The Effects of Streptomycin Derivatives on Sensitive and Dependent Strains of *Escherichia coli*

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SUMMARY

In order to clarify the role of the guanido groups in streptomycin activity, derivatives of dihydrostreptomycin in which both guanido groups were replaced with either amino groups, ureido groups, or aminopyrimidine moieties were tested for their effects on streptomycin-sensitive, -resistant, and -dependent strains of *Escherichia coli*. In addition, a monoguanido derivative (bluensomycin), streptidine, and guanidine were tested.

The same order of effectiveness (streptomycin = dihydrostreptomycin > ureido derivative > aminopyrimidine derivative) was noted when inhibition of protein synthesis, effect on RNA and DNA synthesis, or loss of viability was determined in streptomycin-sensitive bacteria. In all cases there was a temporal relationship between cessation of protein synthesis and loss of viability. The amino derivative, streptidine, and guanidine were inactive.

In cell-free systems, using ribosomes from streptomycin-sensitive strains, the amino derivative had no effect on the polyuridylic acid-directed polymerization of phenylalanine. The aminopyrimidine and ureido derivatives were effective only at relatively higher concentrations.

Mutants selected for resistance to bluensomycin, dihydrostreptomycin, or the ureido derivative were resistant to all the derivatives. Strains selected for resistance to the aminopyrimidine derivative fell into two classes, one showing cross-resistance and the other sensitivity to dihydrostreptomycin and its derivatives.

INTRODUCTION

The streptomycin molecule is composed of streptobiosamine, a disaccharide containing a methylamino group, and streptidine, an inositol ring bearing two guanido groups. Polglase (1) has shown that the antibacterial action of the drug is lost when the guanido groups are converted to primary amino groups or when the glycosidic linkage between streptidine and the sugars is cleaved. The oxidation of the aldehyde of the sugar to the acid form or the introduction of an adenosine monophosphate moiety on the glucosamine sugar (2) also results in loss of activity.

The present study was undertaken to clarify the role of the guanido groups in the action of streptomycin and to determine whether changes in the streptomycin molecule result in concomitant changes in the antibacterial effects for streptomycin-sensitive strains and in the stimulation of growth for streptomycin-dependent strains. Where-

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as substitution of amino for guanido groups resulted in a loss of activity by all parameters measured (killing of streptomycin-sensitive strains, stimulation of streptomycin-dependent strains, effects on protein synthesis *in vivo* and *in vitro*), substitution of ureido or aminopyrimidine moieties for the guanido groups resulted in retention of biological activity.

MATERIALS AND METHODS

Bacterial strains and medium. K-100 is a streptomycin-sensitive strain of *Escherichia coli* K-12. K-105 is a mutant of K-100 which requires 50 μg of streptomycin per milliliter for optimal growth. K-119 is a streptomycin-resistant mutant of K-100 which tolerates streptomycin concentrations up to 2000 μg/ml. The strains have been described in detail (3). Bacteria were grown in Tris-maleate medium (4) supplemented with 0.2% glucose and 0.05% Casamino acids. Viable counts were determined by plating samples on tryptose-phosphate-agar plates (Difco tryptose-phosphate broth solidified with 1.5% Bacto-agar).

Streptomycin derivatives. Bluensomycin (U-12,898D) was a gift of the Upjohn Company. The amino, aminopyrimidine, and ureido derivatives of dihydrostreptomycin, described in Fig. 1, were prepared by the method of Bodanszky (5). Elemental analysis for carbon, nitrogen, and hydrogen and the infrared spectrum of each compound agreed with the structures shown in Fig. 1. No guanido groups were detectable with the Sakaguchi reaction. Thin layer chromatography in an ethanol–1 N HCl–chloroform (5:2:3 by volume) solvent system showed less than 1 % contamination with dihydrostreptomycin. Concentrations of streptomycin and its derivatives are expressed in terms of the free base.

