nirenberg–Leder technique.[4]

The radioactive materials used in these studies were purchased from the following sources: L-^{14}C-phenylalanine, 366 mc/m-mole, from New England Nuclear; L-^{14}C-proline, 125 mc/m-mole, from Nuclear Chicago, and ^{3}H-poly U, 12·7 mc/m-mole, and ^{3}H-poly C, 6·07 mc/m-mole, from Schwarz BioResearch.

RESULTS

Effect on polyribonucleotide-directed amino acid incorporation system. It was demonstrated earlier that poly U-directed ^{14}C-phenylalanine incorporation is moderately inhibited by relatively high concentrations of steffimycin in a cell-free *E. coli* amino acid incorporation system.[1] An antibiotic concentration of 50 μg/ml causes approximately 30 per cent inhibition of poly U-directed phenylalanine incorporation. These findings are now found to apply to a poly C-directed proline incorporation

TABLE 1. EFFECT OF STEFFIMYCIN ON POLY C-DIRECTED PROLINE INCORPORATION SYSTEM *in vitro**

Addition (per ml reaction mixture)	(cpm/activity mg 30S protein)	Per cent of control
None, control	884·7	100·0
Control, 0 min	29·8	3·3
Less poly C	44·1	4·9
Steffimycin, 50 μg	515·8	58·3
25 μg	721·9	81·5
10 μg	852·2	96·3

* Assay mixtures (0·25 ml) were those described by Nirenberg[2] with the following exceptions: Poly C, 15 μg; 30S protein, 1 mg. The mixtures were incubated at 37° for 15 min. The reaction was stopped by the addition of 3 ml of cold 10% TCA. The acid-insoluble product remaining after heating the samples for 20 min at 95° was collected on 0·45 μ millipore filters and counted by liquid scintillation spectrometry.

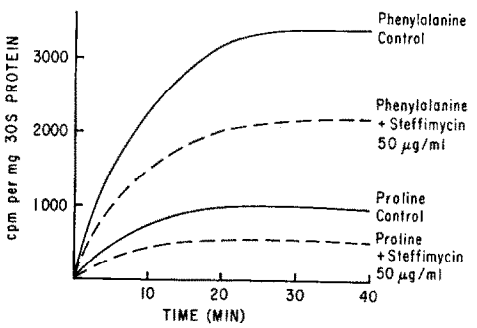

FIG. 1. Effect of steffimycin on polyribonucleotide-directed phenylalanine and proline incorporation. Assay mixtures (0·25 ml) were those described in reference 2 with the following exceptions: Poly U or poly C, 15 μg; 30S protein, 1 mg. The samples were incubated and assayed for incorporated label as described in Table 1. Incorporation of label in the absence of polynucleotide was 1–3 per cent of the control values.

system as well. In this latter case, a concentration of 50 μg/ml of antibiotic causes approximately 40 per cent inhibition of the reaction (Table 1). A concentration of 10 μg/ml, which is sufficient to cause approximately 50 per cent inhibition of RNA synthesis in a Chamberlain–Berg RNA polymerase system,[1] shows only negligible inhibition of poly C-directed proline incorporation. These results were derived from assays run for a constant length of time (15 min). Consequently, they do not allow one to conclude whether the observed inhibitions represent a reduction of the rate of peptide synthesis only, or whether the total amount of peptide formed is reduced as well. Time studies were therefore done with both the phenylalanine and proline incorporation systems. As shown in Fig. 1, the total amounts of amino acid incorporation in the presence of 50 μg/ml of steffimycin were reduced by 23 per cent in the case of phenylalanine and by 44 per cent in the case of proline. The inhibitory activity of the antibiotic results therefore in a net reduction of both the biosynthetic rate and the amount of product formed.

Steffimycin interacts with di- and trivalent cations and probably forms chelates with these ions.[5] The proper functioning of amino acid incorporation systems *in vitro*

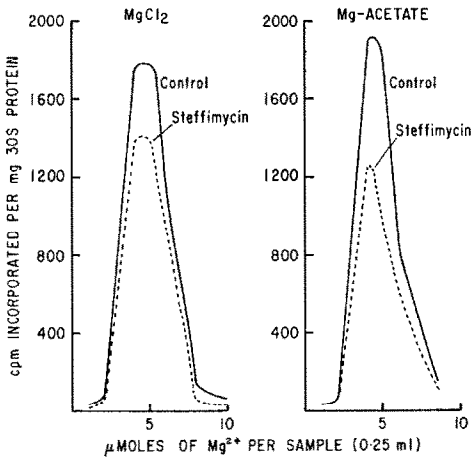

FIG. 2. Effect of Mg^{2+} concentration on steffimycin-induced inhibition of poly C-directed proline incorporation in a cell-free *E. coli* system. The assay conditions are those described in the legend of Table 1. Solid line = control; broken line = steffimycin, 50 μg/ml.

