Biochemistry

 Volume 17, Number 7 April 4, 1978

Aminoglycoside Antibiotics and Eukaryotic Protein Synthesis: Structure–Function Relationships in the Stimulation of Misreading with a Wheat Embryo System†

James M. Wilhelm,*,‡ Susan E. Pettitt, and John J. Jessop

ABSTRACT: Aminoglycoside antibiotics have been assayed for the capacity to stimulate translation errors in a cell-free protein synthesis sytem from wheat embryos. The principal test of misreading was the stimulation of leucine incorporation with a poly(U) template. Misreading was observed with certain antibiotics; others had little or no activity. The highly active molecules had clear structural similarities: (1) the presence of the aminocyclitol 2-deoxystreptamine; (2) a 6′-hydroxyl group on the amino sugar which is linked to the 4-position of 2-deoxystreptamine. For example, paromomycin and kanamycin C strongly stimulated misreading but neomycin and kanamycin B had much lower activity. Drugs in the streptomycin family had no significant activity. Paromomycin was investigated for stimulation of a range of errors. With the poly(U) template, the incorrect amino acids incorporated were leucine, isoleucine, serine, tyrosine, and valine. With a poly(U,G) template, incorrect amino acids incorporated were isoleucine, alanine, serine, tyrosine, arginine, aspartate, and glutamate. The pattern is consistent with a drug-induced misreading of single uridylate residue in the first or second position of the codon. The uridylate residue can be misread as cytidylate, adenylate, or guanylate, but the uridylate to cytidylate error is more frequent than the others. The misreading occurs with the cytoplasmic (80S) ribosome of the plant extract and cannot be attributed to any contaminating organelle or bacterial ribosomes. Thus, antibiotic stimulation of errors in protein synthesis is not limited to prokaryotic systems, but extends to eukaryotic systems, as long as the antibiotic has certain precise structural elements.

Many of the aminoglycoside antibiotics promote mistranslation with bacterial ribosomes (Davies et al., 1964; Friedman & Weinstein, 1964); this property is presumably the basis for the phenotypic suppression of certain mutations by these drugs (for review, see Gorini, 1974). There have been some studies on mistranslation with cytoplasmic (80S) ribosomes of animal cells (Weinstein et al., 1966; Friedman et al., 1968) and cytoplasmic and mitochondrial ribosomes of chick embryo (Kurtz, 1974). Streptomycin was the antibiotic usually tested and it did not promote mistranslation with cytoribosomes; mitoribosomes, however, were responsive to the drug.

Our laboratory has undertaken a systematic study of the action of many aminoglycosides in a variety of protein synthesis systems and in one whole organism, the protozoan, *Tetrahymena*. This communication presents evidence for stimulation of misreading in a system from wheat embryos.

Materials and Methods

Materials. Wheat germ was from Niblack Foods, Rochester, N.Y. Biochemicals were products of Sigma. Radiolabeled amino acids (specific activity in Ci/mmol) were obtained as follows: [^{3}H]phenylalanine (16.1), -proline (26.6), -tyrosine (49.5), -valine (12.5), NEN; [^{3}H]lysine (7), ICN; [^{14}C]arginine (0.310), -isoleucine (0.306), -leucine (0.303), -serine (0.162), NEN; [^{35}S]methionine (491), NEN; ^{14}C-labeled amino acid mixture (individual from 0.102 to 0.420; contained no asparagine, cysteine, glutamine, methionine, or tryptophan), NEN.

Source of antibiotics was as follows: streptomycin and neomycin (Sigma); paromomycin, paromamine, and butirosin (Dr. H. Machamer, Parke, Davis and Co.); neamine (Dr. G. Whitfield, The Upjohn Co.); lividomycins A and B (Dr. K. Price, Bristol Laboratories); gentamicins A, B, B_1, C_1, C_2, and C_{1a} (Dr. G. Wagman, Schering Corp.); bluensomycin, neomycins B and C, kanamycins, A, B, and C (Dr. H. Taber, University of Rochester). When used alone, the symbols Pm and Nm imply the usual commercial forms of the drugs. For Pm, this is a mixture of PmI and PmII. For Nm, this is a mixture of NmB and NmC with a small amount of neamine (also called NmA).

Cell-Free Systems. The cytoplasmic extract (30 000*g* supernatant) of wheat germ was prepared by established pro-

† From the Department of Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642. *Received July 22, 1977.* Supported by National Institutes of Health Grant AI-11427. Preliminary accounts of portions of this work have been communicated (Wilhelm & Jessop, 1975; Wilhelm & Pettitt, 1976).

‡ Recipient of National Institutes of Health Research Career Development Award 5-K04-CA-0061.

