Resistance to the Antibiotics Viomycin and Capreomycin in the *Streptomyces* Species Which Produce Them

By RICHARD H. SKINNER* AND ERIC CUNDLIFFE

Department of Biochemistry, University of Leicester, Adrian Building,
University Road, Leicester LE1 7RH

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Viomycin and capreomycin, antibiotics produced by *Streptomyces vinaceus* and *S. capreolus* respectively, are potent inhibitors of bacterial protein synthesis. Although these organisms are highly tolerant of their own products *in vivo*, their ribosomes are fully sensitive to the action of the drugs *in vitro*. However, they possess novel, antibiotic-inactivating enzymes (viomycin phosphotransferase, capreomycin phosphotransferase, capreomycin acetyltransferase) which, in addition to possible biosynthetic roles, may contribute to the resistances observed *in vivo*.

INTRODUCTION

Viomycin and capreomycin are tuberculostatic peptide antibiotics (Fig. 1) produced by *Streptomyces vinaceus* and *S. capreolus*, respectively (Caltrider, 1967; Herr *et al.*, 1960). Viomycin inhibits both the initiation and elongation phases of bacterial protein synthesis (Liou & Tanaka, 1976) and in the latter context appears to be a specific inhibitor of translocation (Modolell & Vazquez, 1977). Despite the fact that recent structural studies have revealed considerable differences between viomycin and the capreomycins (Nomoto *et al.*, 1977), a conceptual association has long been established between these drugs and they are commonly assumed to act similarly.

Two sets of observations prompted us to examine the organisms which produce viomycin and capreomycin. Firstly, in various mutants of *Mycobacterium smegmatis*, resistance to both drugs could be determined by some undefined property of the RNA of either ribosomal subunit (Yamada *et al.*, 1978). Secondly, in this laboratory (Cundliffe, 1978; Cundliffe & Thompson, 1979) another antibiotic-resistance mechanism involving the state of ribosomal RNA (rRNA) had been elucidated. In this case *S. azureus*, which produces thiostrepton, defends itself by methylation of a specific ribose residue in 23S rRNA. Accordingly, we wished to determine whether (and, if so, how) *S. vinaceus* and *S. capreolus* defend themselves against viomycin and capreomycin.

Here we report that these organisms are indeed resistant to their own toxic products, but in a manner radically different from that observed in *S. azureus*. They do not possess drug-resistant ribosomes; rather, they elaborate novel, antibiotic-modifying enzymes which can inactivate viomycin and capreomycin *in vitro* and thus have the potential to participate in self-defence *in vivo*.

METHODS

Bacterial strains and media. *Streptomyces coelicolor* A(3)2 was provided by Professor D. A. Hopwood of the John Innes Institute, Norwich. *Streptomyces vinaceus* (NCIB 8852) and *S. capreolus* (NCIB 9801) were obtained from the National Collection of Industrial Bacteria, Aberdeen. Stock cultures of all organisms were maintained on 2% (w/v) agar plates containing, per litre: 10 g glucose, 2 g yeast extract (Difco), 2 g

Fig. 1. The structures of viomycin and the capreomycins (Nomoto *et al.*, 1977). Capreomycins IIA and IIB are minor components which differ from IA and IB in that they lack the β -lysine residue. The relative proportions of IA and IB vary, but together they usually account for at least 90 % of the total capreomycin produced.

Casamino acids (Difco), 1 g Lab-Lemco beef extract (Oxoid). The pH value of the medium was adjusted to 7 with KOH. In liquid culture, *S. vinaceus* and *S. capreolus* were grown in the above medium without agar, and *S. coelicolor* in a medium containing, per litre: 25 g nutrient broth no. 2 (Oxoid), 10 g glucose, 6 g K_2HPO_4 , 3 g KH_2PO_4 , 5 g Casamino acids (Difco).

Growth of cells and preparation of extracts. Batch cultures were grown at 30 °C (16 h for *S. vinaceus* and *S. coelicolor*, 4 d for *S. capreolus*) and cells were collected and washed either by centrifugation at 10000 *g* (*S. vinaceus* and *S. capreolus*) or by filtration through Whatman no. 1 paper on a Buchner funnel (*S. coelicolor*). Cells were washed twice by resuspension in RS buffer (10 mM-Tris/HCl pH 7.6 at 20 °C, 10 mM- $MgCl_2$, 50 mM- NH_4Cl , 3 mM-2-mercaptoethanol) containing 0.5 mM-EDTA, followed by re-collection as above.