Synthesis in vivo of DNA, RNA, and protein. Protein synthesis was measured as incorporation of a mixture of ^{14}C-valine, ^{14}C-leucine, ^{14}C-arginine, and ^{14}C-lysine (Schwarz BioResearch protein labeling mixture) into hot trichloracetic acid-insoluble material. RNA synthesis was measured as incorporation of ^{14}C-uridine into cold trichloracetic acid-precipitable material. DNA synthesis was determined either by incorporation of ^{3}H-thymidine into a cold trichloracetic acid-precipitable fraction or by Burton's (6) modification of the diphenylamine reaction.

Cell-free amino acid-incorporating system. For the cell-free system ribosomes were prepared by the $(NH_4)_2SO_4$ precipitation method of Kurland (7). The supernatant fraction (SF II) was prepared according to the method of Wood and Berg (8). The reaction mixtures are described in Table 2. Samples were prepared for counting as previously described (3).

RESULTS

Effect of streptomycin and derivatives on the viability of streptomycin-sensitive and -dependent strains. Streptomycin, dihydrostreptomycin, or their derivatives were added to cultures of K-100 (a streptomycin-sensitive strain, growing exponen-

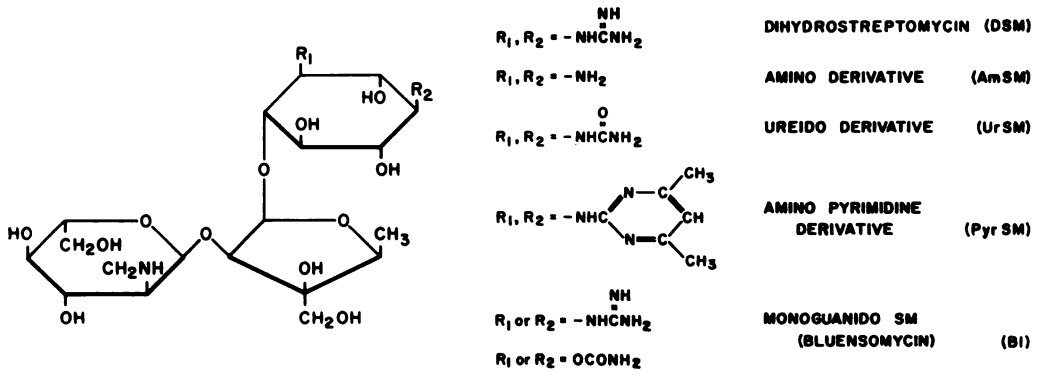

FIG. 1. *Derivatives of dihydrostreptomycin*

tially in Tris-maleate medium). Loss of viability, as measured by colony formation on tryptose-phosphate-agar plates, proceeded at the same rate in the presence of 100 μg/ml of streptomycin (not shown), dihydrostreptomycin, or bluensomycin (Fig. 2). The onset of killing was delayed when ureido-dihydrostreptomycin (100 μg/ml) or aminopyrimidine-dihydrostreptomycin (150 μg/ml) was added; however, a significant drop in the number of viable cells was noted after the initial lag. Amino-dihydrostreptomycin (at concentrations up to 500 μg/ml), guanidine (500 μg/ml), and streptidine (500 μg/ml) had no effect on the growth of K-100.

The same gradient of effectiveness was observed when the derivatives were tested for their ability to support the growth of the streptomycin-dependent strain, K-105 (Fig. 3). Amino-dihydrostreptomycin, streptidine, and guanidine at concentrations of 500 μg/ml could not replace streptomycin in the growth medium. Dihydrostreptomycin and bluensomycin, at concentrations of 50 μg/