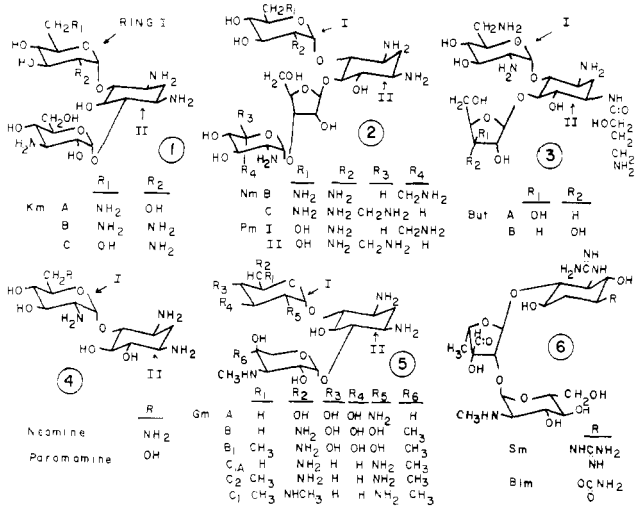

FIGURE 1: The structures of some aminoglycoside antibiotics. Parts 1–5 are the 2-deoxystreptamine-containing antibiotics. Ring I is numbered 1′–6′ from the anomeric carbon. Ring II (2-deoxystreptamine) is numbered 1–6, counterclockwise, such that the methylene carbon is position 2. (1) The kanamycins. (2) The neomycins, paromomycins, and lividomycins. Lividomycin B is 3′-deoxy PmI. LmA is identical except for a mannosyl residue linked to the position adjacent to R_3, R_4. (3) The butirosins. (4) Neamine and paromamine. (5) The gentamicins. (6) Streptomycin and bluensomycin.

cedures (Roberts & Paterson 1973) with one modification: the germ was homogenized with crushed glass rather than sand. The extract was adjusted to 100 A_{260} units/mL. Eighty percent of this material was ribosomes.

The cell-free system from *Escherichia coli* was prepared as described (Wilhelm & Haselkorn, 1971).

In Vitro Protein Synthesis. For the wheat system, the standard assay (20 μL) contained: one-quarter volume of wheat germ extract, 20 mM Hepes[1] (pH 7.6), 2 mM dithiothreitol, 8 mM magnesium acetate, 150 mM KCl, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 40 μg/mL creatine phosphokinase, 125 μg/mL poly(U) or poly(U,G), 20 μM of 19 appropriate unlabeled amino acids, and 2.5 μCi/mL ^{14}C or 25 μCi/mL ^{3}H of one labeled amino acid. The concentration of the labeled amino acid was, or was adjusted to, 5–8 μM.

The conditions for the *E. coli* system have been described (Wilhelm & Haselkorn, 1971). Amino acids and poly(U) were as above.

The systems were incubated (wheat, 25 °C; *E. coli,* 30 °C) for 60 min. Reaction mixtures were made 5% in Cl_3CCOOH and heated in boiling water for 10 min. The precipitates were filtered onto nitrocellulose filters, washed with Cl_3CCOOH and with 80% ethanol. Radioactivity incorporated into the Cl_3CCOOH-insoluble form was determined in scintillation spectrometers. Where incorporations are reported in pmol, these were calculated from a radioactivity measurement of the precursor under the same conditions.

Amino Acid Incorporation from a Radioactive Mixture. The standard assay was modified as follows: total volume was 100 μL; only unlabeled asparagine, cysteine, glutamine, methionine, and tryptophan were added; 10 μCi/mL of the other 15 amino acids were added from a ^{14}C mixture. After incubation, Cl_3CCOOH-insoluble radioactivity was assayed on a 5-μL portion. The remainder of the mixture was made 0.15 N in NaOH and held at 37 °C for 18 h. Cl_3CCOOH was added to 10%; the precipitate was collected, washed with 5% Cl_3CCOOH twice, and hydrolyzed with 200 μL of 6 N HCl at 110 °C for 48 h in a sealed ampule. The solution was diluted with water to 1 mL and then evaporated, dissolved, and reevaporated 5–6 times. The residue was dissolved in 50 μL of water. A 5-μL portion was applied to a cellulose thin-layer sheet (Eastman Chromagram) and developed in one dimension with 2-propanol:butanone:1 N HCl (60:15:25; v/v) and in the second with chloroform:methanol:17% ammonia (2:2:1; v/v). Radioactive amino acids were detected by exposing the chromatograms to x-ray film for 3–4 weeks. The radioactivity was associated with a specific amino acid by comparison of the autoradiogram to the original chromatogram, where amino acids were visualized by the ninhydrin reaction, and comparison of these to standards. The chromatography clearly resolved all of the 15 relevant amino acids except for leucine and isoleucine, which formed distinct spots but with an overlap of about one-third of their areas.

Sedimentation Analysis. After incubation under the conditions indicated in the text, reaction mixtures were layered on 5-mL linear 10–30% sucrose gradients containing 20 mM Tris-HCl (pH 7.6), 10 mM magnesium acetate, 150 mM KCl. Gradients were centrifuged for 90 min at 45 000 rpm in the Beckman SW 50.1 rotor (5 °C). Tube contents were removed from the top, monitored for ribosomes by passage through the flow cell of a spectrophotometer, and collected as fractions (approximately 120 μL) on paper disks. The disks were washed in hot and cold 5% Cl_3CCOOH and in 80% ethanol, and assayed for radioactivity with appropriate double-label corrections.