Cell-free extracts (designated S30) were prepared by grinding with alumina followed by treatment with deoxyribonuclease and centrifugation at 30000 *g* as described elsewhere (Cundliffe *et al.*, 1979).

Preparation of ribosomes and cell-free synthesis of protein. Ribosomes were prepared by layering the S30 over 0.33 vol. RS buffer containing 40 % (w/v) sucrose, followed by centrifugation at 100000 *g* for 14 h and resuspension in RS buffer. Postribosomal supernatant (designated S100) was dialysed at 0 °C against a minimum of 500 vol. RS buffer and both ribosomes and S100 were rapidly frozen and samples stored at -70 °C. Portions of *S. capreolus* S100 to be used as a potential source of antibiotic-modifying enzymes were treated with 5 % (w/v) activated charcoal (Sigma) at 0 °C for 5 min followed by centrifugation at 12000 *g* for 2 min. This treatment was necessary in order to reduce the high 'background' in assays of acetyltransferase activity and probably involved removal of endogenous capreomycin.

Ribosomes and S100 were used to synthesize polyphenylalanine in response to polyuridylic acid [poly(U)] as described previously (Cundliffe *et al.*, 1979) except that ribosomes were present at a final concentration of 0.4 μ M and incubation was at 30 °C. In all experiments, an incorporation of 10000 c.p.m. represented approximately 2.5 residues of [^{14}C]phenylalanine polymerized per ribosome.

Antibiotic-susceptibility tests. Suspensions of spores in 3 ml of growth medium containing 0.5 % (w/v) agar were poured over 9 cm diam. nutrient plates (as above) and allowed to set. Paper discs (Whatman AA, 6 mm diam.) containing various antibiotics were placed on the plates which were incubated for 3 d at 30 °C.

Inactivation of viomycin and capreomycin IA by phosphorylation. Assay mixtures contained, in a final volume of 25 μ l RS buffer, 7.5 μ l *S. coelicolor* S100 or 2 μ l *S. vinaceus* S100 or 10 μ l *S. capreolus* S100 (previously treated with activated charcoal as above), 4 or 30 μ M-antibiotic, and 2.5 mM-MgATP or β , γ -methylene adenosine triphosphate (AMPPCP). The time of incubation at 30 °C was 10 min, except for assays containing *S. vinaceus* S100 and capreomycin IA, or *S. capreolus* S100 and viomycin, which were incubated for 60 min. The reaction was stopped by heating at 100 °C for 5 min, followed by centrifugation at 12000 *g* for 1 min. Samples were removed and the residual drug activity was estimated following dilution (threefold) with the components required for *in vitro* protein synthesis. These included ribosomes and S100 from *S. coelicolor*.

Reactivation of phosphoviomycin by alkaline phosphatase. Viomycin was inactivated by incubation at 30 °C for 3 h in 100 μ l RS buffer containing 50 μ l *S. vinaceus* S100, 5 mM-MgATP and 13 μ M-viomycin sulphate. A parallel incubation lacking drug was also carried out. The reaction was stopped by heating at 100 °C for 5 min, followed by centrifugation at 12000 *g* for 1 min. Two samples were removed from each incubation mixture and diluted 15-fold with RS buffer lacking NH_4Cl . To one of each pair of samples, *E. coli* alkaline phosphatase [Sigma, 100 units ml^{-1} in 2.5 M- $(NH_4)_2SO_4$] was added to a final concentration of 1 unit ml^{-1} . An equal amount of $(NH_4)_2SO_4$ was added to the remaining samples. After 20 h at 30 °C, the

Fig. 2. Sensitivity of *S. coelicolor*, *S. vinaceus* and *S. capreolus* to viomycin and capreomycins *in vivo*. Discs contained: (a) blank; (b) 100 µg viomycin; (c) 100 µg capreomycin IA; (d) 100 µg capreomycin IB.

antibiotic activity in each sample was estimated following dilution (threefold) with components required for *in vitro* protein synthesis, as above. Incubation was continued for a further 30 min at 30 °C and the hot TCA-insoluble radioactivity in 10 µl samples was determined.