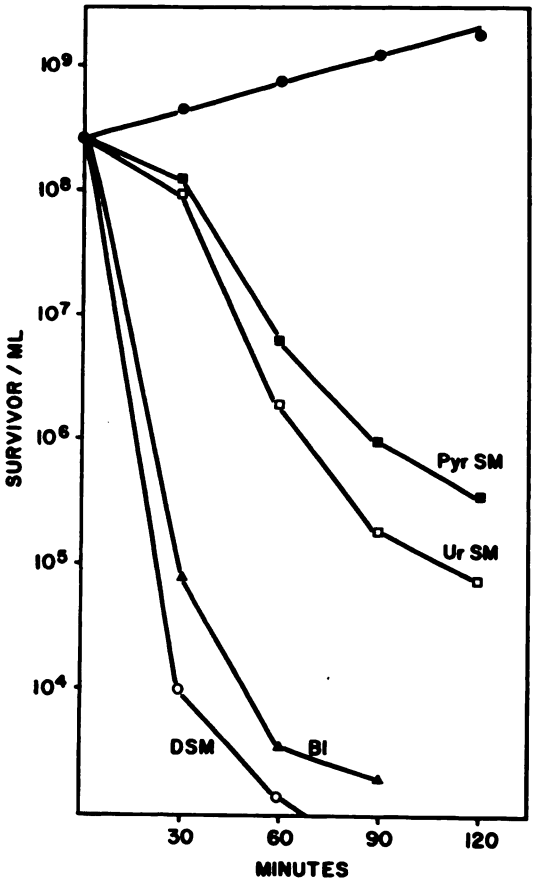

FIG. 2. *Effect of streptomycin derivatives on viability of a streptomycin-sensitive strain*

The drugs were added at zero time to cultures of K-100 growing exponentially in Tris-maleate medium with vigorous aeration. Viability was measured as the number of colonies formed on tryptose-phosphate-agar plates. ●——●, no additions; ○——○, dihydrostreptomycin, 100 μg/ml; ■——■, aminopyrimidine-dihydrostreptomycin, 150 μg/ml; □——□, ureido-dihydrostreptomycin, 100 μg/ml; ▲——▲, bluensomycin, 100 μg/ml. Drug concentrations are expressed in terms of the free base.

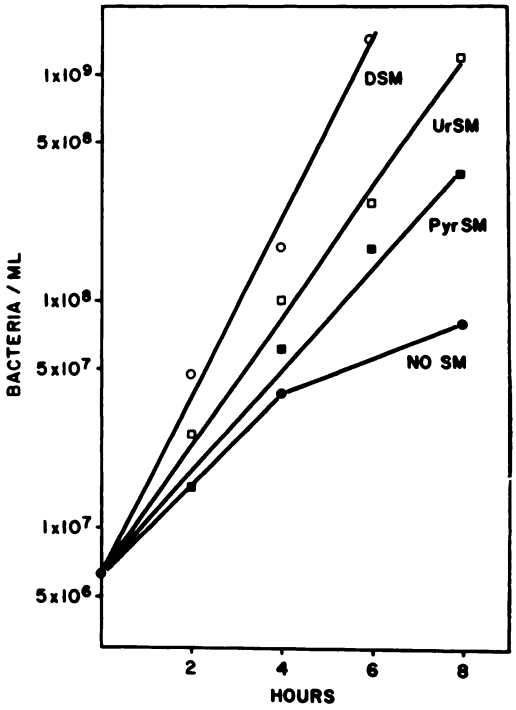

FIG. 3. *Effect of streptomycin derivatives on the growth of a streptomycin-dependent strain*

When a culture of K-105 growing exponentially in Tris-maleate medium reached a concentration of 10^8 cells/ml, the bacteria were washed three times with streptomycin-free medium and diluted in medium containing the streptomycin derivatives. All incubations were conducted at 37° with vigorous aeration. ●——●, no additions; ○——○, dihydrostreptomycin, 50 μg/ml; ■——■, aminopyrimidine-dihydrostreptomycin, 75 μg/ml; □——□, ureido-dihydrostreptomycin, 50 μg/ml.

ml, were as effective as streptomycin in stimulating growth. Ureido-dihydrostreptomycin and aminopyrimidine-dihydrostreptomycin allowed exponential multiplication of the culture, but the generation times were greater than in streptomycin. None of the derivatives had any effect on the growth of K-119, a streptomycin-resistant strain.