Results

Misreading with Aminoglycosides: Structure–Function Relationship. The structures of the antibiotics used in the study are shown as Figure 1. Several have a common aminocyclitol subunit, 2-deoxystreptamine (2-DOS). Exceptions are streptomycin (Sm) and bluensomycin (Blm), which have the cyclitols streptidine and bluensidine. The 2-DOS antibiotics usually have a single amino sugar substituent at position 4 of the cyclitol. For consideration of the data to follow, it may be emphasized that paromomycin (Pm), paromamine, kanamycin C (KmC), and gentamicin A (GmA) have a common 4-substituent, 2-amino-2-deoxy-D-glucose, and the lividomycins (Lm) have the 3-deoxy analogue of that sugar.

The various antibiotics were assayed for effects on protein synthesis in cell-free systems from wheat embryos. The incorporation of phenylalanine into polypeptide was strongly stimulated by poly(U) in these systems. Without the template, [^{3}H]phenylalanine incorporation by a 20-μL cell-free system was 600–900 cpm (0.5–0.7 pmol); with poly(U), phenylalanine incorporation was 40 000–65 000 cpm (28–52 pmol). The antibiotics could be placed in two groups on the basis of effects on phenylalanine incorporation. One group, Sm, the Km's, butirosin (But), and the Gm's either did not affect, or slightly stimulated, incorporation. The other group, Nm, Pm, and the Lm's gave modest inhibitions (20% or less) at concentrations up to 70 μM; above 70 μM, only Nm was more active (60% inhibition at 140 μM). Several aminoglycosides were assayed in a cell-free system from *Escherichia coli.* All inhibited incorporation at least 90% at 70 μM, and Nm, KmA, and KmB inhibited to this extent at 2.8 μM.

Poly(U) does not normally stimulate the incorporation of

[1] Abbreviations used: Pm, paromomycin; Nm, neomycin; Km, kanamycin; Lm, lividomycin; Gm, gentamicin; Sm, streptomycin; Blm, bluensomycin; But, butirosin; poly(U), poly(uridylic acid); poly(U,G), poly(uridylic, guanylic acid); 2-DOS, 2-deoxystreptamine; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

TABLE I: Misreading of a Poly(U) Template; the Incorporation of Leucine into Polypeptide in the Presence of Combinations of Paromomycin with Other Aminoglycoside Antibiotics.

Expt	Conditions	[^{14}C]Leucine incorp[a] cpm	% of Pm alone
1.	− poly(U)	590	
	+ poly(U)	1 100	
	+ poly(U), + Pm (14 μM)	18 300	100
	+ poly(U), + Pm (14 μM), + KmA (85 μM)	14 300	78
	+ poly(U), + Pm (14 μM), + KmB (85 μM)	10 800	59
2.	− poly(U)	340	
	+ poly(U)	670	
	+ poly(U), + Pm (14 μM)	14 800	100
	+ poly(U), + Pm (14 μM), + GmB (88 μM)	9 800	66
	+ poly(U), + Pm (14 μM), + GmC_{1a} (88 μM)	8 100	54
	+ poly(U), + Pm (14 μM), + Sm (70 μM)	14 900	101
	+ poly(U), + Pm (14 μM), + Sm (140 μM)	14 700	99

[a] The protein synthesis system was a cytoplasmic extract of wheat embryos; the values are incorporations in a 20-μL system. Complete experimental details are given in Materials and Methods.

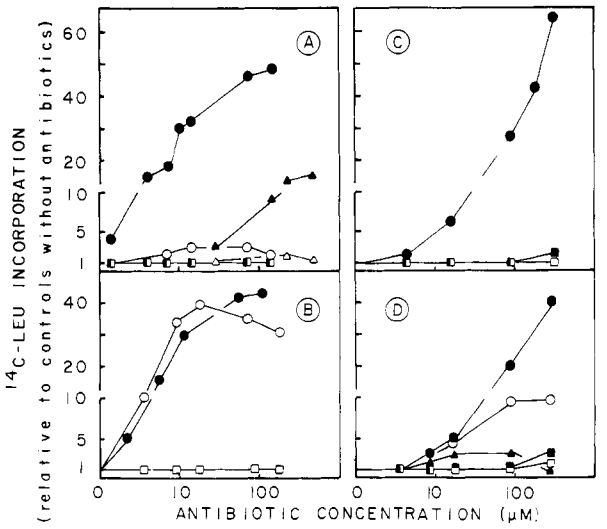

FIGURE 2: Misreading of a poly(U) template; the incorporation of leucine into polypeptide in the presence of aminoglycoside antibiotics. The protein synthesis system was a cytoplasmic extract of wheat embryos. Experimental details are given in Materials and Methods. Numerical values for control incorporations are cited in the text and in Table I. For each drug concentration, the value of the incorporation is expressed relative to a control without antibiotics. Note change in scale on ordinates. A: (●) Pm; (○) Nm; (■) Sm; (□) Blm; (▲) paromamine; (△) neamine (also called NmA). B: (●) LmA; (○) LmB; (□) But (mixture of A and B). C: (●) KmC; (■) KmB; (□) KmA. D: (●) GmA; (○) GmB_1; (▲) GmC_2; (■) GmC_1; (□) GmB (data for GmC_{1a} was identical with GmB).