Antibiotic-modification assays. Incubation mixtures contained, in a total volume of 25 µl RS buffer, 10 µl *S. vinaceus* or *S. capreolus* S100 (the latter previously treated with activated charcoal as above), 30 µM antibiotic and either 0.2 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10 mCi mmol⁻¹, 370 MBq mmol⁻¹) or 0.2 mM- $[1\text{-}^{14}\text{C}]\text{acetyl-coenzyme A}$ (55 mCi mmol⁻¹, 2.03 GBq mmol⁻¹). After 30 min at 30 °C, samples (20 µl) were delivered on to strips (3 × 1 cm) of Whatman P81 phosphocellulose paper. These were washed four times with deionized water to remove unreacted radioactive cofactor and then twice with acetone. Radioactivity retained on the papers (representing modified antibiotic) was estimated by liquid-scintillation spectrometry. Background values obtained from control incubations lacking antibiotic were subtracted.

Purification of $[^{14}\text{C}]\text{acetylcapreomycins IA and IB}$. Capreomycins IA and IB were acetylated separately as described above, except that the final volume of the reaction mixture was 1 ml, the specific radioactivity of the $[1\text{-}^{14}\text{C}]\text{acetyl-coenzyme A}$ was 7 mCi mmol⁻¹ (260 MBq mmol⁻¹), and the time of incubation was 21 h. The reaction mixtures were then applied to columns containing 1 ml Amberlite CG-50 Type I (NH₄⁺ form) which were washed with 4 ml water to remove unreacted cofactor. Radioactive material eluted from each column with 2 ml 2 M-acetic acid was collected, lyophilized, taken up in a small volume of water and applied to separate tracks on Whatman 3MM paper. The chromatogram was then developed by descending chromatography with *n*-butanol/acetic acid/water/pyridine (15:3:12:10, by vol.) for 16 h. After drying, the tracks of the chromatogram were cut into 1 cm strips and the radioactivity in each was determined by liquid-scintillation spectrometry. The *R_F* values of capreomycins IA and IB were 0.10 and 0.12, respectively, and those of the acetylated drugs were 0.16 and 0.39. Appropriate strips from each track were washed twice with toluene and then with acetone before being eluted at 37 °C with 2 % acetic acid. The eluates, purified $[^{14}\text{C}]\text{acetylcapreomycins IA and IB}$ respectively, were lyophilized and dissolved in small volumes of water.

Materials. Antibiotics were obtained from Sigma. Capreomycins IA and IB were purified by the method of Herr & Redstone (1966) as modified by Nomoto *et al.* (1977). AMPPCP was a gift of Dr Ron Cooper, University of Leicester.

RESULTS

Antibiotic-resistance in vivo

In conventional disc-plate assays (Fig. 2), *S. vinaceus* was found to be highly resistant (but not entirely so) to viomycin, partially resistant to capreomycin IA and sensitive to capreomycin IB. In contrast, *S. capreolus* was almost completely resistant to all these drugs. Also included in Fig. 2 are results obtained with *S. coelicolor* (our standard reference actinomycete) which is sensitive to viomycin and the capreomycins.

Fig. 3. *Streptomyces vinaceus* S100 determines resistance to viomycin. Polyphenylalanine synthesis was assayed as described in Methods: ○, control; ●, plus 1 µg viomycin ml⁻¹. (a) *S. coelicolor* ribosomes and *S. coelicolor* S100; (b) *S. vinaceus* ribosomes and *S. vinaceus* S100; (c) *S. coelicolor* ribosomes and *S. vinaceus* S100; (d) *S. vinaceus* ribosomes and *S. coelicolor* S100.

Fig. 4. Sensitivity of ribosomes of *S. coelicolor* and *S. capreolus* to capreomycins IA and IB. Cell-free protein synthesis was measured as described in Methods, using S100 from *S. coelicolor* and ribosomes from either *S. coelicolor* (○) or *S. capreolus* (●). The ribosomes had been washed in buffer containing 1 M-NH₄Cl as previously described (Cundliffe *et al.*, 1979). After 30 min, control incorporation was 18000 c.p.m. and 2600 c.p.m., respectively.