To determine the relative activities of the two derivatives (ureido- and aminopyrimidine-dihydrostreptomycin) which showed antibiotic activity, the effects of various concentrations of the drugs were studied. Different concentrations of dihydrostreptomycin and its derivatives were added to exponentially growing cultures of K-100 when the bacterial concentration was 1×10^8 cells/ml. After exposure to the drug for 60 min, the bacteria were diluted and plated on tryptose-phosphate-agar plates. Table 1 shows the lowest concentration of each drug which resulted in a loss of viability in the culture in 1 hr. In the presence of 5×10^{-7} M dihydrostreptomycin, no loss of viability was noted after the 60-min exposure. At a concentration of 1×10^{-6} M dihydrostreptomycin, 1% of the bacterial population remained viable after 1 hr. The concentrations of the ureido and aminopyrimidine derivatives of dihydrostreptomycin required to kill 99% of the population of streptomycin-sensitive cells in 1 hr were 5×10^{-6} M and 5×10^{-5} M, respectively.

TABLE 1
Minimal inhibitory concentrations of dihydrostreptomycin and its derivatives

Concentration	Survival[a]		
	Dihydrostreptomycin	Ureido-dihydrostreptomycin	Aminopyrimidine-dihydrostreptomycin
M	%	%	%
5×10^{-7}	100	100	
1×10^{-6}	1	60	
5×10^{-6}		1	200
1×10^{-5}			100
2×10^{-5}			70
5×10^{-5}			1

[a] Percentage of bacteria viable after a 1-hr exposure of an exponentially growing culture to the drug.

Effect of ureido and aminopyrimidine derivatives of dihydrostreptomycin on synthesis of macromolecules in a streptomycin-sensitive strain. When streptomycin is added to growing cultures of sensitive bacteria, inhibition of protein synthesis is among the earliest effects noted. This cessation of protein synthesis precedes any gross effect on RNA or DNA synthesis and seems related, in time, to the onset of cell death (9–12).

Figure 2 shows that the time at which cell death begins varies with the streptomycin derivative added. In a similar experiment, radioactive amino acids, uridine, or thymidine was added to different portions of a culture growing logarithmically in Tris-maleate medium. The labeled precursors were added when cell concentration reached 2.0×10^8/ml. Figure 4 shows the effects of the two streptomycin derivatives on synthetic activities in a streptomycin-sensitive strain. In each case there is a clear temporal relationship between the cessation of protein synthesis and the onset of killing. Effects on RNA and DNA follow the inhibition of protein synthesis. As thymidine incorporation may not be an accurate measure of DNA synthesis in *E. coli* (13), the synthesis of DNA was also determined using the diphenylamine reaction. This determination showed that there was no significant effect on DNA synthesis for at least 45 min after addition of any of the derivatives or of streptomycin. The effects of bluensomycin on synthetic activity were indistinguishable from the effects