leucine since it does not contain leucine codons (CUN and UUpurine). Under the ionic conditions of the experiments (8 mM magnesium acetate, 150 mM potassium chloride), poly(U) stimulated leucine incorporation only very slightly. In typical experiments, [^{14}C]leucine incorporation was 200–600 cpm (0.4–1.0 pmol) without template, and 300–1000 cpm (0.5–1.7 pmol) with template. Thus, the ratio of the net template-dependent leucine incorporation to that for phenylalanine was generally 0.02 or less.

As a test of misreading potential, the aminoglycosides were assayed for stimulation of poly(U)-dependent leucine incorporation. The results of these experiments are shown as Figure 2. Sm and Blm did not produce significant changes in the leucine incorporation. By contrast, some of the 2-DOS-containing drugs stimulated mistranslation markedly. In particular, Pm was highly active as were LmA and LmB, which are closely related to Pm (Figure 2, parts A and B). At 70 μM Pm, the incorporations of leucine and phenylalanine were almost equal on a pmole basis.

The activity of commercial Nm was much lower; this drug stimulated leucine errors to only about 6% of the level seen with Pm; purified NmB and NmC produced similar small stimulations. Butirosin, which is more like Nm than Pm, had no activity.

Neamine and paromamine are fragments of Nm and Pm, containing only 2-DOS and the amino sugar at the 4-position. Paromamine elevated misreading, although only at higher concentrations than Pm. Neamine was much less active than paromamine.

In the kanamycin group (Figure 2C), KmA and KmB did not stimulate misreading whereas KmC definitely did. In comparison with Pm and Lm, about three fold higher concentrations of KmC were required to achieve equivalent levels of misreading.

Experiments with the gentamicins are shown as Figure 2, part D. A strong stimulation of misreading was found with GmA; this drug was active at very nearly the same concentrations as KmC. Misreading was not stimulated by GmB or by GmC_{1a}. However, other members of the group, GmB_1, $-C_2$, and $-C_1$, had low but definite misreading activities. GmB_1 was the most active; it stimulated leucine incorporation to about one-quarter the level seen with GmA.

It is clear that certain aminoglycosides neither reduced phenylalanine incorporation with poly(U) nor stimulated significant incorporation of an incorrect amino acid. It was of interest, however, to determine if these molecules had any potential for counteracting the misreading effect of an active molecule, such as Pm. The data of Table I show that KmA, KmB, GmB, and GmC_{1a} did partially reverse the Pm stimulation of leucine incorporation. On the other hand, Sm did not reduce the Pm stimulation, even when present at tenfold the concentration of Pm.

Significant Misreading Not Observed with Simple Polyamines. The aminoglycosides are molecules with multiple amino groups and are thus somewhat similar to a simple polyamine, such as spermine. In a bacterial system, polyamines were shown to stimulate misreading (Friedman & Weinstein, 1964). The polyamines were tested for stimulation of poly(U)-dependent leucine incorporation in the wheat embryo system (Table II, experiment 1); these compounds clearly were not active. Also, D-glucosamine has no significant misreading activity (Table II, experiment 2). This compound was tested because the antibiotics with high activity have in common this moiety, or the related 3-deoxy-D-glucosamine (LmA and LmB). In additional experiments, the polyamines and D-glucosamine did not reverse the misreading effect of Pm, even when present in 20-fold excess.

Low Levels of Misreading Observed with Elevated Magnesium Ion Concentrations and Alcohols. In bacterial systems, translation errors can be promoted by elevated magnesium ion concentrations (Davies et al., 1964; Friedman & Weinstein, 1964) and by ethanol and methanol (So & Davie, 1964). In addition, elevated magnesium ion concentrations stimulate leucine errors in the translation of poly(U) by reticulocyte ri-

TABLE II: Misreading of a Poly(U) Template; the Incorporation of Leucine into Polypeptide in the Presence of Paromomycin, Polyamines, and D-Glucosamine.