Antibiotic-resistance in vitro

Cell-free extracts (S30), active in the poly(U)-directed synthesis of polyphenylalanine, were prepared from *S. coelicolor* and *S. vinaceus*. The rate of synthesis in extracts of *S. coelicolor* was decreased 50 % by 50 ng viomycin ml⁻¹ whereas extracts of *S. vinaceus* were 200 to 300 times less sensitive to the drug. The crude extracts were then fractionated into ribosomes and postribosomal supernatant (S100) and reciprocal combinations of these were tested for their sensitivity to viomycin as above (Fig. 3). The results showed that resistance to viomycin was determined exclusively by the S100 fraction from *S. vinaceus* and that the ribosomes of this organism were intrinsically sensitive to the antibiotic. In the light of these results, ribosomes were prepared from *S. capreolus* and tested (using S100 from *S. coelicolor*) for sensitivity of polyphenylalanine synthesis to capreomycins IA and IB (Fig. 4). Again,

Table 1. *Inactivation of viomycin and capreomycin IA*

Viomycin or capreomycin IA at the concentrations specified were incubated with S100 from the organisms listed, with or without cofactor, and their subsequent inhibitory activity against cell-free polyphenylalanine synthesis was determined as described in Methods (final concentrations of antibiotics were one-third of the initial concentrations). In drug-free controls, synthesis proceeded linearly for at least 30 min at which time an average of 22000 c.p.m. of [^{14}C]phenylalanine [5.5 pmol (pmol ribosomes) $^{-1}$] had been incorporated per sample.

Source of S100	Cofactor	Antibiotic (initial concn, μM)	Inhibition of polyphenylalanine synthesis (%)
<i>S. coelicolor</i>	—	Viomycin (4)	90
<i>S. coelicolor</i>	ATP	Viomycin (4)	93
<i>S. vinaceus</i>	—	Viomycin (4)	91
<i>S. vinaceus</i>	ATP	Viomycin (4)	14
<i>S. vinaceus</i>	AMPPCP	Viomycin (4)	88
<i>S. vinaceus</i>	—	Capreomycin IA (4)	72
<i>S. vinaceus</i>	ATP	Capreomycin IA (4)	4
<i>S. capreolus</i>	—	Capreomycin IA (30)	92
<i>S. capreolus</i>	ATP	Capreomycin IA (30)	20
<i>S. capreolus</i>	—	Viomycin (30)	94
<i>S. capreolus</i>	ATP	Viomycin (30)	23

Table 2. *Restoration of antibiotic activity of inactivated viomycin by alkaline phosphatase*

Viomycin (13 μM) was inactivated by incubation with *S. vinaceus* S100 and ATP as described in Methods. Inactivated viomycin was then incubated with and without alkaline phosphatase, before its inhibitory effect against cell-free protein synthesis was determined (as in Table 1). Drug-free controls were included to compensate for the inhibitory effect of alkaline phosphatase on polyphenylalanine synthesis. In other controls, ATP was omitted from the initial incubation in order to show the effect of non-inactivated viomycin. The final concentration of viomycin (modified or unmodified) was 0.3 μM .

Components of inactivation incubation	Phosphatase	Polyphenylalanine synthesis	
		(%)	(c.p.m.)
S100+ATP	—	100	22800
S100+ATP+viomycin	—	108	24580
S100+viomycin	—	11	2450
S100+ATP	+	100	16210
S100+ATP+viomycin	+	40	6540
S100+viomycin	+	8	1340

ribosomes of the producing organism were indistinguishable from those of *S. coelicolor* in their response to these antibiotics.

Since much evidence suggests that viomycin (and, by analogy, capreomycin) acts directly on ribosomes (e.g. Misumi & Tanaka, 1978), it seemed unlikely that resistance to these drugs would be determined by any of the supernatant factors involved in protein synthesis. Rather, it seemed more probable that the producing organisms might contain enzymes capable of inactivating viomycin and capreomycin.

Inactivation of viomycin and capreomycin IA

The biological activity of viomycin or capreomycin IA (i.e. inhibition of cell-free protein synthesis) could be abolished by incubation with ATP together with S100 from either *S. vinaceus* or *S. capreolus* (Table 1). Moreover, in the inactivation of viomycin, ATP could not be replaced by its non-hydrolysable analogue AMPPCP, nor could *S. vinaceus* S100 be replaced by that from *S. coelicolor*. These results suggested that viomycin and capreomycin IA were being inactivated by phosphorylation, a conclusion substantiated by the further

Table 3. *Substrate specificity of viomycin- and capreomycin-modifying enzymes*

Antibiotics (600 pmol) were incubated with *S. vinaceus* or *S. capreolus* S100 and either [γ - ^{32} P]ATP or [1 - ^{14} C]acetyl-coenzyme A and the extent of modification was determined as described in Methods.