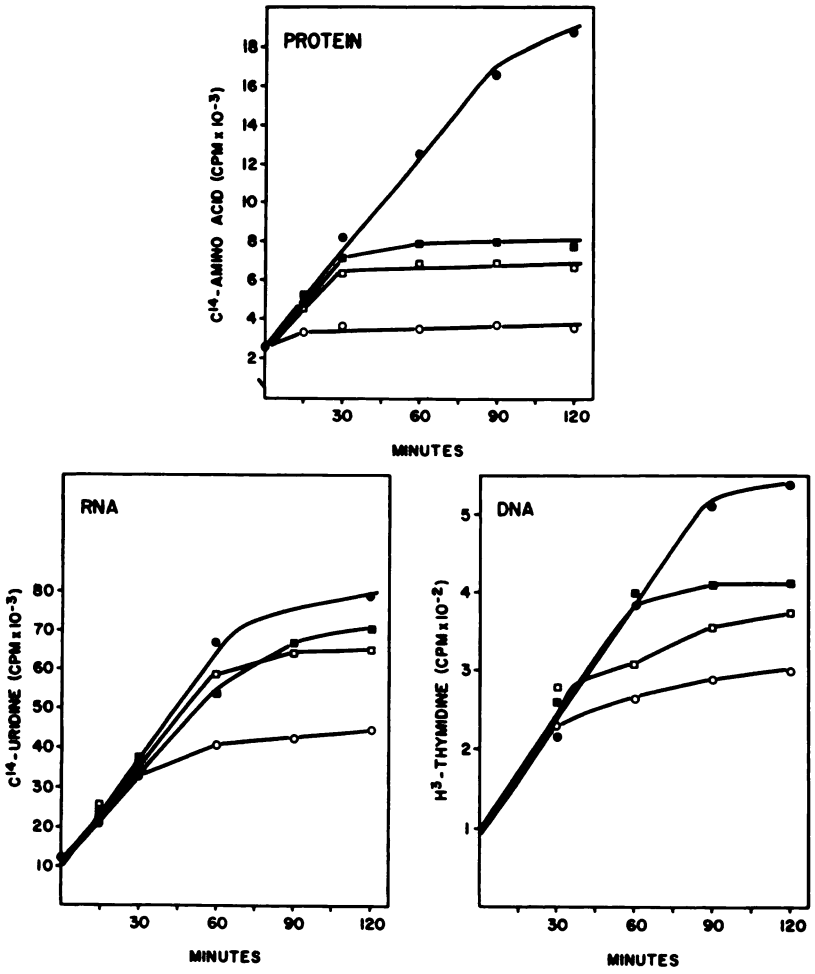

FIG. 4. *Effect of streptomycin derivatives on the synthesis of protein, RNA, and DNA*

^{14}C-Amino acids, ^{14}C-uridine, or ^{3}H-thymidine was added to an exponentially growing culture of K-100 at minus 10 min. Growth conditions are described in the legend to Fig. 2. The concentration of cells at the time of addition of drugs was 2×10^8/ml. Drugs were added at zero time. ●——●, no addition; ○——○, dihydrostreptomycin, 100 μg/ml; ■——■, aminopyrimidine-dihydrostreptomycin, 150 μg/ml; □——□, ureido-dihydrostreptomycin, 100 μg/ml.

of streptomycin and dihydrostreptomycin at concentrations of 100 μg/ml.

Effects of dihydrostreptomycin derivatives on cell-free protein synthesis. The gradient of effectiveness observed *in vivo* using equimolar concentrations of the drugs (streptomycin = dihydrostreptomycin = bluensomycin > ureido-dihydrostreptomycin > aminopyrimidine-dihydrostreptomycin) may result from differences in the permeability of the bacterial cell to the various compounds rather than different effects on the protein-synthesizing complex of the cell. To test this possibility, the streptomycin derivatives were studied in cell-free systems containing ribosomes from streptomycin-sensitive, -resistant, or -dependent strains. Table 2 shows the effects of the drugs on the poly U-primed polymerization of phenylalanine, leucine, and isoleucine. At the concentrations studied, all five compounds inhibited the polymerization of phenylalanine to approximately the same extent when the ribosomes of the system

TABLE 2

Effect of streptomycin derivatives on poly U-directed polymerization of phenylalanine, leucine, and isoleucine

Addition[a]	Streptomycin-sensitive ribosomes			Streptomycin-resistant ribosomes			Streptomycin-dependent ribosomes		
	Phe	Ile	Leu	Phe	Ile	Leu	Phe	Ile	Leu
None	100[b]	100	100	100	100	100	100	100	100
Streptomycin	25	200	237	105	105	125	110	102	300
Ureido-dihydrostreptomycin	18	220	230	98	95	115	170	120	180
Aminopyrimidine-dihydro-streptomycin	30	185	190	108	100	100	140	110	140
Amino-dihydrostreptomycin	100	80	100				110	105	100
Bluensomycin	15	240	200	93	110	120	102	90	260
Guanidine	100	80	90	102	90	90	90	100	90