Expt	Conditions	[^{14}C]leucine incorp[a] cpm	Relative to poly(U) alone (control)
1.	− poly(U)	450	
	+ poly(U), (control)	670	(1.00)
	+ poly(U), + putrescine (14 μM)	580	0.87
	+ poly(U), + putrescine (280 μM)	540	0.81
	+ poly(U), + spermidine (14 μM)	870	1.30
	+ poly(U), + spermidine (280 μM)	710	1.06
	+ poly(U), + spermine (14 μM)	630	0.95
	+ poly(U), + spermine (280 μM)	540	0.82
2.	− poly(U)	380	
	+ poly(U), (control)	920	(1.00)
	+ poly(U), + Pm (28 μM)	33 800	36.4
	+ poly(U), + D-glucosamine (50 μM)	880	0.95
	+ poly(U), + D-glucosamine (1 mM)	1 100	1.20

[a] See footnote to Table I for details.

bosomes (Friedman et al., 1968). In the wheat system, magnesium ion concentrations above 10 mM decreased poly(U)-dependent phenylalanine incorporation and increased mistranslation to leucine (data not shown). Maximal stimulation was found at 15 mM, but was less than one-half of the maximal Pm stimulation.

Ethanol and methanol inhibited phenylalanine incorporation and detectably stimulated the incorporation of leucine. The maximal stimulation, achieved with 1.1 M ethanol, was about 20% of the maximal Pm misreading.

Pattern of Errors Produced by Paromomycin during Translation of Poly(U). Pm was chosen as representative of the active antibiotics; the drug was assayed for stimulation of misreading with nine individual amino acids. In these experiments, the concentration of the particular radioactive amino acid was 5–10 μM and the other 19 amino acids were present in nonradioactive form at 20 μM. Table III shows the data obtained for two concentrations of Pm. In the order of decreasing response, mistranslation to leucine, isoleucine, serine, valine, and tyrosine was observed; however, stimulation of arginine, lysine, proline, and methionine errors was not found.

The observations on the error pattern were confirmed and extended in a different type of experiment (Table IV). The polypeptides made in vitro were labeled with a mixture of 15 ^{14}C-labeled amino acids; this material (along with the protein component of the extract) was hydrolyzed and the resultant amino acids were separated and identified by thin-layer chromatography. Radioactivity was detected by autoradiography. In the absence of poly(U), barely visible spots of arginine and alanine were detected in the hydrolysate. The posttranslational addition of arginine to protein has been described for eukaryotic cells (Soffer, 1973); this may account for a portion of the arginine background. Such a phenomenon has not been reported for alanine; however, alanine was the strongest spot detected by ninhydrin reaction in the separated hydrolysates. Since the endogenous incorporation presumably represents synthesis of wheat proteins, alanine may be the only amino acid for which background incorporation through actual protein synthesis can be detected by autoradiography. At any rate, the incorporation of arginine and alanine was not visibly changed by poly(U) and/or Pm. Essentially only phenylalanine was found in the product of poly(U) translation, and with the addition of the antibiotic, the only detectable incorrect amino acids incorporated were leucine, isoleucine, serine, tyrosine, and valine.

Pattern of Errors Produced by Paromomycin during Translation of Poly(U,G). Misreading of this random copolymer was investigated by chromatography as described for poly(U); the data are seen in Table IV. Poly(U,G) has codons for phenylalanine, leucine, valine, glycine, cysteine, and tryptophan. The labeled product of poly(U,G) translation contained the first four of those amino acids (the radioactive mixture did not contain cysteine or tryptophan; these were present in nonradioactive form). Pm stimulated a template-dependent incorporation of the additional amino acids, isoleucine, serine, tyrosine, aspartate, and glutamate, and clearly increased arginine and alanine over background levels.

Misreading Is Associated with Cytoplasmic Ribosomes. For the experiment in Figure 3, part A, two wheat systems were set up with poly(U) as template. In one, the polypeptide product was labeled with [^{3}H]phenylalanine; in the other, Pm was included to stimulate mistranslation and the product was labeled with [^{14}C]leucine. These two systems were mixed after protein synthesis and analyzed by velocity sedimentation in a sucrose gradient. The incorporated amino acids were found to cosediment, and both labels were found predominantly with the absorbance peak of the monoribosomes. In a second ex-

TABLE III: The Pattern of Misreading of Poly(U) with Paromomycin; Incorporation of Individual Amino Acids into Polypeptide.

Amino acid	pmol of amino acid incorp[a] − poly(U)	+ poly(U)	+ poly(U) net[b]	+ poly(U) + Pm (28 μM)	+ poly(U) + Pm (28 μM) net[b]	+ poly(U) + Pm (140 μM)	+ poly(U) + Pm (140 μM) net[b]
Phe	0.60	52.0	51.4	44.0	43.4	31.8	31.2
Leu	0.50	0.80	0.30	33.0	32.5	36.0	35.5
Ile	0.60	0.45	<0	8.9	8.3	16.0	15.4
Ser	1.0	0.95	<0	2.3	1.3	7.3	6.3
Tyr	0.25	0.22	<0	0.53	0.28	0.90	0.65
Val	0.26	0.28	0.02	0.5	0.24	1.95	1.69
Arg	2.3	2.1	<0	2.1	0	[c]	[c]
Lys	0.69	0.71	0.02	0.71	0.02	[c]	[c]
Pro	0.27	0.33	0.06	0.34	0.07	0.32	0.05
Met	0.31	0.34	0.03	0.31	0.0	0.23	<0

[a] See footnote to Table I and complete details in Materials and Methods. [b] Obtained by subtraction of − poly(U) value. [c] Not determined.