TABLE 2. EFFECT OF INCREASING AMOUNTS OF 30S FRACTION OR POLY U ON STEFFIMYCIN INHIBITION OF PHENYLALANINE INCORPORATION*

30S protein (mg/sample)	Poly U (μg/sample)	Incorporation (cpm/sample)		Inhibition (%)
		Control	Steffimycin (100 μg/ml)	
1·2		5866·0	3542·2	39·6
0·8		3130·9	1586·7	49·4
0·4		1002·3	588·3	41·2
	30	4036·5	2083·6	48·4
	15	2945·2	1526·8	48·2
	7·5	2645·0	1351·5	49·0

* Assay conditions were as described under Table 1.

depends upon the presence of divalent cations (for pertinent references see Moore,[3] and Asano[6]). It is thus possible that the interference of steffimycin with polypeptide synthesis might be due to substantial removal of cations (particularly Mg^{2+}) from the system by chelation. The results of experiments designed to test this possibility (Fig. 2) show that the optimal Mg^{2+} concentration in the poly C-directed proline incorporation system amounts to 4–5 μmole/sample of either $MgCl_2$ or magnesium acetate. In each case, maximal inhibition of proline incorporation by steffimycin is evident at optimal magnesium concentrations. Inhibition was 20 per cent in the system containing $MgCl_2$ and 35 per cent in the one containing magnesium acetate.

Variation of the 30S fraction (1·2, 0·8 and 0·4 mg protein/sample) in the poly U-directed phenylalanine incorporation system resulted in inhibitions of 40, 49 and 41 per cent respectively in the presence of 100 μg/ml of steffimycin (Table 2). In the presence of various amounts of poly U in the same system (30, 15 or 7·5 μg/ml poly U/sample), inhibitions of 48 per cent were obtained in each case (Table 2). Thus steffimycin inhibition of polypeptide biosynthesis is not reversed by increasing concentrations of 30S fraction or messenger RNA (poly U). Addition of tRNA (50–400

TABLE 3. EFFECT OF INCREASING CONCENTRATIONS OF tRNA ON STEFFIMYCIN INHIBITION OF PHENYLALANINE OR PROLINE INCORPORATION*

System	rRNA (μg/ml)	Incorporation (cpm/mg 30S protein) Control	Steffimycin (50 μg/ml)	Inhibition (%)
Poly U–phenyl-alanine		4160·2	2048·6	50·8
	400	5719·3	3663·3	36·0
	200	4802·6	3339·5	30·5
	100	4758·6	3261·1	31·5
	50	4592·0	2625·7	42·9
Poly C–proline		5711·3	4612·8	19·3
	400	18,343·1	13,998·6	23·7
	200	11,958·2	10,037·7	16·1
	100	8057·1	7718·0	4·3
	50	6567·5	6099·1	7·2

* The remainder of the assay systems were as described under Table 1.

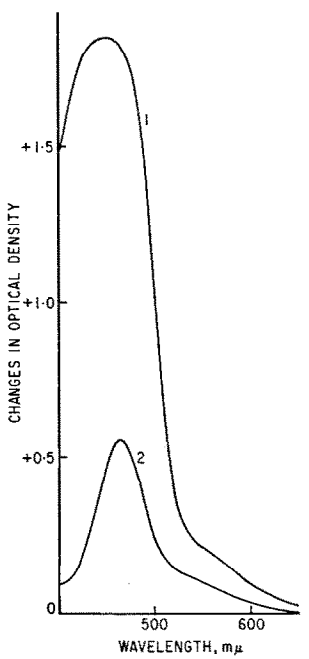

FIG. 3. Difference spectrum of steffimycin and tRNA. Both substances were dissolved in 0·01 M Tris · HCl buffer, pH 7·3, containing 0·01 M NaCl. Curve 1 = regular spectrum of steffimycin (100 μg/ml) read against buffer blank; curve 2 = a 1-ml amount of antibiotic solution containing 400 μg/ml mixed with 1 ml of a 10 mg/ml solution of tRNA.

μg/ml) to the poly U-directed phenylalanine incorporation system reduced steffimycin inhibition from 50 per cent to approximately 30 per cent (Table 3). For unknown reasons, 200 μg/ml of tRNA in the assay system reversed more effectively (30 per cent inhibition) than 400 μg/ml (36 per cent inhibition). Similar tRNA additions to the poly C-directed proline incorporation system caused partial reversal of steffimycin inhibition, but this effect was only apparent at low tRNA concentrations (50–100

μg/ml). Higher tRNA concentrations did not seem to interfere (Table 3). These results were reproducible with both the poly U-phenylalanine and poly C–proline incorporation systems.