[a] Ribosomes (25 A_{260} units), isolated by the method of Kurland (7) from K-100, K-119, and K-105, were added to the following reaction mixture (final volume, 0.125 ml), containing 0.01 M Tris (pH 8.0), 0.05 M KCl, and 0.008 M $MgSO_4$: 0.025 μmole of each amino acid not being studied; 3 μmoles of mercaptoethanol; 0.25 μmole of ATP; 0.15 μmole of GTP; 20 μg of poly U; 250 μg of soluble fraction II of Wood and Berg (8); 5 A_{260} units of sRNA; 0.5 μg of heated salmon sperm DNA; and 0.01 μmole of ^{14}C-labeled amino acid. Drugs were added to the reaction mixture at the start of the reaction: streptomycin, ureido-dihydrostreptomycin, and bluensomycin at a concentration of 10 μg/ml; aminopyrimidine-dihydrostreptomycin at 15 μg/ml; and amino-dihydrostreptomycin and guanidine at 50 μg/ml.

[b] Incorporation is expressed as a percentage of control values (control = reaction mixture without addition of drugs). 100% = 0.2 μμmole of phenylalanine, 0.01 μμmole of isoleucine, and 0.04 μμmole of leucine incorporated in 30 min at 35°.

came from the streptomycin-sensitive strain, K-100. None of the drugs inhibited phenylalanine incorporation using ribosomes from the streptomycin-resistant or -dependent strain. In the presence of heated salmon sperm DNA, added to maximize error frequency (14), these same derivatives stimulated incorporation of isoleucine and leucine with streptomycin-sensitive ribosomes. Amino-dihydrostreptomycin, guanidine, and streptidine had little or no effect on the poly U-directed amino acid incorporation.

These results suggest that streptomycin, ureido-dihydrostreptomycin, bluensomycin, and aminopyrimidine-dihydrostreptomycin are equally effective *in vitro* at the relatively high concentrations used. However, at lower concentrations (Table 3), the aminopyrimidine and ureido derivatives of di-

TABLE 3

Effect of drug concentration on inhibition of poly U-directed phenylalanine incorporation

The reaction mixtures and conditions of incubation were the same as those in Table 2. The ribosomes were isolated from K-100. In the absence of drugs, 0.2 μmole of phenylalanine was incorporated.

Concentration	Inhibition			
	Dihydrostreptomycin	Bluensomycin	Ureido-dihydro-streptomycin	Aminopyrimidine-dihydrostreptomycin
μg/ml	%	%	%	%
15	70	69	82	70
1.5	66	64	62	35
0.75	60	58	40	5
0.15	5	3	2	5

hydrostreptomycin are less effective in inhibiting phenylalanine incorporation than are streptomycin and bluensomycin. These findings suggest that aminopyrimidine- and ureido-dihydrostreptomycin are less effective inhibitors of protein synthesis than streptomycin, and that this decreased effectiveness is not the result of decreased permeability of the cell to the compound.

Cross-resistance of mutants to streptomycin derivatives. All mutants of *E. coli* selected for resistance to high levels of streptomycin (> 100 μg/ml) were resistant to all of the derivatives described. Similarly, those mutants selected for resistance to high levels of bluensomycin or ureido-dihydrostreptomycin (100 μg/ml) were resistant to streptomycin and all of the derivatives. However, two types of mutants were found among those selected for the capacity to grow on agar plates containing aminopyrimidine-dihydrostreptomycin at 100 μg/ml. One group was sensitive to streptomycin, dihydrostreptomycin, ureido-dihydrostreptomycin, and bluensomycin; the other was resistant to these compounds (Table 4). In liquid culture the first group of aminopyrimidine-dihydrostreptomycin-resistant mutants was killed rapidly by 100 μg of streptomycin per milliliter; loss of viability in medium containing 100 μg of ureido-dihydrostreptomycin per milliliter was very slow.