Substrate	[^{32} P]Phosphate transferred (pmol)		[^{14} C]Acetate transferred (pmol)
	<i>S. vinaceus</i> S100	<i>S. capreolus</i> S100	<i>S. capreolus</i> S100
Viomycin	76	445	7
Capreomycin IA	293	604	29
Capreomycin IB	1	2	49
Capreomycin IIA	287	488	27
Capreomycin IIB	0	8	61

Fig. 5

Fig. 6

Fig. 5. Inactivation and phosphorylation of viomycin by S100 from *S. vinaceus*. The following components (in 300 μ l RS buffer) were incubated together at 30 $^{\circ}$ C: 30 μ l *S. vinaceus* S100, 16 μ M-viomycin sulphate, 0.5 mM-[γ - ^{32} P]ATP. At intervals, 20 μ l samples were removed and phosphoviomycin was estimated by the phosphocellulose binding technique as described in Methods. Also, 5 μ l samples were diluted 15-fold with 10 mM-imidazole/HCl pH 6.8 at 20 $^{\circ}$ C, heated at 100 $^{\circ}$ C for 5 min and assayed for inhibition of polyphenylalanine synthesis using components from *S. coelicolor*. The residual amounts of active drug in the samples were estimated by comparison with the effects of known amounts of viomycin. ●, Residual active viomycin; ○, [^{32} P]phosphate transferred to viomycin.

Fig. 6. Effect of acetylcapreomycins on cell-free polyphenylalanine synthesis by ribosomes and S100 from *S. coelicolor*: ○, control; □, plus 0.5 μ M-[^{14} C]acetylcapreomycin IA; ▲, plus 0.5 μ M-[^{14} C]-acetylcapreomycin IB; ■, plus 0.5 μ M-capreomycin IA; ▼, plus 0.5 μ M-capreomycin IB.

observation that inactivated viomycin was reactivated by incubation with alkaline phosphatase (Table 2). Subsequently, phosphotransferase activity was assayed directly using the phosphocellulose binding assay (Ozanne *et al.*, 1969). Positively charged antibiotics, but not negatively charged radiolabelled ATP, are adsorbed to phosphocellulose paper. Using [γ - ^{32} P]ATP and *S. vinaceus* S100, it was found that viomycin was radioactively phosphorylated, and, furthermore, there was a reciprocal correlation between the extent of phosphorylation and the residual biological activity (Fig. 5). It appeared that approximately one molecule of phosphate was transferred per molecule of viomycin inactivated.

Substrate specificity of antibiotic-modifying enzymes

In addition to viomycin, capreomycins IA and IIA were substrates for the phosphotransferases of *S. vinaceus* and *S. capreolus* (Table 3). In this experiment, complete conversion of the antibiotics to phosphorylated product was not achieved because of the need to use

less ATP (employed here in radiolabelled form) and more antibiotic than in the drug-inactivation experiments described above. Significantly, neither enzyme modified aminoglycosides such as streptomycin, neomycin, kanamycin or gentamicin (results not given) although these latter drugs are subject to inactivation by phosphorylation in other systems (for a review, see Davies & Smith, 1978). We therefore conclude that the viomycin/capreomycin phosphotransferases of *S. vinaceus* and *S. capreolus* are novel, antibiotic-inactivating enzymes.

The results of Table 3 also show that all the capreomycins can be acetylated by extracts of *S. capreolus*. Moreover, when the acetylated forms of capreomycins IA and IB were purified by paper chromatography (see Methods), they were found to be inactive against protein synthesis *in vitro* (Fig. 6). Thus, 'capreomycin acetyltransferase' activity could contribute to the observed resistance of *S. capreolus* to both capreomycins IA and IB. In contrast, however, the 'capreomycin phosphotransferase' could only protect against capreomycin IA (and the minor component IIA). Although the IA component can be modified either by phosphorylation or by acetylation, these processes may be mutually exclusive since phosphocapreomycin IA could not be acetylated (results not given).