Difference spectroscopy with antibiotic–tRNA mixtures. Difference spectra obtained with DNA–steffimycin mixtures show an absorption shift from 440 to 480 mμ, as described previously.[5] No significant interaction of tRNA and steffimycin was detected at that time. The observed partial reversal of steffimycin inhibition during polypeptide biosynthesis prompted a reevaluation of these findings with much higher tRNA and antibiotic concentrations: difference spectra obtained with mixtures containing 5 mg/ml of tRNA and 200 μg/ml of steffimycin as compared to 0·5 mg/ml tRNA and 50 μg/ml of antibiotic, described in reference 1, show an absorption shift from 440 to approximately 460 mμ (Fig. 3). This indicates that steffimycin indeed interacts with tRNA.

Effect on charging of amino acids to tRNA. The experiments discussed above suggested that the property of steffimycin to bind to tRNA might be responsible for the drug's interference with polypeptide biosynthesis. It was thus of interest to evaluate the effect of steffimycin on amino acyl–tRNA formation. Synthesis of phenylalanyl–tRNA was stimulated approximately 50 per cent in the presence of high antibiotic concentrations (Table 4). The extent of stimulation varied somewhat from experiment to experiment. Formation of prolyl–tRNA was not affected significantly in the experiment shown in Table 4, but showed minor stimulation in other experiments.

TABLE 4. EFFECT OF STEFFIMYCIN ON CHARGING ACTIVITY OF AMINO ACIDS TO tRNA

Sample *	Steffimycin (μg/ml)	Activity (cpm/sample)	Per cent of control
Phenylalanyl–tRNA formation	none	3116·7	100
	100	5252·8	168·5
	50	5023·6	161·1
	25	4808·1	154·2
	10	3434·4	110·1
Prolyl–tRNA formation	none	5418·8	100
	100	5607·8	103·4
	50	4853·3	89·2
	25	5653·5	104·3
	10	5546·5	102·3

* Samples contained in a total volume of 0·5 ml: 10 μmole Tris.HCl buffer, pH 7·4, 5 μmole magnesium acetate; 5 μmole KCl; 0·25 μmole mercaptoethanol; 5 μmole phosphoenol pyruvate (trisodium salt); 1·5 μmole ATP; 0·1 μmole each of 19 unlabeled amino acids minus either phenylalanine or proline; 0·1 μc of either proline-^{14}C or phenylalanine-^{14}C; 10 μg pyruvate kinase (Calbiochem); 100 μg tRNA and dialyzed 100S fraction containing 275 μg of protein. Samples were run and assayed for radioactivity as decribed under Methods.

Effect on polynucleotide attachment to ribosomes. Attachment of either ^{3}H-poly U or ^{3}H-poly C to ribosomes was assessed by the millipore filtration technique. Self-adsorption of poly U necessitates that the ribosomes be adsorbed first to the filter. The filter is washed with bovine serum albumin to reduce available binding sites for poly U on the filter *per se* and the poly U solution containing the antibiotic is run through the filter last. Poly C does not bind to the filters. Ribosomes and poly C can be mixed directly prior to filtration through millipore filters. The results of these binding studies (Table 5) show that steffimycin does not induce any marked changes of the binding capacity of ribosomes toward either poly U or poly C.

TABLE 5. EFFECT OF STEFFIMYCIN ON POLY U AND POLY C-ATTACHMENT TO RIBOSOMES*

Sample	Polynucleotide	Activity (cpm/sample)	Per cent of control
Complete, control	Poly U	1427·6	100
Steffimycin, 20 μg/sample	Poly U	1545·1	108
10 μg/sample	Poly U	1255·5	88
Less ribosomes	Poly U	306·1	21
Complete, control	Poly C	2467·5	100
Steffimycin, 10 μg/sample	Poly C	2567·8	104
5 μg/sample	Poly C	2610·5	106
Less ribosomes	Poly C	46·0	2

* In the binding assay with Poly U, 2·4 O.D.$_{260}$ units of ribosomes were adsorbed on a 0·45 μ millipore filter; the filter was then washed with 1 ml of a 5 mg/ml bovine serum albumin solution to reduce nonspecific binding of poly U, and 0·015 μć(10 μg) of ^{3}H-poly U was passed through the filter. In the binding experiments with poly C, ribosomes and 0·015 μć(10 μg) of ^{3}H-poly C were mixed in 0·15 ml buffer, diluted and then adsorbed on millipore filters. Further details of the assay are given under Methods.

