EVIDENCE FOR THE NON-ESSENTIALITY OF TRANSLATION ERRORS (MISREADING) IN MUTAGENESIS BY AMINOGLYCOSIDE ANTIBIOTICS

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In this communication we report the results of experiments bearing on the hypothesis that misreading of the genetic code may be responsible for the action of streptomycin and related antibiotics (Davies, et al., 1964). The recent discovery (Davies, et al., 1965b) that spectinomycin, an aminoglycoside antibiotic related to streptomycin, inhibits protein synthesis in bacteria without causing errors in the translation of messenger RNA, provides a simple and direct method for evaluating the contribution of misreading to the mutagenic action of these antibiotics.

In bacteria, streptomycin and other aminoglycoside antibiotics interfere with the accuracy of translation of synthetic polyribonucleotides (Davies, et al., 1964; 1965a; Friedman and Weinstein, 1965; Pestka, et al., 1965) or natural messenger RNA (Schwartz, 1965), resulting in the formation of nonfunctional proteins (Brownstein, 1964; Krueger, 1965). The site of sensitivity of streptomycin has been localized on the 30S ribosomal subunit (Cox, et al., 1964; Davies, 1964).

In algae such as Chlamydomonas, streptomycin has been recognized as a mutagen for non-chromosomal genes (Sager, 1962; Sager and Tsubo, 1962). Treatment of Euglena with aminoglycoside antibiotics results in the deletion of extrachromosomal genetic determinants that confer photosynthetic activity upon plastids (Provasoli, et al., 1948; Zahalsky, et al., 1962).

To determine if the misreading hypothesis proposed for bacterial systems is relevant to the heritable changes observed in algae, we have attempted to

obtain evidence on the requirement for translation errors in aminoglycoside-induced mutagenesis, through a comparative study of streptomycin and spectinomycin action in Euglena. In contrast to streptomycin and other aminoglycoside antibiotics, spectinomycin is not bacteriocidal, causes no detectable errors in the translation of synthetic polynucleotides, and is an effective inhibitor of misreading produced by other aminoglycoside antibiotics (Davies, personal communication). Spectinomycin has been reported to selectively inhibit protein synthesis, and to act at the same ribosomal site as streptomycin. Since this antibiotic is bacteriostatic rather than bacteriocidal, it serves to protect sensitive bacteria against the lethal effects of streptomycin (Davies, et al., 1965b).

If error in the translation process is a requisite condition for mutagenesis by aminoglycoside antibiotics, then spectinomycin should be inactive as a mutagen; moreover, spectinomycin should protect cells against streptomycin mutagenicity because it acts at the same ribosomal site. Alternatively, if translation errors are not essential for aminoglycoside mutagenesis, and mutants can arise by a mechanism not involving errors in the translation of the RNA code, then spectinomycin should be similar to other aminoglycoside antibiotics in its mutagenic action. Accordingly we should anticipate a high frequency of mutants unable to develop a normal photosynthetic apparatus; in addition, spectinomycin should afford no protection against the mutagenic action of streptomycin.

MATERIALS AND METHODS

Euglena gracilis strain Z was grown in a synthetic medium (Hutner, et al., 1956) under conditions where the presence of a functional photosynthetic apparatus confers no selective advantage. Dark-grown cells were preincubated with the antibiotic for two hours prior to exposure to light; samples were withdrawn during the incubation period, appropriately diluted and plated. The experimental conditions and plating procedure described previously (Lyman, et. al., 1961) were employed for determining viability and mutant frequency.

Streptomycin was obtained from Calbiochem, Inc., Los Angeles, California; Spectinomycin sulfate was generously supplied by Dr. W. I. Sokolski of the Upjohn Company, Kalamazoo, Michigan.

RESULTS AND DISCUSSION

Initial experiments were performed to determine if spectinomycin was inhibitory to Euglena at concentrations that prevent the growth of sensitive bacteria. No apparent inhibition of growth was observed, and no marked reduction in viability was detected following exposure of growing cells to 1.0 mg/ml of spectinomycin for periods up to 48 hours. Euglena tolerates streptomycin equally well.

When the mutagenic properties of these antibiotics were compared, no striking differences in mutagenicity were noted. Table 1 presents evidence that mutant production by spectinomycin is similar to streptomycin in its concentration and time dependence. In experiments not tabulated here, stable chlorophyll-less mutants were generated with frequencies approaching 100%. A large number of spectinomycin-induced mutants have been isolated, and have thus far maintained their mutant identity through numerous subcultures. Failure of chlorophyll-less mutants to revert when grown in the absence of the antibiotic provides support for their genetic stability. By analogy with streptomycin-induced chlorophyll-less mutants that lack chloroplast DNA (Edelman, et al., 1965; Ray and Hanawalt, 1964; 1965), we postulate that spectinomycin-induced chlorophyll-less mutants no longer contain the DNA uniquely associated with the potentiality to form chloroplasts. The stability of spectinomycin mutants is consistent with the proposal that extranuclear determinants necessary for normal photosynthetic function are deleted as a consequence of aminoglycoside mutagenesis. Like other aminoglycoside mutants that lack the ability to develop chlorophyll when exposed to light (Gibor and Granick, 1962), spectinomycin mutants continue to synthesize carotenoids. This has been provisionally interpreted as evidence that the genetic determinants for carotenoid synthesis are not linked to the aminoglycoside-sensitive extranuclear determinants that con-