Sites of phosphorylation of viomycin and capreomycin IA

Phosphorylated viomycin is restored to antibiotic activity by incubation with alkaline phosphatase (Table 2). Also (results not given), the phosphate group is removed from [³²P]phosphoviomycin and, at a lower rate, from [³²P]phosphocapreomycin IA, by the same enzyme. Since phosphoamides are not substrates for bacterial alkaline phosphatase (Reid & Wilson, 1971), these results eliminate the possibility that *N*-phosphorylation occurred. This conclusion is supported by the observation that radiolabelled phosphate was not released from phosphoviomycin by treatment with 1 M-HCl at 100 °C for 60 min, under which conditions *N*-phosphorylated compounds would be readily hydrolysed. Evidently, inactivation of these antibiotics involves *O*-phosphorylation. Radiolabelled phosphoviomycin and phosphocapreomycin IA were then subjected to partial hydrolysis in 2 M-HCl at 110 °C for 4 h followed by high voltage paper electrophoresis at pH 1.9 (Jones & Rodnight, 1971) or paper chromatography in solvent containing butanol/acetic acid/water (50:11:25, by vol.). In all hydrolysates, a substantial proportion of the total radioactivity co-migrated with *O*-phospho-L-serine which had been added prior to hydrolysis, while most of the remainder was identified as free phosphate (results not given). We therefore concluded that inactivation of viomycin and capreomycin IA resulted from phosphorylation of serine residues. In the case of capreomycin IA there is no ambiguity, but we cannot specify which of the two serines in viomycin is modified.

Sites of acetylation of capreomycins

The sites of acetylation of the capreomycins have not been unequivocally established. There are four potential sites: the α -amino group of α,β -diaminopropionic acid, the β - and ϵ -amino groups of β -lysine, and the guanidinium group. In an attempt to distinguish between these possibilities, we compared the electrophoretic mobilities of acetylcapreomycins IA and IB with those of the unmodified antibiotics at various pH values. The rationale behind these experiments was that acetylation would result in a charge change of -1 at pH values below the pK_a of the modified group and would reduce the electrophoretic mobility relative to unmodified drug. As shown in Table 4, the pK_a value of the group acetylated in acetylcapreomycin IA was greater than 11 compared with about 8 for the group modified in acetylcapreomycin IB. Apparently, therefore, different groups were acetylated in the different capreomycins. We conclude that the guanidinium group is the probable site of acetylation in capreomycin IA; however, comparison of the published pK_a values for capreomycin (Herr, 1963) with those of viomycin, β -lysine and α,β -diamino-

Table 4. *Electrophoretic mobilities of [¹⁴C]acetylcapreomycins at various pH values*

[¹⁴C]Acetylcapreomycins IA and IB, prepared as described in Methods, were subjected to high voltage paper electrophoresis in the buffers indicated (50 mM, pH measured at 20 °C) and their mobilities relative to the corresponding unmodified capreomycins were measured. Electrophoresis was at 33 V cm⁻¹ for 2 to 4 h. Markers were detected by u.v. absorbance or by the ninhydrin reaction. Distribution of radioactivity was measured by cutting the paper into 1 cm strips followed by liquid-scintillation spectrometry.

Buffer	Relative mobility	
	[¹⁴ C]Acetylcapreomycin IA	[¹⁴ C]Acetylcapreomycin IB
Tris/maleate pH 5.2	0.6	0.8
Tris/maleate pH 7.2	0.6	0.7
Glycine/NaOH pH 9.2	0.6	1.0
Triethanolamine/HCl pH 11	0.4	1.0

propionic acid (Dyer *et al.*, 1965) suggests that the β -amino group of β -lysine (pK_a 7.9) could be the site of acetylation in capreomycin IB.

Finally (results not given), we confirmed that [1-¹⁴C]acetyl-coenzyme A acted as a source of acetyl and not carboxymethyl groups in these experiments. Total acid hydrolysis (6 M-HCl, 100 °C, 24 h) of the presumptive [¹⁴C]acetylcapreomycins released radioactivity in a form (presumably acetic acid) which did not bind to phosphocellulose paper at pH 2.5. [¹⁴C]Carboxymethylcapreomycins would have yielded radioactive degradation products that bound to phosphocellulose.

DISCUSSION

Antibiotic-producing organisms are frequently resistant to their products *in vivo* (Okami *et al.*, 1969) but there have been relatively few detailed studies of the biochemical bases of such resistance. This is especially so in the case of those organisms which produce inhibitors of ribosome function, although *Streptomyces erythreus* and *S. azureus* possess ribosomes which fail to bind erythromycin and thiostrepton, respectively, and are thereby resistant to their own products (Teraoka & Tanaka, 1974; Cundliffe, 1978). Otherwise, it is less clear how self-defence is achieved since, in all other cases so far examined, the ribosomes of the producing organisms were shown to be fully sensitive to the antibiotics in question. This latter group includes the producers of streptomycin (Cella & Vining, 1974; Piwowarski & Shaw, 1979), chloramphenicol (Malik & Vining, 1972), and, as reported here, viomycin and capreomycin.