DISCUSSION

Streptomycin appears to act by binding to the 30 S ribosomal subunit and interfering with some step in protein synthesis. A recent review article summarizes the evidence suggesting such a role for the antibiotic (15). Although the structure of the streptomycin molecule is known and a three-dimensional model has been proposed (16), the active group or groups have not been determined.

Studies *in vivo* and *in vitro* show that the aldehyde group can be changed to either $—H_2$ or —OH without loss of activity (17); however, oxidation to the acid form results in complete loss of activity. Further cleavage of the glycosidic linkage (17) destroys most of the activity of the molecule, as does removal of the guanido groups (dideguanyl-dihydrostreptomycin) (1) or carbobenzyloxylation of the secondary amine (1). These findings, along with the demonstration of the biological activity of bluensomycin (18, 19), a dihydrostreptomycin derivative in which one of the guanido groups is replaced with a carbamoyl moiety

TABLE 4
Effect of streptomycin derivatives on growth of drug-resistant mutants

Strain of *E. coli*	Concentration of drug (μg/ml)					
	No drug	Streptomycin (20)	Streptomycin (100)	Bluensomycin (100)	Ureido-dihydrostreptomycin (100)	Aminopyrimidine-dihydrostreptomycin (100)
K-100	+[a]	−	−	−	−	−
Streptomycin-resistant	+	+	+	+	+	+
Ureido-dihydrostreptomycin-resistant	+	+	+	+	+	+
Aminopyrimidine-dihydrostreptomycin-resistant—1	+	−	−	−	−	+
Aminopyrimidine-dihydrostreptomycin-resistant—2	+	+	+	+	+	+

[a] + = growth on tryptose-phosphate-agar plates; − = no growth after 48 hr of incubation.

(18), suggest that both the sugar moieties and guanido groups of the inositol ring play a key role in the biological activity of streptomycin.

The present study confirms the observations that a cleavage product of the molecule (streptidine) is inactive *in vivo* and *in vitro*. Furthermore, the observation of Polglase (1) that substitution of amino groups for guanido groups results in inactivity has been confirmed *in vivo* and extended into the system *in vitro*. However, a recent study (20), using misreading rather than inhibition of phenylalanine incorporation as a measure of antibiotic activity in poly U-primed cell-free systems, suggests that streptidine and dideguanyl-dihydrostreptomycin have low levels of activity at high concentrations. The activity of ureido- and aminopyrimidine-dihydrostreptomycin, compounds in which both the net charge and the size of the substituent have been changed, stresses the importance of the carbon atom of the guanido groups or stereochemically similar structures for biological activity, but diminishes the importance of the net positive charge.

The results suggest that the biological activity of the streptomycin molecule (killing of streptomycin-sensitive cells, stimulation of growth of streptomycin-dependent strains, inhibition of polypeptide synthesis *in vivo* and in cell-free systems, and "misreading") is preserved when one guanido group is present (bluensomycin) or when both guanido groups are replaced by ureido or aminopyrimidine groups.

The studies *in vitro* using ribosomes from the streptomycin-sensitive strain suggest that the diminished activity of the ureido and aminopyrimidine derivatives is related to their effect on protein synthesis rather than to an inability of the cell to take up these compounds from the medium. Studies on the binding of streptomycin and its derivatives[4] suggest that ureido and aminopyrimidine derivatives have lower affinities for the 70S ribosome than does dihydrostreptomycin. It cannot be determined from the studies *in vitro* described in this paper whether these derivatives display the same primary effects as dihydrostreptomycin on protein synthesis. It has been suggested (21) that streptomycin acts by interfering with the initiation of polypeptide synthesis. The study of the effects of streptomycin derivatives on cell-free systems utilizing natural messenger RNAs may help to clarify the role of the various groups of the streptomycin molecule in the inhibition of protein synthesis.

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