Table I. Mutant Frequency as a Function of Aminoglycoside Concentration and Incubation Time

		Average Number of Mutants Colonies /10^2 Cells	
A.	Concentration of Antibiotic	Spectinomycin	Streptomycin
	0	0	0
	0.1 mg/ml	0	0.5
	0.5 mg/ml	15.1	14.8
	1.0 mg/ml	83.0	94.4
B.	Incubation Time (Hours)		
	0	0	0
	2	0.6	0.9
	8	2.4	1.6
	24	24.6	39.8
	48	84.1	96.7

Conditions: Inoculum (10^4 cells/ml) was incubated with antibiotic at 24-24°C in continuous light (~200 ft. candles); samples (1.0 ml) were diluted to ~200 cells/0.2 ml; cells were spread on antibiotic-free medium + 2% agar; plates were incubated 5 days in darkness, then returned to light to induce chlorophyll synthesis in wild type cells. Colonies lacking chlorophyll were scored as mutants. Total number of colonies scored for each value was ~330. A: Incubation time 48 hours; B: Antibiotic concentration 1.0 mg/ml.

Table II. Frequency of Chlorophyll-less Mutants Induced by Aminoglycoside Antibiotics Alone and in Combination

Additions to Medium		Average Number of Mutant Colonies /10^2 Cells
Spectinomycin	Streptomycin	
0	0	0
0.5 mg/ml	0	5.6
0	0.5 mg/	1.4
0.5 mg/ml	0.5 mg/ml	21.6
1.0 mg/ml	0	20.2
0	1.0 mg/ml	21.2
1.0 mg/ml	1.0 mg/ml	64.1

Incubation time in these experiments was 24 hours; other conditions were as described in Table I.

fer photosynthetic activity upon plastids.

To determine if spectinomycin offers protection against streptomycin, mutant frequencies were determined for each of the antibiotics both singly and in com-

bination. It is clear from the results presented in Table II that spectinomycin does not protect Euglena against streptomycin mutagenicity, rather, in the presence of both antibiotics, mutant production appears to be greatly enhanced, and not simply additive.

Misreading in cell free systems can be promoted by increasing the cation concentration (Davies, et al., 1964; Friedman and Weinstein, 1964; So, et al., 1964; Szer and Ochoa, 1964; Van Knippenberg, et al., 1964, 1965a, 1965b; Grunberg-Manago and Dondon, 1965). The effect of excess Mg^{++} and Ca^{++} ions on mutagenicity of aminoglycoside antibiotics is presented in Table III. In

Table III. Effect of Divalent Cations on Aminoglycoside Mutagenesis.

Additions to Medium		Average Number of Mutant Colonies /10^2 Cells
Antibiotic	Cation	
0	0	0
0	$MgCl_2$	0
0	$CaCl_2$	0
Spectinomycin	0	59.9
Streptomycin	0	69.6
Spectinomycin	$CaCl_2$	25.0
Streptomycin	$MgCl_2$	44.7
Streptomycin	$CaCl_2$	13.1

Incubation time in this experiment was 48 hours. Antibiotic concentration, 1.0 mg/ml; cation concentration, 50 mM; other conditions as described in Table I.

striking contrast to cell-free systems where aminoglycoside misreading is enhanced by divalent cations, mutagenicity of spectinomycin and streptomycin for Euglena is strongly suppressed by these cations.

We have presented evidence that mutations can be induced by an aminoglycoside antibiotic that inhibits protein synthesis without causing misreading. These results indicate that mutants can arise by a mechanism not involving errors in the translation of the genetic code. The high frequency of mutant production, the mutagenic specificity for extranuclear genetic determinants of the photosynthetic apparatus and the characteristics of chlorophyll-less mutants induced by this antibiotic support the idea that spectinomycin shares

mutagenic properties with streptomycin and other aminoglycoside antibiotics. However, the enhancement of mutagenesis resulting from the combination of streptomycin and spectinomycin leads us to infer that the sites of action of these antibiotics in Euglena may not be identical. This inference is strengthened by the report that spectinomycin and streptomycin differ in misreading properties and in their lethality to bacteria (Davies, et al., 1965b). If no permeability barrier prevents entry of these cations into cells of Euglena (there is experimental support for this premise) then the evidence for suppression of mutagenicity by Mg^{++} and Ca^{++} implies a mechanism of action not compatible with aminoglycoside misreading as noted in bacterial systems.

Taken together, the results of this comparative study of aminoglycoside antibiotics argue against the idea that translation errors are essential for aminoglycoside mutagenesis in Euglena. The recent observation (Sager and Toback, personal communication) that aminoglycoside antibiotics fail to enhance translation errors in Chlamydomonas is consistent with the view that the misreading hypothesis proposed as a mechanism of aminoglycoside antibiotic action in bacteria may not be relevant for the action of these antibiotics in algae. Finally, the results of these experiments concerned with misreading and mutagenesis call attention to striking differences between in vivo and in vitro systems.

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