TABLE IV: The Pattern of Misreading of Poly(U) and Poly(U,G) with Paromomycin; Incorporation of an Amino Acid Mixture into Polypeptide.

A. Incorporation Data

Conditions	cpm of [^{14}C]amino acid mixture incorp[a]
1. − template	380
− template, + Pm (28 μM)	290
+ poly(U)	2480
+ poly(U), + Pm (28 μM)	6430
2. − template	260
− template, + Pm (28 μM)	270
+ poly(U,G)	1130
+ poly(U,G), + Pm (28 μM)	3500

B. Chromatographic Analysis of Incorp Material after Acid Hydrolysis
Radioact. detected at position of amino acid[b]

Amino acid	− template	− template + Pm (28 μM)	+ poly(U)	+ poly(U) + Pm (28 μM)	+ poly(U,G)	+ poly(U,G) + Pm(28 μM)
Phe	−	−	++++	++++	+++	+++
Leu[c]	−	−	±	++++	+++	+++
Ile[c]	−	−	−	+++	−	++
Ser	−	−	−	++	−	+
Tyr	−	−	−	+	−	+
Val	−	−	−	+	+++	+++
Pro	−	−	−	−	−	−
Gly	−	−	−	−	++	++
Thr	−	−	−	−	−	−
His	−	−	−	−	−	−
Arg[d]	±	±	±	±	±	++
Ala[d]	±	±	±	±	±	++
Asp	−	−	−	−	−	+
Lys	−	−	−	−	−	−
Glu	−	−	−	−	−	+

[a] Assay system is described in Materials and Methods sections. Values given are amounts determined in 5-μL portions. [b] Details are given in Materials and Methods. Radioactivity was detected by autoradiography of chromatogram on x-ray film. (−) No detectable exposure; (±) barely visible spot; (+ to ++++) easily visible darkened spots with number of +'s assigned in order of increasing degree of exposure. [c] These two amino acids could be distinguished but formed spots which partially overlapped. [d] See discussion in text of radioactivity incorporated in the absence of template.

periment (Figure 3, part B), the system labeled with [^{3}H]-phenylalanine was prepared from *Escherichia coli*. The [^{3}H]phenylalanine in the monoribosome region clearly sedimented more slowly than the [^{14}C]leucine. Also note that a large fraction of the in vitro product of the bacterial system was apparently released from ribosomes and found at the top of the gradient, whereas little of the product in the wheat system was released.

The responses of the wheat system to standard inhibitors of protein synthesis were compared in experiments not shown. Poly(U)-dependent phenylalanine incorporation was insensitive to 1 mM chloramphenicol, an inhibitor of bacterial protein synthesis. In contrast, 1 mM cycloheximide inhibited phenylalanine incorporation, and the leucine incorporation that occurred in the presence of Pm, by 97%.

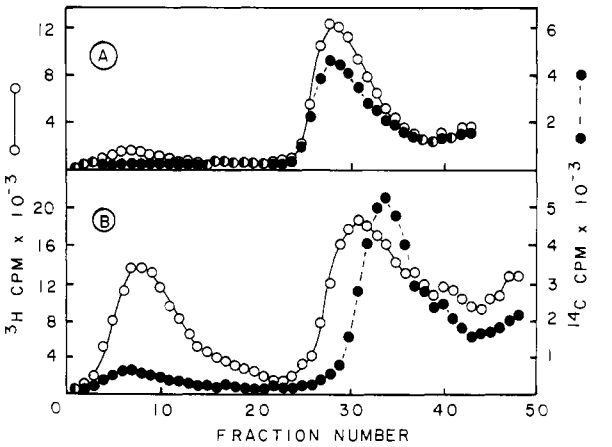

FIGURE 3: Sedimentation analysis of protein synthesis systems after amino acid incorporation with poly(U). Standard assay systems and conditions for sedimentation in sucrose gradients are described in Materials and Methods. Incubation volumes were 100-μL. (Part A) One wheat system was labeled from [^{3}H]Phe; a second contained Pm (28 μM) and was labeled from [^{14}C]Leu. After incubation, further incorporation was inhibited by addition of cycloheximide to 0.5 mM. The reaction mixtures were mixed and analyzed on a sucrose density gradient. (●) ^{14}C cpm; (○) ^{3}H cpm. (Part B) As described for part A, except for the following changes: the cell-free system labeled from [^{3}H]Phe was prepared from *Escherichia coli;* after incubation, further incorporation in this system was inhibited by addition of Pm to 28 μM. (●) ^{14}C cpm; (○) ^{3}H cpm.