Several streptomycetes which produce aminoglycoside antibiotics contain aminoglycoside-inactivating enzymes similar to those which exist as drug-resistance determinants in clinical isolates (Benveniste & Davies, 1973). The question of whether any such enzyme plays a biosynthetic or protective role (or both) cannot, however, be resolved in the absence of other information. Relevant factors include the sensitivity of the ribosomes to the drug in question, the permeability properties of the cell membrane, and the possibility of intracellular compartmentalization. The present work shows that streptomycetes producing viomycin and capreomycin possess ribosomes which are entirely sensitive to these antibiotics. From this we infer that viomycin and capreomycin do not exist in these organisms in significant concentrations in the same cellular compartments as the ribosomes. Possibly the drugs are synthesized there as inactive precursors, or alternatively they may be produced in separate compartments. In the event that viomycin and capreomycins IA and IIA are produced intracellularly in phosphorylated form, one might predict the existence of phosphatases capable of restoring antibiotic activity extracellularly. In this context, it is noteworthy that the addition of inorganic phosphate to the culture medium of a viomycin-producing streptomycete severely inhibited antibiotic production with a concomitant repression of

alkaline phosphatase activity (Pass & Raczyńska-Bojanowska, 1968). One wonders whether, under these conditions, viomycin accumulated as its phosphorylated derivative.

Under appropriate culture conditions, *S. vinaceus* and *S. capreolus* produce high external concentrations of viomycin and capreomycin, respectively, and, given that the ribosomes of these organisms are drug-sensitive, it seems logical to suppose that their cell membranes may be relatively impermeable to the drugs. As to the role of the antibiotic-modifying enzymes, they may be involved in the biosynthesis and/or export of the drugs and, since they require ATP or acetyl-coenzyme A as cofactors, we assume that they are either intracellular enzymes or are associated with the cytoplasmic membrane. These enzymes may additionally serve to protect the organisms from any antibiotic which re-enters the cells. If so, resistance is the result of a balance between uptake and inactivation such that the intracellular concentration of antibiotic remains subinhibitory. In this context, we note that *S. vinaceus* is considerably more resistant to capreomycin IA than to capreomycin IB (Fig. 2) and that its phosphotransferase can inactivate the former, but not the latter. On the other hand, *S. vinaceus* is substantially more sensitive to capreomycin IA than to viomycin (Fig. 2), even though the former antibiotic is a better substrate for the phosphotransferase (Table 3). This observation would be accounted for if the cell membranes of *S. vinaceus* were more permeable to capreomycin IA than to viomycin.

Regarding 'capreomycin acetyltransferase', it is not clear whether this activity is attributable to a single enzyme. Indeed, our results suggest that the sites of acetylation of capreomycins IA and IB may be different, which raises the possibility that there may be different acetyltransferases specific for different components of the capreomycin complex. However, our tentative identification of the sites of acetylation of these molecules requires confirmation by bulk synthesis and physico-chemical analysis. The minor components, capreomycins IIA and IIB, are also acetylated and we assume that they are thereby inactivated, although we have no information regarding the sites of modification.

Antibiotic-modifying enzymes occur widely in drug-resistant bacteria isolated in clinical situations (Davies & Smith, 1978) and an attractive hypothesis suggests that plasmids encoding these enzymes may have acquired the relevant genes directly or indirectly from antibiotic-producing organisms (Walker & Walker, 1970; Benveniste & Davies, 1973). Viomycin and capreomycin have been used as tuberculostatic agents for some years and there are many reports of the clinical isolation of strains of *Mycobacterium tuberculosis* resistant to these antibiotics (Tsukamura, 1969; Algeorge & Petre, 1970; McClatchy *et al.*, 1977). It would be interesting to know whether any of these resistant strains contain antibiotic-modifying enzymes.

Note added in proof. In a 'shotgun' cloning experiment, resistance to viomycin was cloned from *S. vinaceus* into *S. lividans* on the vector SLP 1.2. Recombinant clones of *S. lividans* resistant to high levels of viomycin were cross-resistant to capreomycin IA but not to capreomycin IB, and extracts of these strains contained viomycin phosphotransferase activity. This enzyme was not, however, detected in the viomycin-sensitive parental strain of *S. lividans* (C. J. Thompson, J. M. Ward and D. A. Hopwood, unpublished results).

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