TABLE 6. EFFECT OF STEFFIMYCIN ON ^{14}C-AMINO ACYL-tRNA-mRNA-RIBOSOME COMPLEX FORMATION

Sample*	Activity (cpm/sample)	Per cent of control
Poly U, complete, control	10,561·3	100·0
Steffimycin, 100 μg/ml	10,357·2	98·6
50 μg/ml	10,477·4	99·1
Less poly U	645·6	6·1
Poly C, complete, control	1102·9	100·0
Steffimycin, 100 μg/ml	1120·6	102·0
50 μg/ml	1148·1	104·0
Less poly C	20·3	1·8

* Samples (0·1 ml) contained 0·1 M Tris-HCl buffer, pH 7·2; 0·02 M magnesium acetate; 0·05 M KCl; 2·6 O.D.$_{260}$ units of washed ribosomes; 10 μg poly U or poly C; 100 μg of either ^{14}C-phenylalanyl-tRNA or ^{14}C-prolyl-tRNA. Incubation was at room temperature for 20 min. The tubes were then chilled in ice, diluted with 3 ml of cold Tris-magnesium acetate-KCl buffer, filtered through 0·45 μ millipore filters and assayed for radioactivity as described by Nirenberg and Leder.[4]

Attachment of ^{14}C-amino acyl-tRNA to ribosomes. The formation of amino acyl-tRNA-mRNA-ribosome complexes was tested in two systems leading to the formation of either phenylalanyl-tRNA-poly U-ribosome complexes or prolyl-tRNA-poly C-ribosome complexes. As shown in Table 6, high concentrations of steffimycin (100 or 50 μg/ml) failed to affect significantly the polynucleotide-directed binding of amino acyl-tRNA to ribosomes. This observation pertains to both poly U- and polyC-mediated binding.

DISCUSSION

The main inhibitory effect of steffimycin is its inhibition of RNA synthesis demonstrated in whole bacteria and in bacterial cell-free systems. As a secondary effect, protein synthesis is also impaired by the drug. In whole bacterial cells, protein synthesis and RNA synthesis are suppressed at equal antibiotic concentrations. Polypeptide synthesis mediated by synthetic messengers in cell-free systems is impaired only at relatively high antibiotic concentrations. Chelation of inorganic cations

by the antibiotic does not appear to be responsible for the impairment of polypeptide synthesis in cell-free systems, since increasing Mg^{2+} concentrations up to 40 μmole/ml did not reverse its inhibitory activity. Steffimycin inhibition of polypeptide synthesis is not reversed by increasing concentrations of 30S fraction or messenger RNA (poly U). Steffimycin inhibition of polypeptide biosynthesis is partially reversed by tRNA, but not by 30S fraction or messenger RNA. This suggests that steffimycin interacts with tRNA and thereby inhibits amino acid incorporation. Steffimycin binding to tRNA was substantiated by difference spectroscopy. Aggregation of the ternary amino acyl–tRNA–messenger–ribosome complex and messenger attachment to ribosomes are insensitive to the antibiotic. However, contrary to our expectations, synthesis of phenylalanyl–tRNA and to as light degree also prolyl–tRNA formation was stimulated by steffimycin. The magnitude of this stimulatory effect varied with both amino acids tested and was almost absent in some experiments, but could amount to as much as a 1·5-fold increase in others. The stimulation of amino acylation of tRNA by steffimycin is difficult to assess. At present, we like to think that steffimycin partially inhibits amino acylation of specific tRNA species (e.g. proline acceptor tRNA). On the other hand, tRNA species other than the ones specific for phenylalanine or proline might undergo structural changes by binding with the antibiotic. These structural changes in turn might be sufficient to reduce the acceptor specificity for a given tRNA species and allow for the binding of amino acids which the species would normally not accept. Incorporation of amino acids derived from such faulty amino acyl–tRNA complexes into polypeptide would then be prevented during further reactions occurring prior to peptide bond formation.

The interaction of steffimycin with tRNA suggests that this agent is related to a group of compounds with similar properties such as proflavine, nogalamycin, and Miracil D. All of these agents bind preferentially to double-stranded DNA and inhibit RNA synthesis, but interact also with tRNA.[7,8] In the case of the tRNA–proflavine interaction, Weinstein and Finkelstein[7] demonstrated that this effect leads to inhibition of amino acid incorporation.

Acknowledgement—The technical assistance of Mrs. M. A. Conklin is gratefully acknowledged.

REFERENCES

1. F. REUSSER, *J. Bact.* **93**, 65 (1967).
2. M. W. NIRENBERG, *Meth. Enzym.* **6**, 17 (1963).
3. P. B. MOORE, *J. molec. Biol.* **18** 8 (1966).
4. M. W. NIRENBERG and P. LEDER, *Science* **145**, 1399 (1964).
5. F. REUSSER, *Archs Biochem. Biophys.* **121**, 399 (1967).
6. P. B. MOORE and K. ASANO, *J. molec. Biol.* **18**, 21 (1966).
7. B. WEINSTEIN and I. H. FINKELSTEIN, *J. biol. Chem.* **242**, 3757 (1967).
8. B. K. BHUYAN and C. G. SMITH, *Proc. natn. Acad. Sci. U.S.A.* **54**, 566 (1965).