Discussion

The data of this paper show that mistranslation can be stimulated in a plant system. It is critically important to establish that the mistranslation is actually a property of the cytoplasmic ribosomes. First, cytoplasmic ribosomes are inhibited by cycloheximide, but not by chloroamphenicol, while bacterial ribosomes and those of mitochondria and chloroplasts display just the reverse sensitivities (reviewed by Kroon et al., 1972). Cycloheximide virtually eliminated translation in our

system. Secondly, translation in the wheat system was associated with monoribosomes of a sedimentation coefficient clearly greater than bacterial ribosomes (Figure 3). Chloroplast and mitochondrial ribosomes of plants have been studied (Vasconcelos & Bogorad, 1971); the sedimentation coefficients of the organelle monoribosomes were less than those of cytoplasmic ribosomes, and very close to those of bacterial ribosomes.

It is reasonable to assume that mistranslation occurs on the ribosome, and that errors in aminoacylation are not stimulated. This is not proved by the data presented, and experiments are in progress to determine if Pm has any effect on the fidelity of aminoacylation. Preliminary experiments (Wilhelm & Feuer, unpublished) do show that Pm binds to ribosomes at concentrations where mistranslation is stimulated; this is consistent with an action of the drug on the ribosome itself.

There is an obvious structure–function relationship among the active antibiotics (Figures 1 and 2): those drugs that have high misreading activity share a 2-deoxystreptamine nucleus with a 4-substituent bearing a 2′-amino and a 6′-hydroxyl function. The replacement of the 6′-hydroxyl function by an amino function leads to a great reduction in activity: perhaps the hydroxyl group forms a critical hydrogen bond to some part of the ribosome, and this interaction is not readily formed by the amino group which is capable of also forming an ionic linkage. Note that the moiety associated with high misreading activity is simply a D-glucosamine ring; this compound itself, however, has no activity (Table II). Moreover, the structure–function data clearly argue for an interaction with ribosomes which is more specific than the interaction of simple polyamines (see Table II).

There are certain parts of the antibiotics which are not necessary for misreading. The 3′-hydroxyl function is not critical since LmA and LmB, which are 3′-deoxy, are as active as Pm. The substituents at position 5 or 6 of 2-DOS are not necessary because paromamine itself is active. Note, however, that much higher concentrations of paromamine are required than of Pm. Therefore, the amino group(s) on the 5- or 6-substituents may stabilize the interaction of the drug with ribosomes through ionic linkages. This suggestion is consistent with the observation that Pm, LmA, and LmB stimulate misreading at lower concentrations than KmC or GmA. The former antibiotics have two amino groups on a 5-substituent while the latter have only a single such function on a 6-substituent.

Gentamicin B_1 is an interesting exception to the structure–function relationship. This molecule is about one-quarter as active as GmA, yet has a 2′-hydroxyl and a 6′-amino function; however, the 6′-position bears an additional C-methyl group. In addition, a low level of misreading activity is associated with GmC_1 and GmC_2; each bears a C-methyl at position 6′. Possibly the methyl group causes the drugs to take a configuration which brings amino and hydroxyl functions into congruent positions with those of GmA. Inspection of models does not, however, make it readily apparent how or why this can occur.

The structure–function relationships for activity against living bacteria and in bacterial systems are presented elsewhere (Price et al., 1974; Benveniste & Davies, 1973). The most active molecules in the 2-DOS family are those bearing a 6′-amino group rather than a 6′-hydroxyl. Also, stimulation of misreading in bacterial systems is a *general* property of drugs in the streptomycin or 2-DOS families. In fact, 2-DOS itself is sufficient for misreading activity (Tanaka et al., 1966).

Although our data demonstrate that some members of the 2-DOS family do not stimulate much misreading in a eukaryotic system, this does not necessarily mean that these do not interact with the ribosomes. In particular, weakly active members of the kanamycin and gentamicin groups appear to partially reverse the misreading effect of Pm (Table I). This result suggests competitive interactions among the drugs. One may speculate that most, or all, of the antibiotics containing 2-DOS interact with eukaryotic ribosomes, perhaps at a common site(s).

The streptomycin antibiotics, however, stimulate no misreading and do not reverse the effect of Pm (Table I). Therefore, if the streptomycin antibiotics interact at all with eukaryotic ribosomes, the site(s) may be distinct from that for the 2-DOS compounds. These two general classes of aminoglycosides interact differently with prokaryotic ribosomes. First, members of the 2-DOS family do not compete with streptomycin in binding to ribosomes (Chang & Flaks, 1972). Secondly, bacterial strains that are resistant to streptomycin are not necessarily cross-resistant to the 2-DOS family, and the ribosomes of such resistant cells, although not stimulated to misread by streptomycin, are still responsive to the other drugs (Davies et al., 1964).

The patterns of incorporation errors are consistent with some general rules (Tables III and IV). First, codons are misread only one base at a time; secondly, U residues may be misread as C, A, or G. Also, U is more frequently misread as C than as A or G. If the leucine errors arise only by misreading UUU as CUU, then the data further suggest that the 5′ position of the codon is misread more frequently than the internal. However, leucine errors could also arise by UUU read as UUpurine; since leucine errors are the highest observed, perhaps either the 5′ or the 3′ position can be misread. In any case, the misreading of a single base at a time in a UUU codon has also been observed with bacterial cell-free systems (Davies et al., 1965). One may postulate that the antibiotics distort the ribosomes so that an incorrect mRNA–tRNA interaction is stabilized in which only two of the three codon–anticodon base pairs need be in the usual form. There is, in fact, a natural flexibility, or wobble, which arises through formation of nonstandard base pairs in reading the 3′ position of the codon (Crick, 1966; Topal & Fresco, 1976). One should note that misreading of U as C can arise simply by formation of the wobble pair U-G; this base pair *must* form during normal reading of codons with 3′-U residues, because A is not found in 5′ position of anticodons (Jukes, 1973). The misreading of U as A or G by base pairing would require the nonstandard pairs U-U or U-C. There is at least some evidence for the former: a modified U in the anticodon 5′ position may pair naturally with U in addition to A or G (reviewed by Nishimura, 1972).

Because poly(U,G) is a random copolymer, it is neither possible to determine if the purine base is misread nor to assign misreading of U unambiguously. The errors with poly(U,G) are, however, explainable by the misreading of a single U as C or A (Table IV). For example, Pm stimulated arginine errors which may be UGU read as CGU, or UGG read as CGG or AGG.

Other work from this laboratory suggests that drug-induced misreading occurs in a variety of eukaryotic systems. In particular, the antibiotics that are active in the plant system also elicit misreading of natural messenger RNA in a system from cultured human cells (Wilhelm et al., 1978). Recently, we have begun experiments with a lower eukaryote, *Tetrahymena thermophila*. The active antibiotics identified in this paper are potent inhibitors of growth, and the same drugs inhibit protein synthesis and elicit misreading in cell-free extracts of the organism (Palmer & Wilhelm, 1978). These findings encourage us in the hope that certain aminoglycoside antibiotics may be

useful agents for the study of the consequences of protein synthesis errors in vivo and for the investigation of the genetics of ribosomes in a lower eukaryote.

Acknowledgments

The authors wish to thank the individuals named in Materials and Methods for generous gifts of antibiotics.

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Aminoglycoside Antibiotics and Eukaryotic Protein Synthesis: Stimulation of Errors in the Translation of Natural Messengers in Extracts of Cultured Human Cells†

James M. Wilhelm,*,‡ John J. Jessop, and Susan E. Pettitt

ABSTRACT: Aminoglycoside antibiotics were tested for the capacity to stimulate misreading in a cell-free protein synthesis system derived from human cells (line KB). The stimulation of leucine incorporation with a poly(U) template in preincubated extracts was employed as one test of misreading. Some of the 2-deoxystreptamine-containing antibiotics stimulated misreading; high misreading activity was correlated with the presence of a paromamine (or 3-deoxyparomamine) moiety in the molecule. Thus, paromomycin and lividomycin B stimulated considerably more misreading than neomycin. Members of the streptomycin family had no activity in this system. The effects of paromomycin were further examined in a system from cells infected with type 5 adenovirus. In the system, protein synthesis is dependent on endogenous messengers and the translation product, which is composed of adenovirus polypeptides, can assemble into multimeric protein complexes, characteristic of viral substructures (Wilhelm, J. M., & Ginsberg, H. S. (1972) *J. Virol. 9,* 973). The presence of paromomycin, during protein synthesis, markedly reduced the capacity of the polypeptides made to participate in the assembly reaction. Furthermore, evidence was obtained which suggests that paromomycin may cause the insertion of methionine into a particular protein which normally contains little or none of that amino acid. The results suggest that an aminoglycoside antibiotic can stimulate misreading of natural messengers in a human cell system.

Many of the aminoglycoside antibiotics promote mistranslation with bacterial ribosomes; in contrast, the specific antibiotic, streptomycin, does not stimulate protein synthesis errors with cytoplasmic ribosomes of eukaryotic cells (Weinstein et al., 1966; Friedman et al., 1968; Kurtz, 1974). Our laboratory has undertaken a systematic study of aminoglycosides and protein synthesis in eukaryotic systems. Evidence has been presented (Wilhelm et al., 1978) on the stimulation of mistranslation by a specific subset of aminoglycosides, namely, paromomycin and related molecules containing the fragment, paromamine. Those studies demonstrated errors in

† From the Department of Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642. *Received July 22, 1977.* Supported by National Institutes of Health Grant AI-11427. A preliminary account of portions of this work has been communicated (Wilhelm & Jessop, 1975).

‡ Recipient of National Institutes of Health Career Development Award 5-KO5-CA-0061.

0006-2960/78/0417-1149$01.00/0